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Cholinerge Regulation der ATP-vermittelten Freisetzung des pro-inflammatorischen Zytokins Interleukin-1β

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I. Hecker A., Küllmar M., Wilker S., **Richter K.**, Atanasova S., Timm T., Zakrzewicz A., Klein J., Kaufmann A., Bauer S., Padberg W., Kummer W., Jaciauskiene S., Fronius M., Schweda E.K.H., Lochnit G., Grau V. (2015). Phosphocholine-modified macromolecules and canonical nicotinic agonists inhibit ATP-induced IL-1β release. *Journal of Immunology* 195 (5), S. 2325-34. DOI: 10.4049/jimmunol.1400974. (*Reproduced with permission from Journal of Immunology*)

II. Zakrzewicz A.*, **Richter K.***, Agné A., Wilker S., Siebers K., Fink B., Krasteva-Christ G., Althaus M., Padberg W., Hone A.J., McIntosh J.M., Grau V. (2017). Canonical and novel non-canonical cholinergic agonists inhibit ATP-induced release of monocytic interleukin-1β via different combinations of nicotinic acetylcholine receptor subunits α7, α9 and α10. *Frontiers in cellular neuroscience* 11:189. DOI: 10.3389/fncel.2017.00189. (*Published under an open access Creative Common CC BY license*)

III. **Richter K.**, Mathes V., Fronius M., Althaus M., Hecker A., Krasteva-Christ G., Padberg W., Hone A.J., McIntosh J.M., Zakrzewicz A., Grau V. (2016). Phosphocholine – an agonist of metabotropic but not of ionotropic functions of α9-containing nicotinic acetylcholine receptors. *Scientific reports* 6:28660. DOI: 10.1038/srep28660. (*Reproduced with permission from Springer Nature*)

IV. Backhaus S., Zakrzewicz A., **Richter K.**, Damm J., Wilker S., Fuchs-Moll G., Küllmar M., Hecker A., Manzini I., Ruppert C., McIntosh J.M., Padberg W., Grau V. (2017). Surfactant inhibits ATP-induced release of interleukin-1β via nicotinic acetylcholine receptors. *Journal of lipid research* 58(6):1055-1066. DOI: 10.1194/jlr.M071506. (*Published under the terms of the Creative Common CC BY license*)

V. **Richter K.*,** Koch C.*, Perniss A., Wolf P.M., Schweda E.K., Wichmann S., Wilker S., Magel I., Sander M., McIntosh J.M., Padberg M., Grau V. (2018a). Phosphocholine-modified lipooligosaccharides of *Haemophilus influenzae* inhibit ATP-induced IL-1β release by pulmonary epithelial cells. *Molecules* 23(8):1979. DOI: 10.3390/molecules23081979. (*Published under an open access Creative Common CC BY license*)

VI. **Richter K.***, Sagawe S.*, Hecker A., Küllmar M., Askevold I., Damm J., Heldmann S., Pöhlmann M., Ruhrmann S., Sander M., Schlüter K.-D., Wilker S., König I.R., Kummer W., Padberg W., Hone A.J., McIntosh J.M., Zakrzewicz A., Koch C., Grau V. (2018b). C-reactive protein stimulates nicotinic acetylcholine receptors to control ATP-mediated monocytic inflammasome activation. *Frontiers in immunology* 9:1604. DOI: 10.3389/fimmu.2018.01604. (*Published under an open access Creative Common CC BY license*)

VII. **Richter K.***, Ogiemwonyi-Schaefer R.*, Wilker S., Chaveiro A.I., Agné A., Hecker M., Reichert M., Amati A.-L., Schlüter K.-D., Manzini I., Schmalzing G., McIntosh J.M., Padberg W., Grau V., Hecker A. (2020). Amyloid beta peptide ($A\beta_{1-42}$) reverses the cholinergic control of monocytic IL-1β release. *Journal of clinical medicine* 9(9):2887. DOI: 10.3390/jcm9092887. (*Published under an open access Creative Common CC BY license*)

VIII. **Richter K.**, Asci N., Singh V.K., Yakoob S.H., Meixner M., Zakrzewicz A., Liese J., Hecker A., Wilker S., Stumpf S., Schlüter K.-D., Rohde M., Gödecke A., Padberg W., Manzini I., Schmalzing G., Grau V. (2023a). Activation of endothelial NO synthase and P2X7 receptor modification mediates the cholinergic control of ATP-induced interleukin-1β release by mononuclear phagocytes. *Frontiers in immunology* 14:1140592. DOI: 10.3389/fimmu.2023.1140592. (*Published under an open access Creative Common CC BY license*)

IX. **Richter K.***, Papke R.L.*, Stokes C., Roy D.C., Espinosa E.S., Wolf P.M.K., Hecker A., Liese J., Singh V.K., Padberg W., Schlüter K.-D., Rohde M., McIntosh J.M., Morley B.J., Horenstein N.A., Grau V., Simard A.R. (2022). Comparison of the anti-inflammatory properties of two nicotinic acetylcholine receptor ligands, phosphocholine and *p*CF3-diEPP. *Frontiers in cellular neuroscience* 16:779081. DOI: 10.3389/fncel.2022.779081. (*Published under an open access Creative Common CC BY license*)

X. **Richter K.**, Herz S.M., Stokes C., Damaj M.I., Grau V., Papke R.L. (2023b). Pharmacological profiles and anti-inflammatory activity of *p*CN-diEPP and *m*CN-diEPP, new alpha9alpha10 nicotinic receptor ligands. *Neuropharmacology* 240:109717. DOI: 10.1016/j.neuropharm.2023.109717. (*Copyright Elsevier 2023*)

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Abkürzungen

AAT	α1-Antitrypsin
α-Bun	α-Bungarotoxin
Aβ	Amyloid-β
Abb.	Abbildung
ACh	Acetylcholin
AIM2	*absent in melanoma* 2
APP	*amyloid precursor protein*
ArIB	[V11L;V16D]ArIB
ASC	*apoptosis-associated speck-like protein containing a* CARD
ATP	Adenosintriphosphat
BzATP	2'-3'-O-(4-Benzoylbenzoyl)Adenosin-5'-Triphosphat
CARD	Caspase-Rekrutierungsdomaine
CAPS	Cryopyrin-assoziierte periodische Syndrome
CD14	*cluster of differentiation* 14
CFA	*complete Freund's adjuvant*
CLRs	*C-type* Lektin-Rezeptoren
COPD	*chronic obstructive pulmonary disease*
CRP	C-reaktives Protein
DAMPs	Schaden-assoziierte molekulare Muster
DPPC	Dipalmitoylphosphatidylcholin
ELISA	*enzyme-linked immunosorbent assay*
G-PC	Glycerophosphocholin
H. influenzae	*Haemophilus influenzae*
IL	Interleukin
LPC	Lysophosphatidylcholin
LPS	Lipopolysaccharid
Mec	Mecamylamin
MHC	Haupthistokompatibilitätskomplexmoleküle
nAChRs	nikotinische Acetylcholinrezeptoren
NK-Zellen	natürliche Killerzellen
NLRs	*NOD-like*-Rezeptoren
NLRP3	NACHT, LRR und PYD-Region enthaltendes Protein 3
NOD	*nucleotide-binding oligomerization domain and leucine-rich repeat-containing receptors*
P2RX7	P2X7-Rezeptor
PAMs	positive allosterische Modulatoren
PAMPs	pathogen-assoziierte molekulare Muster
PBMCs	*peripheral blood mononuclear cells*
PC	Phosphocholin

PC-BSA	PC-modifiziertes Bovines Serumalbumin
PC-LOS	PC-modifizierte Lipooligosaccharide
PRRs	*pattern recognition*-Rezeptoren
PYD	Pyrin
RIC3	*resistance to inhibitors of cholinesterase* 3
RLRs	*RIG-I-like*-Rezeptoren
sAPP	sezeniertes *amyloid precursor protein*-α
SLPI	*secretory leukocyte protease inhibitor*
SLURPs	*secreted ly6/uPAR-related proteins*
Strych	Strychnin
TLRs	*Toll-like*-Rezeptoren
TNF-α	Tumornekrosefaktor-α
vgl.	vergleiche
X. laevis	*Xenopus laevis*
z.B.	zum Beispiel

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1. Einleitung

1.1 Das pro-inflammatorische Zytokin Interleukin (IL)-1β: von der Infektabwehr zur systemischen Inflammation

Der Entzündungsmediator Interleukin (IL)-1β ist ein hoch potentes pro-inflammatorisches Zytokin des angeborenen Immunsystems. Erstmal 1977 entdeckt und von Charles A. Dinarello et al. als Pyrogen klassifiziert (Dinarello et al. 1977), ist es bis heute noch von großem wissenschaftlichem und klinischem Interesse. IL-1β, welches hauptsächlich von mononukleären Phagozyten (Kaufmann 2009) wie Monozyten und Makrophagen gebildet wird, spielt für das angeborene Immunsystem eine bedeutende Rolle bei der Infektabwehr gegen eine Vielzahl von Pathogenen (Vladimer et al. 2013). Vermittelt über den IL-1-Rezeptor-I, welcher von fast allen menschlichen Zellen exprimiert wird, dient IL-1β als immunologischer Botenstoff, der weitere Lymphozyten anlocken, stimulieren und/oder deren Proliferation steuern kann (Dinarello 2013). So ist IL-1β auch ein wichtiger Mediator der sogenannte Akute-Phase-Reaktion, einer unspezifischen systemischen Reaktion des Körpers auf Störungen seiner Homöostase verursacht durch diverse pathophysiologische Situationen wie Infektionen, Autoimmunerkrankungen, Traumata und Gewebeschädigungen (Dinarello 1988; Gabay und Kushner 1999; Dinarello 2018). Aufgrund dieser potenten, ubiquitären Funktion muss die Synthese und Freisetzung von IL-1β strikt reguliert werden und hängt meist von zwei "Gefahrensignalen" ab. Die biologisch inaktive pro-Form von IL-1β (pro-IL-1β) wird, im Gegensatz zum pro-inflammatorischen Zytokin IL-18, in Immunzellen nicht konstitutiv exprimiert (Gross et al. 2011). Es bedarf zunächst einer *priming*-Phase in der pro-IL-1β gebildet wird (Gross et al. 2011; Piccioli und Rubartelli 2013). Dieses *priming* kann durch Pathogene induziert werden, welche durch ihre Pathogen-assoziierten molekularen Muster (englisch: *pathogen-associated molecular patterns*, PAMPs) von verschiedenen *pattern recognition*-Rezeptoren (PRRs) der Immunzellen wahrgenommen werden (Vladimer et al. 2013; Broz und Dixit 2016). Zu den PRRs zählen *Toll-like*-Rezeptoren (TLRs), *RIG-I-like*-Rezeptoren (RLRs), *C-type* Lektin-Rezeptoren (CLRs), sowie die *NOD-like*-Rezeptoren (englisch: *nucleotide-binding oligomerization domain and leucine-rich repeat-containing receptors,* NLRs) (Bauernfeind et al. 2011; Gross et al.

2011). Auch Schaden-assoziierte molekulare Muster (englisch: *danger/damage-associated molecular patterns*, DAMPs), d.h. endogene Molekülstrukturen, die von geschädigten Zellen freigesetzt werden, wie Hitzeschockproteine oder mitochondriale DNA, können durch PRRs detektiert werden und führen zur intrazellulären Anreicherung von pro-IL-1β (Bauernfeind et al. 2011; Piccioli und Rubartelli 2013; Vladimer et al. 2013; Broz und Dixit 2016). Alle PRRs können verschiedene Signalwege induzieren, um zur Transkription verschiedener Zytokine, Chemokine oder anderer inflammatorischer Proteine zu führen (Piccioli und Rubartelli 2013). Mononukleäre Phagozyten können Pathogene zudem durch Phagozytose abtöten (Kaufmann 2009) und als "professionelle" antigenpräsentierende Zellen die Pathogenfragmente nach intrazellulärer Prozessierung auf ihrer Zelloberfläche über die Haupthistokompatibilitätskomplexmoleküle (englisch: *major histocompatibility complex molecule*, MHC) MHCI und MHCII den T-Lymphozyten präsentieren und somit das erworbene Immunsystem aktivieren (Iwasaki und Medzhitov 2015; Abualrous et al. 2021).

Für die Freisetzung von IL-1β muss pro-IL-1β zunächst proteolytisch gespalten werden, was eines zweiten "Gefahrensignals" bedarf. Dabei spielt eine besondere Untergruppe der intrazellulären NLRs eine wichtige Rolle, welche NLRP1, NLRP3, NLRC4, *absent in melanoma* 2 (AIM2) und Pyrin umfasst (Bauernfeind et al. 2011; Gross et al. 2011; Broz und Dixit 2016). Die Aktivierung dieser NLRs führt zunächst zur Formierung zytosolischer Multiproteinkomplexe, die sogenannten Inflammasome (Bauernfeind et al. 2011; Gross et al. 2011; Broz und Dixit 2016). Inflammasome wurden erstmals 2002 von der Arbeitsgruppe Tschopp beschrieben (Martinon et al. 2002). Das bis heute am besten untersuchte Inflammasom ist das NLRP3-Inflammasom. Es wird durch eine Vielzahl verschiedener Stimuli aktiviert, von bakteriellen und viralen Nukleinsäuren und Lipopolysaccharid (LPS) über diverse DAMPs wie extrazelluläres ATP, Harnsäure und Amyloid-β (Aβ)-Peptide (Bauernfeind et al. 2011; Santana et al. 2016).

Die Aktivierung NLRs führt zur Rekrutierung des Adaptorproteins ASC, welches aus einer Pyrin (PYD) und einer Caspaserekrutierungsdomaine (CARD) besteht und so die Inflammasomformierung erlaubt (Bauernfeind et al. 2011; Vladimer et al. 2013; Broz und Dixit 2016). Das Inflammasom dient dann als Plattform zur Aktivierung der biologisch inaktiven Form der Protease Caspase-1 (pro-Caspase-1). Die aktivierte

Caspase-1 spaltet proteolytisch das biologisch inaktive pro-IL-1β zu gereiftem IL-1β, welches dann aus den Zellen freigesetzt wird (Vladimer et al. 2013; Broz und Dixit 2016). Neben IL-1β können weitere Zytokine wie IL-18, IL-33 und IL-37 Inflammasom-abhängig von der Caspase-1 aktiviert werden (Dinarello 2018). Die Caspase-1 kann zudem Pyroptose induzieren, einen programmierten Zelltod, der morphologisch durch Zellschwellung und Zerstörung der Plasmamembran charakterisiert ist (Tan et al. 2021). Durch Pyroptose werden nicht nur pro-inflammatorische Zytokine wie IL-1β freigesetzt, sondern eine Vielzahl weiterer DAMPs wie HMGB1 und Laktatdehydrogenase (Tan et al. 2021). Dadurch kommt es zu einer Amplifizierung der Entzündungsreaktion und Rekrutierung weiterer Immunzellen (Lamkanfi et al. 2010; Tan et al. 2021).

Die Produktion von IL-1β ist keineswegs auf Monozyten, Makrophagen beschränkt, sondern erfolgt auch durch dendritische Zellen und diverse Epithelzellen (Santana et al. 2016). Die Freisetzung von IL-1β und die damit einhergehenden Entzündungsprozesse haben eine protektive und regenerative Funktion, da so eine Eliminierung infektiöser Faktoren sowie die Wiederherstellung der Gewebeintegrität, eingeleitet wird (Santana et al. 2016; Tan et al. 2021). Entzündungsreaktionen, welche durch Trauma, Ischämie-Reperfusionsschaden, Verbrennungen oder chemisch induziert werden, laufen meist ohne infektiöse Faktoren ab und werden als deshalb als “sterile Entzündung“ bezeichnet (Bortolotti et al. 2018). Auch die sterile Inflammation ist charakterisiert durch die Rekrutierung von Immunzellen wie Makrophagen, sowie die Freisetzung pro-inflammatorischer Zytokine wie IL-1 (Bortolotti et al. 2018). Fehlregulationen und erhöhte systemische IL-1β-Konzentrationen können hingegen schwere inflammatorische und autoimmune Erkrankungen hervorrufen (Mantovani et al. 2019).

Trotz intensiver Forschung führen Polytraumata und große chirurgische Eingriffe auch heute noch zu lebensbedrohlichen systemischen Entzündungen mit einer nicht akzeptablen hohen Gesamtletalität (Westphal und Kampmeier 2015; Bortolotti et al. 2018). In diesem Zusammenhang spielen die sterile Inflammation und IL-1β eine zentrale Rolle. Durch massive Gewebeschädigung kommt es zu einer überschießenden Zytokin-Ausschüttung und folglich zur traumainduzierten Immundysfunktion, welche von systemischen Entzündungen, Schock, Multiorganversagen bis hin zur Sepsis führen kann (**Abb. 1**). Nach Verletzungen wird

die Reifung und Ausschüttung von pro-inflammatorischen Zytokinen wie IL-1β aus mononukleären Phagozyten durch das Gefahrensignal extrazelluläres ATP stimuliert, welches von aktivierten Zellen freigesetzt oder aus dem Zytoplasma geschädigter Zellen stammt. Freigesetztes ATP wird dann über den ATP-sensitiven Rezeptor P2X7 (P2RX7; Gen: *P2RX7*) wahrgenommen, welcher von mononukleären Phagozyten exprimiert wird (Gross et al. 2011; Broz und Dixit 2016; Mantovani et al. 2019). Durch die Aktivierung des ionotropen P2RX7 kommt es dann zu Änderungen der intrazellulären Ionenkonzentration, Formierung des NLRP3-Inflammasoms, Aktivierung der Caspase-1 und letztlich Freisetzung von IL-1β (Gross et al. 2011; Broz und Dixit 2016; Bortolotti et al. 2018; Mantovani et al. 2019).

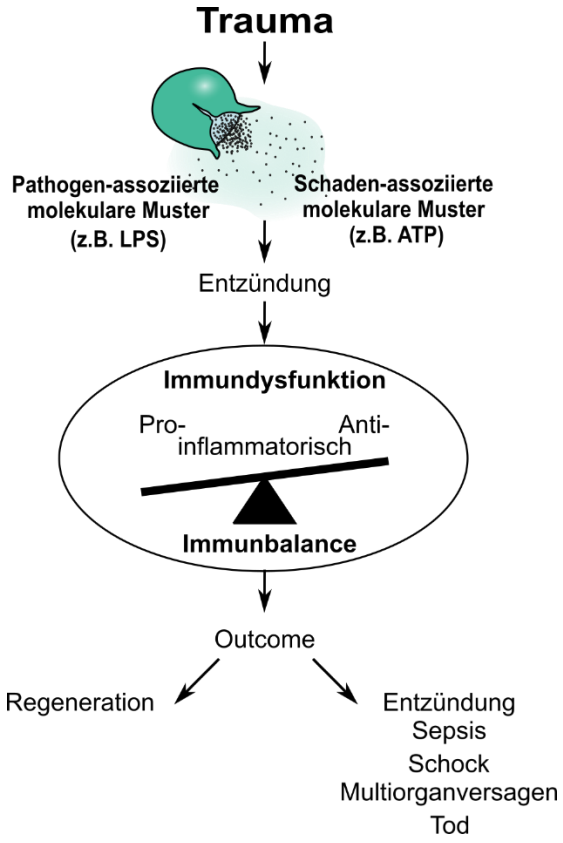

Abbildung 1. Die traumainduzierte Immundysfunktion. Bei schweren Traumata und großen chirurgischen Eingriffen kann es zu einer Immundysfunktion kommen, die von systemischen Entzündungen bis hin zum Tod der Patienten führen kann.

Aktuell gibt es, neben der Gabe von immunsupprimierenden Steroiden, welche ein breites Nebenwirkungsspektrum haben (Schäcke et al. 2002), kaum geeigneten Behandlungen für die traumainduzierte Immundysfunktion. Bisher wurden mehrere "*biologicals*" entwickelt, die am IL-1-System angreifen, um eine überschießende Entzündungsreaktion zu verhindern (Dinarello 2018), wie beispielsweise der

rekombinante IL-1-Rezeptor-Antagonist Anakinra, Anti-Zytokin-Therapien wie anti-IL-1β (Canakinumab) oder rekombinante IL-1-Rezeptor-Fusionsproteine (Rilonacept) (Bortolotti et al. 2018). Diese Therapien werden zur Behandlung von entzündlichen Erkrankungen wie Gicht, Diabetes Typ 1 und Cryopyrin-assoziierten periodischen Syndromen (CAPS) eingesetzt (López-Castejón und Pelegrín 2012; Bortolotti et al. 2018). Anti-Zytokin-Therapien wie Canakinumab erwiesen sich in klinischen Studien zur traumainduzierten Immundysfunktion jedoch als nicht effektiv. Für eine direkte Hemmung der IL-1β-Sekretion in Form von NLRP3-Inflammasom-Antagonisten gibt es bis heute zwar vielversprechende Ansätze, aber keine zugelassenen Therapien (Marchetti et al. 2018). Zudem können entzündungshemmende Therapien die Infektanfälligkeit der Betroffenen maßgeblich erhöhen. Es ist daher von großem, klinischem Interesse, neue Therapieansätze zu finden, welche die sterile Inflammation kontrollieren, ohne dabei die Infektabwehr zu vermindern.

1.2 Nikotinische Acetylcholinrezeptoren (nAChRs) und das non-neuronale cholinerge System

Nikotinische Acetylcholinrezeptoren (nAChRs) sind aus der traditionellen Sicht als Liganden-gesteuerte Ionenkanäle bekannt, welche eine unerlässliche Rolle für die Reizweiterleitung an Neuronen und der motorischen Endplatte spielen (Wessler und Kirkpatrick 2008; Changeux 2012). Ein funktioneller nAChR besteht dabei aus einem pentameren Komplex von Untereinheiten, die sich als Homomere und Heteromere zusammenlagern können (Stokes et al. 2015). Jede Untereinheit besteht dabei aus einer extrazellulären Domäne, die den C- und N-Terminus des Proteins sowie die orthosterische Liganden-Bindungsstelle beinhaltet (Stokes et al. 2015; Gharpure et al. 2020), sowie aus vier Transmembrandomänen und einer variablen zytoplasmatischen Domäne (Gharpure et al. 2020). In der Familie der Säugetiere wurden bisher 16 verschiedene nAChR-Untereinheiten identifiziert. Die nAChR-Untereinheiten werden dabei in die Typ-α (α1 – α7, α9, α10) und nicht-Typ-α (β1-β4, γ, δ, or ε) unterschieden (Changeux 2012; Papke 2014; Stokes et al. 2015). Die orthosterische Liganden-Bindungsstelle befindet sich zwischen zwei benachbarten Untereinheiten (Bouzat et al. 2018). Entsprechend ihrer typischen zellulären Expressionsmuster, können nAChRs in nAChRs vom muskulären Typ und solche vom neuronalen Typ eingeteilt werden. Die nAChRs vom muskulären Typ werden im ausgewachsenen Muskel aus

zwei α1-Unereinheiten sowie jeweils einer β1-, δ-, und ε-Untereinheit gebildet und sind in den neuromuskulären Endplatten zu finden (Cetin et al. 2020). Während der Embryogenese wird anstelle von der ε-Untereinheit eine γ-Untereinheit exprimiert, die später ersetzt wird (Cetin et al. 2020). nAChRs vom neuronalen Typ kommen sowohl als Homopentamere der α7- oder α9-Untereinheiten vor, Heteropentamere aus den Untereinheiten α7 und β2 oder α9 und α10, sowie in zahlreichen weiteren Kombinationen aus zwei α-Untereinheiten (α2 – α4 oder α6) mit drei β-Untereinheiten (β2 oder β4) (Zoli et al. 2018).

Ein endogener Ligand der nAChRs und gleichzeitig einer der wichtigsten Neurotransmitter im cholinergen Nervensystem von Vertebraten und Insekten ist ACh (Horiuchi et al. 2003; Wessler und Kirkpatrick 2008), welches bereits 1921 durch Otto Lewi als Vagusstoff entdeckt und später durch Henry H. Dale als solches identifiziert wurde (Lewi und Dale erhielten 1936 den Nobelpreis für Medizin).

Die Bedeutung von nAChRs für die Regulation des Immunsystems kam vor über zwei Jahrzehnten auf, als der Begriff des “anti-inflammatorischen cholinergen Reflex“ geprägt wurde (Borovikova et al. 2000). Dieser vagale, neuroimmunologische Reflexmechanismus wurde zuerst in Arbeiten von Kevin J. Tracey beschrieben: In anästhesierten Ratten wird die LPS-induzierte Freisetzung pro-inflammatorischer Zytokine durch die Stimulation des Vagusnerv abgemildert (Borovikova et al. 2000). Es wurde weiterhin Hinweise dafür erbracht, dass dieser Reflex über ACh vermittelt wird, welches von T-Lymphozyten der Milz freigesetzt wird (Rosas-Ballina et al. 2011). Das freigesetzte ACh aktiviert dann α7-nAChRs in Makrophagen und Dendritischen Zellen, wodurch die Freisetzung pro-inflammatorischer Zytokine wie IL-1β, IL-6 und Tumornekrosefaktor (TNF)-α abgemildert wird (Borovikova et al. 2000; Rosas-Ballina et al. 2011; Olofsson et al. 2012; Hoover 2017). Das Konzept des “anti-inflammatorischen Reflex” wurde später von der Fachwelt in Frage gestellt und ist bis heute umstritten, nicht zuletzt weil die Milz gar nicht durch den Vagusnerv innerviert wird (Martelli et al. 2014). Dennoch führten diese frühen Arbeiten zum Forschungsfeld der cholinergen anti-inflammatorischen Regulation des Immunsystems, aus dem auch verschiedene therapeutische Ansätze zur Behandlung von inflammatorischen Erkrankungen und neuropathischem Schmerz hervorgegangen sind (Hoover 2017; Halder und Lal 2021; Zhang et al. 2022).

Fast zeitgleich zum cholinergen Reflex wurde mit der Entdeckung des “non-neuronalen cholinergen Systems“ ein neues Kapitel der cholinergen Forschung geschrieben. Untersuchungen zeigten, dass die wesentlichen Komponenten und Rezeptoren des cholinergen Systems wie die Cholinacetyltransferase, ACh-Esterase, ACh, muskarinerge AChRs und nAChRs auch von non-neuronalen Zellen in Säugern exprimiert werden (Wessler et al. 2001; Grando et al. 2007; Wessler und Kirkpatrick 2008). Viel mehr noch können ACh und viele weitere non-neuronale cholinerge Komponenten bereits schon in Bakterien, Algen, Protozoen und primitiven Pflanzen gefunden werden, was das cholinerge System zu einem evolutionär hoch-konservierten und somit phylogenetisch wichtigen System macht (Wessler et al. 1999; Wessler et al. 2001). Zudem wurden Hinweise erbracht, dass dieses non-neuronale System zu grundlegenden Zellfunktionen beiträgt wie Genexpression, Proliferation, Zelldifferenzierung, Zell-Zell-Kommunikation, Zell- und Gewebe-Homöostase sowie immunologische Funktionen (Wessler et al. 2001; Grando et al. 2007; Wessler und Kirkpatrick 2008).

Es wird immer deutlicher, dass das non-neuronale cholinerge System im Allgemeinen und nAChRs im Besonderen das Immunsystem regulieren können. So wirkt die Aktivierung von nAChRs durch endogene Liganden wie ACh oder Nikotin protektiv bei verschiedenen inflammatorischen Erkrankungen wie entzündliche Darmerkrankungen (*inflammatory bowel disease*), rheumatoide Arthritis und Gicht (Hoover 2017; Lei und Duan 2021). Immunzellen wie T- und B-Zellen, Monozyten, Makrophagen und Dendritische Zellen exprimieren die wesentlichen Komponenten des cholinergen Systems zur Synthese, Transport und Freisetzung von ACh, sowie verschiedenste nAChR-Untereinheiten wie α2, α5, α6, α7, α9, α10 und β2 (Fujii et al. 2017a, 2017b; Hecker et al. 2015). Für nAChRs mit der Untereinheit α7 konnte in einer Vielzahl von Studien gezeigt werden, dass ihre Aktivierung zur Abmilderung der Zytokinfreisetzung führt sowie die Expression pro-inflammatorischer Zytokine bereits auf translationaler Ebene hemmen kann (Borovikova et al. 2000; Jonge und Ulloa 2007; Olofsson et al. 2012; Fujii et al. 2017a, 2017b; Stegemann und Böhm 2020; Lei und Duan 2021). In den letzten Jahren zeigte sich jedoch, dass neben α7 auch andere nAChR-Untereinheiten wie α9 und β2 anti-inflammatorische Effekte induzieren können (Simard et al. 2013; Hecker et al. 2015; St-Pierre et al. 2016; Liu et al. 2017).

Die kanonischen Funktionen von nAChRs sind ionotrop. Dabei weisen sie, abhängig von der Zusammensetzung der Untereinheiten, eine Permeabilität für Na^+, K^+ und Ca^{2+} auf (Changeux 2012; Stokes et al. 2015). Eine vollkommen andere Wirkungsweise wurde zuerst für α7-nAChRs gezeigt. In Studien zur cholinergen Modulation von Entzündungsreaktionen zeigten α7-nAChR Liganden-induzierte metabotrope Funktionen und das vollkommen unabhängig von der kanonischen ionotropen Funktion (Jonge und Ulloa 2007; Kabbani et al. 2013; Stokes et al. 2015; Kabbani und Nichols 2018). Interessanterweise scheinen insbesondere sogenannte nAChR "*silent agonists*" ("stumme Agonisten"), d.h. Substanzen, die metabotrope aber keine ionotropen Funktionen an α7-nAChRs induzieren, mit zu den effektivsten Modulatoren von Entzündungsreaktionen zu gehören (Horenstein und Papke 2017). In Mikrogliazellen konnte gezeigt werden, dass der α7-nAChR *silent agonist* NS6740 effektiver die LPS-induzierte Freisetzung von TNF-α inhibierte als der partielle α7-Agonist GST-21 (Thomsen und Mikkelsen 2012). Somit stellen nAChR *silent agonists* einen vielversprechenden therapeutischen Ansatz zur Behandlung von entzündlichen Erkrankungen dar (Horenstein und Papke 2017).

2. Zielsetzung der Arbeit

Bisher wurden zahlreiche Arbeiten zur modulierenden Wirkung des non-neuronalen cholinergen Systems auf das Immunsystem auf transkriptionaler und post-transkriptionaler Ebene durchgeführt (Hoover 2017). Ob das non-neuronale cholinerge System auch die traumainduzierte Freisetzung von IL-1β modulieren und damit auf posttranslationaler Ebene wirken kann, ist Gegenstand der vorliegenden Arbeit. Folgende initiale Hypothese wurde im Rahmen dieser Forschungsarbeit adressiert:

1. Wird die ATP-vermittelte Freisetzung von IL-1β aus Monozyten durch cholinerge Agonisten inhibiert? (**Hecker et al. 2015; Zakrzewicz & Richter et al. 2017; Richter et al. 2016**)
2. Sind Phosphatidylcholine und deren Metabolite wie Phosphocholin (PC) unkonventionelle Agonisten von nAChRs? (**Hecker et al. 2015; Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Backhaus et al. 2017**)
3. Ist der anti-inflammatorische, cholinerge Regulationsmechanismus der ATP-vermittelten Freisetzung von IL-1β auch in Lungenepithelzellen und Lungengewebe aktiv? (**Richter et al. 2018a**)
4. Können Akute-Phase-Proteine wie das C-reaktive Protein (CRP) den anti-inflammatorischen cholinergen Regulationsmechanismus induzieren? (**Richter et al. 2018b**)
5. Hat das $A\beta_{1\text{-}42}$-Peptid einen Einfluss auf den cholinergen Regulationsmechanismus der ATP-vermittelten Freisetzung von IL-1β? (**Richter et al. 2020**)
6. Wie können nAChRs die Aktivität des ATP-sensitiven P2RX7 modulieren? (**Richter et al. 2023a**)
7. "*To channel or not to channel*" – Fungieren nAChRs in mononukleären Phagozyten als metabotrope oder ionotrope Rezeptoren? (**Hecker et al. 2015; Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Backhaus et al. 2017; Richter et al. 2018b**)
8. Sind Liganden von nAChRs mit der Untereinheit α9 geeignete Werkzeuge in der Grundlagenforschung und potenzielle Therapeutika inflammatorischer Erkrankungen und Schmerzen? (**Richter et al. 2022; Richter et al. 2023b**)

3. Ergebnisse der eigenen Arbeiten

3.1 Cholinerge Regulation der ATP-vermittelten Freisetzung des pro-inflammatorischen Zytokins IL-1β

Zunächst wurde die Hypothese überprüft, dass die Aktivierung von monozytären nAChRs zur Inhibition des ionotropen P2RX7 führt und somit die ATP-vermittelte Freisetzung von IL-1β aus Monozyten unterdrückt. Um die Fragestellung zu untersuchen, wurden verschiedene Untersuchungen an monozytären Zellen *in vitro* und *ex vivo* durchgeführt. Die Zellen wurden zunächst mit LPS vorstimuliert, um als erstes Gefahrensignal die Expression von pro-IL-1β zu induzieren (**vgl. Abb. 2**). Anschließend wurde als 2. Gefahrensignal ATP appliziert, meist in Form von P2RX7 spezifischem BzATP (2'-3'-O-(4-Benzoylbenzoyl)adenosin-5'-triphosphat) und die Konzentration von IL-1β im Zell-freien Zellkulturüberstand mittels *enzyme-linked immunosorbent assay* (ELISA) analysiert. Neben der humanen monozytären Zelllinie U937, wurden auch primäre humane Monozyten ($CD14^+$) und humane mononukleäre Zellen des peripheren Blutes (englisch: *peripheral blood mononuclear cells*, PBMCs), die aus Blutproben von freiwilligen Spendern frisch isoliert wurden, für die Untersuchungen verwendet, sowie frisch aufgereinigte murine PBMCs (**Hecker et al. 2015**; **Zakrzewicz & Richter et al. 2017**). In diesen Untersuchungen konnte gezeigt werden, dass die ATP-vermittelte Aktivierung des NLRP3-Inflammasoms und Freisetzung von IL-1β aus LPS-stimulierten monozytären Zellen durch klassische nikotinerge Agonisten wie ACh, Nikotin und Cholin effizient inhibiert wurde (**Hecker et al. 2015**; **Zakrzewicz & Richter et al. 2017;** vgl. **Abb. 2**). Auch die ATP-induzierte Aktivierung und Freisetzung der Caspase-1 wurde durch die cholinergen Agonisten abgemildert (**Hecker et al. 2015**). Die NLRP3-Inflammasom-unabhängigen Zytokine IL-6 und TNF-α wurden hingegen weder durch ATP noch durch nikotinerge Agonisten beeinflusst (**Hecker et al. 2015**). Die spezifische Wirkung des cholinergen Regulationsmechanismus auf den P2RX7 konnte zudem in weiteren Experimenten bestätigt werden. Wurde bei LPS-stimulierten Monozyten das NLRP3-Inflammasom mit dem Poren-bildenden Toxin Nigericin (Graven et al. 1966) und somit über ATP-unabhängige Wege aktiviert, so zeigten die nikotinergen Agonisten keinen Einfluss auf die IL-1β-Freisetzung (**Hecker et al. 2015**). Diese Ergebnisse stehen im Einklang mit

der Hypothese der spezifischen cholinergen Regulation der ATP-abhängigen Aktivierung des NLRP3-Inflammasoms und Freisetzung von IL-1β über den ionotropen P2RX7.

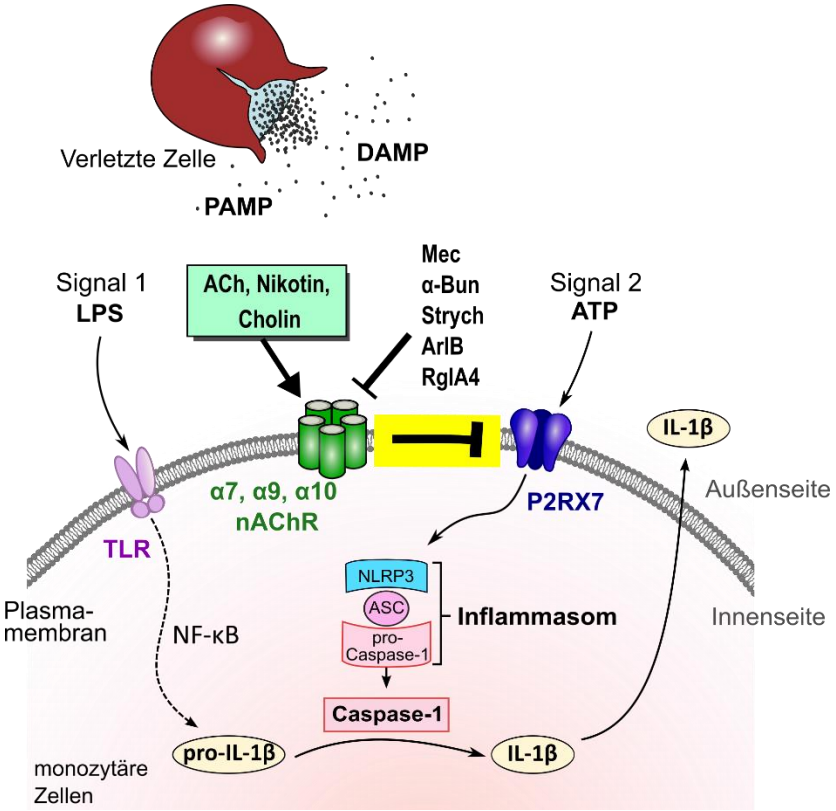

Abbildung 2. Cholinerge Regulation der ATP-vermittelten Freisetzung des pro-inflammatorischen Zytokins IL-1β. Die Synthese und Freisetzung von IL-1β sind streng reguliert und benötigen zweier Gefahrensignale. Ein prototypisches erstes Gefahrensignal ist LPS von Gram-negativen Bakterien, welches vermittelt über TLR die Expression von bioinaktivem pro-IL-1β induziert. Extrazelluläres ATP aktiviert als Schadensignal den P2RX7. Dadurch kommt es zur Assemblierung des NLRP3-Inflammasomes, Aktivierung der Caspase-1 und Spaltung von pro-IL-1β und letztlich zu dessen Freisetzung. Die Aktivierung von nAChRs mit den Untereinheiten α7, α9 und α10 durch klassische Agonisten wie ACh, Nikotin und Cholin führt zur Inhibition des P2RX7 und somit der IL-1β-Freisetzung. Dieser cholinerge Mechanismus wird durch nAChR-Antagonisten wie Mec, α-Bun, Strych und die Conopeptide ArIB (spezifisch für α7) und RgIA4 (spezifisch für α9/α10) aufgehoben. α-Bungarotoxin, α-Bun; Acetylcholin, ACh; *damage-associated molecular patterns*, DAMP; Interleukin-1β, IL-1β; Lipopolysaccharid, LPS; Mecamylamin, Mec; nikotinische ACh-Rezeptoren, nAChRs; P2RX7, P2X7-Rezeptor; *pathogen-associated molecular patterns*, PAMP; Strychnin, Strych; *Toll-like*-Rezeptoren, TLR. (**Hecker et al. 2015; Zakrzewicz & Richter et al. 2017; Richter et al. 2016**).

Interessanterweise wurde die inhibierende cholinerge Wirkung auf die ATP-vermittelte IL-1β-Freisetzung aus Monozyten auch durch Cholin induziert (**Hecker et al. 2015**; **Zakrzewicz & Richter et al. 2017**; **Richter et al. 2016**), welches als selektiver Agonist von nAChRs mit den Untereinheiten α7* (* zeigt das mögliche Vorhandensein weiterer nAChR-Untereinheiten an) und α9* bekannt ist (Papke et al. 1996; Verbitsky et al. 2000; Pereira et al. 2002). Um eine mögliche Beteiligung der nAChR-Untereinheiten α7* und α9* am cholinergen Regulationsmechanismus der ATP-vermittelten Freisetzung von IL-1β weiter zu untersuchen, führten wir zunächst pharmakologische Untersuchungen mit einer Reihe von nAChR-Antagonisten durch. Es zeigte sich, dass die inhibierende Wirkung der nAChR-Agonisten ACh, Nikotin und Cholin in Anwesenheit des unspezifischen nAChR-Antagonist Mecamylamin (Innocent et al. 2008) sowie der α7*- und α9*-nAChR-Antagonisten α-Bungarotoxin und Strychnin (Johnson et al. 1995; Briggs und McKenna 1996; Elgoyhen et al. 2001), aufgehoben wurde (**Hecker et al. 2015**). Die verwendeten Antagonisten erlauben jedoch keine Unterscheidung zwischen α7*- oder α9*-nAChR-Untereinheiten. Durch eine Kooperation mit Prof. J.M. McIntosh (Universität Utah, Salt Lake City, USA) konnten dann zwei spezifische Conopeptide eingesetzt werden: [V11L;V16D]ArIB (ArIB), welches spezifisch für die nAChR-Untereinheit α7 ist (Whiteaker et al. 2008; Hone et al. 2009; Hone et al. 2010; Grau et al. 2018), sowie das α9/α10-spezifische RgIA4 (Christensen et al. 2017; Romero et al. 2017; Grau et al. 2018). Diese Conopeptide wurden wie zuvor beschrieben (Innocent et al. 2008; Romero et al. 2017) synthetisiert und charakterisiert. Interessanterweise wurde die inhibierende Wirkung von ACh, Nikotin und Cholin auf die ATP-vermittelte IL-1β-Freisetzung aus Monozyten durch beide Conopeptide aufgehoben (**Zakrzewicz & Richter et al. 2017**; **Richter et al. 2016**), was auf eine Beteiligung der nAChR-Untereinheiten α7, α9 und/oder α10 hindeutet. Diese Ergebnisse konnten durch *small interfering* RNA (siRNA)-Experimente an monozytären U937-Zellen (**Hecker et al. 2015**; **Zakrzewicz & Richter et al. 2017**), sowie in Experimenten an PBMCs von Mäusen die Gen-defizient für die α7, α9 oder α10 nAChR-Untereinheit waren, untermauert werden (**Zakrzewicz & Richter et al. 2017**; **Richter et al. 2016**).

Die Expression der nAChR-Untereinheiten α7, α9 und α10 konnte unsere Arbeitsgruppe durch den Nachweis von mRNA für *Chrna*7, *Chrna*9 und *Chrna*10 in monozytären U937-Zellen sowie humanen PBMCs erbringen (**Hecker et al. 2015**),

was mit früheren Publikationen übereinstimmt (Kawashima und Fujii 2000; Chernyavsky et al. 2010).

Zusammenfassend konnten ungewöhnliche nAChRs mit den Untereinheiten α7, α9, und α10 in mononukleären Phagozyten identifiziert werden, die effizient die Aktivierung des ATP-sensitiven P2RX7 und damit einhergehend die Aktivierung des NLRP3-Inflammasoms und der ATP-induzierten Freisetzung von IL-1β inhibieren. Dieser Mechanismus reguliert spezifisch die ATP-induzierte Freisetzung von IL-1β, wohingegen die ATP-unabhängige Zytokin-Freisetzung nicht beeinflusst wird.

3.2 Dipalmitoylphosphatidylcholin, Phosphocholin und weitere Metabolite von Phosphatidylcholinen sind unkonventionelle Agonisten von nAChRs

Phosphatidylcholine (Lecithine) sind wichtige Grundbausteine aller eukaryotischen Zellmembranen (Li und Vance 2008). Ein Stoffwechselintermediat, welches bei der Synthese von Phosphatidylcholin entsteht ist das zwitterionisches Molekül PC (Li und Vance 2008). Interessanterweise weisen ACh, Cholin und PC eine hohe strukturelle Ähnlichkeit auf (**Abb. 3**).

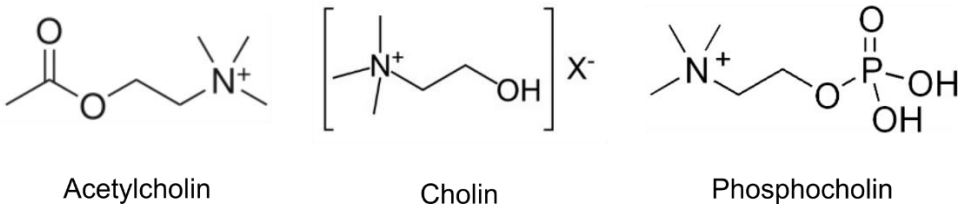

Abbildung 3. Chemische Strukturformeln. Acetylcholin, Cholin und Phosphocholin weisen eine hohe strukturelle Ähnlichkeit auf.

Dies führte zu der Hypothese, dass PC und PC-haltige Moleküle als Liganden von nAChRs fungieren könnten. Wir konnten in verschiedenen Arbeiten zeigen, dass PC ein unkonventioneller Agonist monozytärer nAChRs ist und die ATP-induzierte Freisetzung von IL-1β effizient inhibiert (**Hecker et al. 2015**; **Zakrzewicz & Richter et al. 2017; Richter et al. 2016**). Dabei ist der IC_{50}-Wert von PC mit 10 µM in etwa identisch zu Cholin (**Hecker et al. 2015**). In weiteren Untersuchungen konnten durch Verwendung spezifischer Inhibitoren, siRNA-Experimente an monozytären U937-

Zellen, sowie in Experimenten an PBMCs von Mäusen, die Gen-defizient für die α7-, α9- oder α10-nAChR-Untereinheit waren, nachgewiesen werden, dass der PC-induzierte Mechanismus der nAChR-Untereinheiten α7, α9, und α10 bedarf (**Hecker et al. 2015**; **Zakrzewicz & Richter et al. 2017; Richter et al. 2016**).

Nachfolgend konnten wir zahlreiche andere PC-haltigen Moleküle als bisher unbekannte, unkonventionelle Agonisten von nAChRs identifizieren. Glycerophosphocholin (G-PC), Lysophosphatidylcholin (LPC), sowie auch Dipalmitoylphosphatidylcholin (DPPC) inhibierten über nAChRs-vermittelt effizient die ATP-induzierte Freisetzung von IL-1β aus Monozyten (**Hecker et al. 2015**; **Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Backhaus et al. 2017;** vgl. **Tabelle 1**).

Tabelle 1: Klassische und unkonventionelle Liganden von nAChRs, ihre benötigte Konzentration, die zu einer 50%igen Inhibition (IC_{50}) der ATP-induzierten Freisetzung von IL-1β aus monozytären Zellen führt und die dafür benötigten nAChR-Untereinheiten.

Ligand	IC_{50}	nAChR-Untereinheiten
Cholin	10 µM [I]	α7, α9, α10 [III]
Acetylcholin	1 µM [I]	α7, α9, α10 [III]
Nikotin	10 µM [I]	α7, α9, α10 [III]
PC	10 µM [I]	α7, α9, α10 [III]
PC-BSA	140 nM [I]	α7, α9, and/or α10 [I]
RM-PC-LOS	25 nM [I]	α7, α9, and/or α10 [I]
NTHi-PC-LOS	25 nM [I]	α7, α9, and/or α10 [I]
G-PC	1 µM [III]	α9, α10 [II]
LPC	1 µM [III]	α9, α10 [II]
DPPC	10 µM [IV]	α9 and α7 or α10 [IV]

Dipalmitoylphosphatidylcholin, DPPC; Glycerophosphocholin, G-PC; Lysophosphatidylcholin, LPC; nikotinischer Acetylcholinrezeptor, nAChR; Phosphocholin, PC; PC-modifiziertes bovines Serumalbumin, PC-BSA; PC-modifizierte Lipooligosaccharide, PC-LOS. **[I]** Hecker et al. 2015; **[II]** Zakrzewicz & Richter et al. 2017; **[III]** Richter et al. 2016; **[IV]** Backhaus et al. 2017.

Vielmehr noch ergaben sich in den Untersuchungen merkliche Unterschiede bezüglich des Bedarfs der nAChR-Untereinheiten für den inhibitorischen cholinergen Mechanismus. Während klassische nAChR-Agonisten wie ACh die Untereinheiten α7, α9 und α10 benötigen, erfordert der G-PC- und LPC-induzierte Mechanismus lediglich die nAChR-Untereinheiten α9 und α10 (**Zakrzewicz & Richter et al. 2017; Tabelle 1**). Für den inhibitorischen Effekt von DPPC auf die ATP-induzierte IL-1β Freisetzung ist die α9-Untereinheit essenziell zusammen mit der α7- oder der α10-Untereinheit (**Backhaus et al. 2017; Tabelle 1**).

Wildtypstämme von *Haemophilus influenzae* (*H. influenzae*) besitzen das sogenannte lic1-Operon, um PC-modifizierte Lipooligosaccharide (PC-LOS) synthetisieren zu können (Risberg et al. 1999; Schweda et al. 2000; Månsson et al. 2003; Pang et al. 2008). PC-LOS wirkt auf Wirbeltiere anti-inflammatorisch und erleichtert deren Besiedlung durch *H. influenzae* (Risberg et al. 1999; Schweda et al. 2000; Månsson et al. 2003; Pang et al. 2008). Aufgrund der Entdeckung, dass PC ein Ligand von nAChR ist, stellten wir die Hypothese auf, dass Symbionten und Parasiten den anti-inflammatorischen cholinergen Kontrollmechanismus ausnutzen, um die Immunantwort des Wirts zu umgehen. Durch Kooperation mit Prof. Elke K. H. Schweda (Linköping Universität, Linköping, Schweden) standen uns aufgereinigte PC-LOS Proben von zwei verschiedenen Wildtyp *H. influenzae* Stämmen, RM118 (RM-PC-LOS) und NTHi123323 (NTHi-PC-LOS), zur Verfügung. Als Kontrolle wurden LOS-Proben von zwei von korrespondierenden, mutierten *H. influenzae* Stämmen verwendet, RM7004-lic1 und NTHi1233-lic1, welche durch eine Mutation im lic1-Operon keine PC-LOS-Modifikationen synthetisieren können. Wir konnten zeigen, dass PC-LOS (RM-PC-LOS, NTHi-PC-LOS) Dosis-abhängig die BzATP-induzierte Freisetzung von IL-1β aus LPS-*geprimten* monozytäre Zellen effizient inhibierten (**Hecker et al. 2015**). Die aus den mutierten *H. influenzae* Stämmen gewonnenen PC-freien LOS (RM7004-lic1, NTHi1233-lic1) waren hingegen inaktiv. Dementsprechend beruht die anti-inflammatorische Wirkung auf der PC-Modifikation. Vielmehr noch waren die PC-LOS mit einer IC_{50}-Konzentration von ~25 nM um ein Vielfaches effizienter als PC (siehe **Tabelle 1**). In weiteren Untersuchungen konnte durch den Einsatz verschiedener nAChR-Antagonisten die Beteiligung der nAChR-Untereinheiten α7, α9 und/oder α10 an dem PC-LOS-vermittelten Effekt aufgezeigt werden. Um sezernierte PC-modifizierte Makromoleküle, die von Helminthen synthetisiert und freigesetzt werden können, zu imitieren, wurde PC synthetisch an bovines Serumalbumin (BSA) gebunden (PC-BSA). Auch hier zeigte sich, dass PC-BSA über nAChR-vermittelt die BzATP-induzierte Freisetzung von IL-1β inhibiert (**Hecker et al. 2015**). Interessanterweise resultierte auch hier die Modifizierung von PC mit einem großen Molekül wie BSA in einer Potenzierung des inhibierenden Effekts um den Faktor 10 (IC_{50} = 140 nM; siehe **Tabelle 1**).

Zusammenfassend konnten somit nicht nur bislang unbekannte, endogene Liganden der nAChR-Untereinheiten α7, α9 und/oder α10 identifiziert werden, sondern es wurde

zudem ein Verständnis über die zugrundeliegenden Mechanismen von PC-modifizierten Makromoleküle gewonnen.

3.3 Cholinerge Regulation der ATP-vermittelten Freisetzung von IL-1β aus Lungenepithelzellen und Lungengewebe

Neben Monozyten und Makrophagen können auch Epithelzellen IL-1β synthetisieren und freisetzen (Pinkerton et al. 2017; Message und Johnston 2004; Tian et al. 2017).
Die beschriebenen Erkenntnisse zur cholinergen Funktion von PC-modifizierten Makromolekülen, sowie die Tatsache, dass *H. influenzae* als opportunistisches Bakterium hauptsächlich in den oberen und unteren Atemwegen angesiedelt ist (Jordens und Slack 1995), führte uns zu der Hypothese, dass solche Mechanismen auch in pulmonalen Epithelien ablaufen können. Um die Hypothese zu überprüfen, führten wir Experimente durch an humanen A549-Zellen, welche alveolaren Epithelzellen vom Typ-II ähneln (Giard et al. 1973), sowie der Bronchialepithelzelllinie Calu-3 (Shen et al. 1994). Die Stimulation der Zellen mit LPS und daran anschließende Behandlung mit BzATP induzierte die Freisetzung von IL-1β aus beiden Zelllinien (**Richter et al. 2018a**). Die BzATP-induzierte Freisetzung wurde effizient durch Nikotin und PC sowie die aus *H. influenzae* isolierten PC-LOS (RM-PC-LOS, NTHi-PC-LOS) inhibiert (**Richter et al. 2018a**), wodurch erstmalig der cholinerge Kontrollmechanismus in Epithelzellen nachgewiesen wurde. Die PC-freien LOS aus den *H. influenzae* Mutanten RM7004-lic1 and NTHi1233-lic1 zeigten, wie in der vorherigen Arbeit an monozytären Zellen (**Hecker et al. 2015**), keinen Einfluss auf die IL-1β-Freisetzung. In weiteren Experimenten konnte mittels nAChR-Antagonisten zudem gezeigt werden, dass PC und PC-LOS auch in Epithelzellen als Liganden der nAChR-Untereinheiten α7, α9 und/oder α10 fungieren (**Richter et al. 2018a**).
Um die Komplexität der Lunge zu imitieren, führten wir zudem *ex vivo* Experimente an *precision cut lung slices* durch, welche aus frischen Mauslungen präpariert wurden. Auch hier konnte die inhibierende Wirkung von PC-LOS nachgewiesen (**Richter et al. 2018a**) und somit die physiologische Bedeutung untermauert werden.

3.4 Das Akute-Phase-Protein CRP reguliert die ATP-induzierte Freisetzung von IL-1β - eine negative Rückkopplungsschleife?

Wie in **Kapitel 3.2 – 3.3** beschrieben, können PC und PC-haltige Moleküle die nAChRs in mononukleären Phagozyten aktiveren und darüber vermittelt die ATP-induzierte Freisetzung von IL-1β inhibieren. Wir stellten daher die Hypothese auf, dass natives, pentameres CRP (pCRP), welches mit PC-beladen ist, ebenfalls ein endogener Agonist der non-kanonischen nAChRs ist und den cholinergen Regulationsmechanismus aktivieren kann. Um die Hypothese zu überprüfen, führten wir Experimente an monozytären U937-Zellen und humanen PBMCs durch, die mit LPS vorstimuliert wurden (**Richter et al. 2018b**). Kommerziell erworbenes pCRP, welches aus menschlichen Pleura-Flüssigkeiten isoliert wurde, inhibierte konzentrationsabhängig die ATP-induzierte Freisetzung von IL-1β mit einem IC_{50}-Wert von ~5 µg/ml (**Richter et al. 2018b**). Zudem war der Effekt sensitiv für diverse nAChR-Antagonisten inklusive der spezifischen Conopeptide ArIB und RgIA4, wodurch wir Hinweise auf eine Beteiligung der nAChRs mit den Untereinheiten α7, α9 und/oder α10 erbringen konnten (siehe **Abb. 4**).

Es stellte sich dann die Frage, ob die inhibitorische Wirkung von pCRP von dem Pentraxin selbst, oder von seinem gebunden Liganden, vermutlich PC, induziert wird. Wurde das Pentraxin Serum-Amyloid-P, welches keine PC-Bindungsstellen besitzt (Mantovani et al. 2008) oder rekombinantes CRP (rCRP) aus Bakterien anstelle von pCRP eingesetzt, so zeigte sich keine Wirkung auf die ATP-induzierte IL-1β-Freisetzung (**Richter et al. 2018b**). In weiteren Experimenten konnten wir zeigen, dass die Aktivität von pCRP auf eine Ca^{2+}-abhängigen Bindung mit einem bisher noch nicht identifizierten endogenen Liganden, beruhte. Wurde pCRP mit einem Ca^{2+}-Chelator behandelt, so war es inaktiv. Durch eine anschließende Behandlung mit Ca^{2+} und PC konnte das inaktivierte pCRP hingegen wieder aktiviert werden. Auch rCPR konnte künstlich mit PC beladen und aktiviert werden und führte dann zu einer effizienten Inhibition der ATP-induzierten IL-1β-Freisetzung (**Richter et al. 2018b**). Wir können nur spekulieren, ob PC und/oder PC-haltige Moleküle auch *in vivo* als Ligand an pCRP gebunden sind. Es zeigte sich in unseren Experimenten jedoch noch ein weiterer interessanter Aspekt: Um inaktiviertes pCRP oder rCRP durch Behandlung mit Ca^{2+} und PC wieder zu aktivieren, waren nur geringe PC-Konzentrationen von 1 µM nötig,

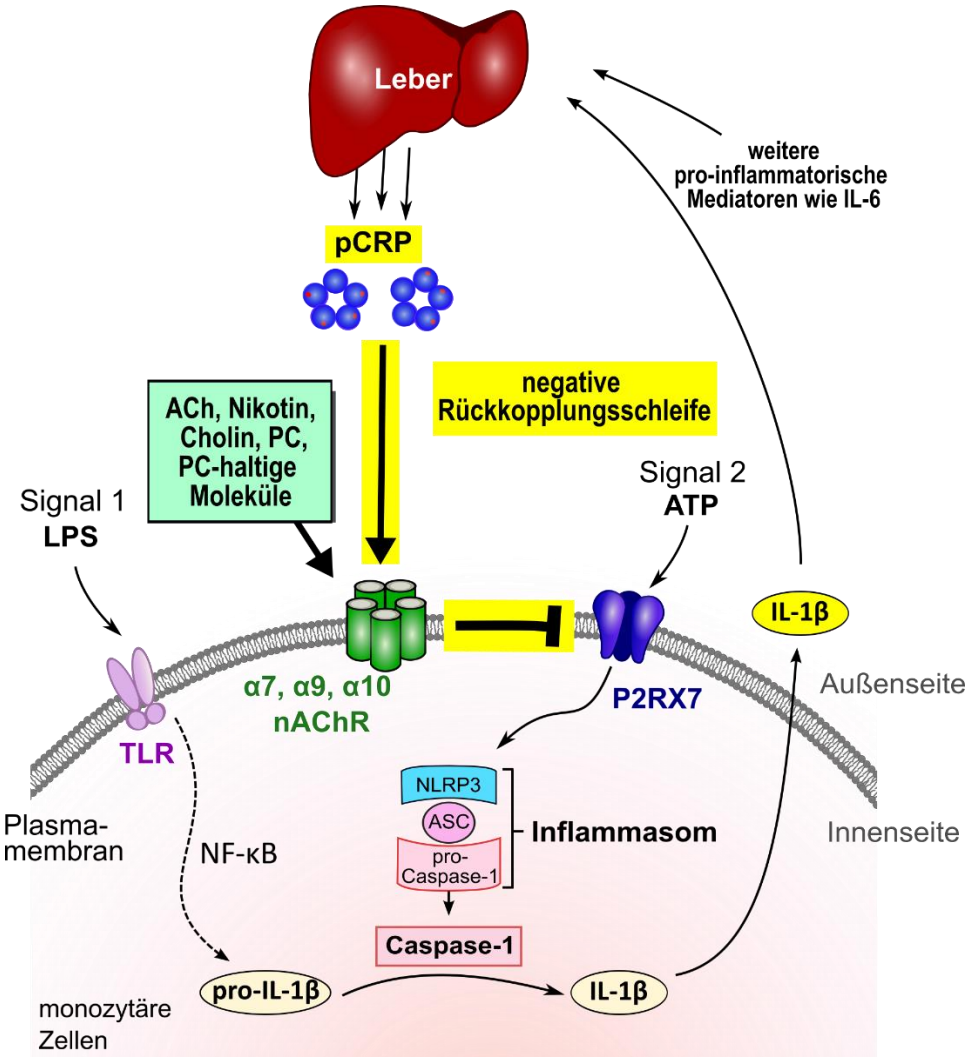

Abbildung 4. Das Akute-Phase-Protein pCRP moduliert die ATP-induzierte Freisetzung von IL-1β in mononukleären Phagozyten über nAChRs. Die Aktivierung von nAChRs mit den Untereinheiten α7, α9 und/oder α10 durch ACh, Nikotin, Cholin, PC oder PC-haltige Moleküle führt zur Inhibition des P2RX7 und somit der IL-1β-Freisetzung. pCRP konnte als neuer Ligand der nAChRs identifiziert werden. Da pro-inflammatorische Mediatoren wie IL-1β die Expression und Freisetzung von pCRP aus der Leber induzieren, könnte pCRP somit Teil einer negativen Rückkopplungsschleife sein, die eine überschießende z.B. verletzungsbedingte IL-1β-Freisetzung verhindert. Acetylcholin, ACh; Interleukin-1β, IL-1β; Lipopolysaccharid, LPS; nikotinische ACh-Rezeptoren, nAChRs; P2RX7, P2X7-Rezeptor; pentameres C-reaktives Protein, pCRP; Phosphocholin, PC; *Toll-like*-Rezeptoren, TLR. (**Hecker et al. 2015; Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Richter et al. 2018b**)

eine PC-Konzentration, die selbst keine inhibitorische Wirkung auf die IL-1β-Freisetzung hatte (**Hecker et al. 2015; Richter et al. 2018b**). Die Bindung von PC an CRP scheint demnach die Wirkung von PC zu potenzieren. Dieses Phänomen zeigte

sich bereits in früheren Arbeiten mit PC-BSA und PC-LOS (**Hecker et al. 2015; Richter et al. 2018a**) (siehe **Tabelle 1**).

Um zu überprüfen, ob der von uns identifizierte pCRP-induzierte Mechanismus auch *in vivo* aktiv ist, führten wir eine kleine, prospektive Studie an weiblichen und männlichen Polytrauma-Patienten durch. Es wurden vom Zeitpunkt der Einlieferung der Patienten in unsere Klinik (Universitätsklinikum Gießen und Marburg, Standort Gießen) täglich Blutproben abgenommen und die Plasmawerte für CRP sowie verschiedener Zytokine bestimmt. In Korrelationsanalysen zeigte sich dann eine negative Korrelation der IL-1β-Plasmakonzentrationen mit CRP (**Richter et al. 2018b**). Die NLRP3-Inflammasom-unabhängigen Zytokine IL-6, IL-18, und TNF-α waren hingegen positiv-korreliert (**Richter et al. 2018b**).

Wir konnten in unserer Arbeit erstmals eine mögliche physiologische Funktion von pCRP aufzeigen (**Richter et al. 2018b**): In mononukleären Phagozyten ist Liganden-beladenes pCRP ein effizienter Agonist von nAChRs mit den Untereinheiten α7, α9 und/oder α10. Da die Expression und Sekretion von CRP durch pro-inflammatorische Zytokine wie IL-1β und IL-6 induziert wird, scheint pCRP in diesem Zusammenhang Teil einer negativen Rückkopplungsschleife zu sein, welche die ATP-induzierte IL-1β-Freisetzung inhibiert, und vor überschießenden Entzündungsreaktionen schützen kann (**Richter et al. 2018b**) (siehe **Abb. 4**).

3.5 Das $A\beta_{1-42}$-Peptid ist ein negativer Regulator des cholinergen Kontrollmechanismus

Bisher wurden das *amyloid precursor protein* (APP) und die Aβ-Peptide im Zusammenhang der Alzheimer-Erkrankung intensiv beforscht (Heneka et al. 2015; Chen et al. 2017; Zhang et al. 2023b). Die biologischen Funktionen dieses ubiquitären Systems liegen jedoch weitgehend im Dunkeln. Das brachte uns zu der Frage, ob das $A\beta_{1-42}$-Peptid einen Einfluss auf den cholinergen Regulationsmechanismus der ATP-vermittelten Freisetzung von IL-1β hat (**Richter et al. 2020**).

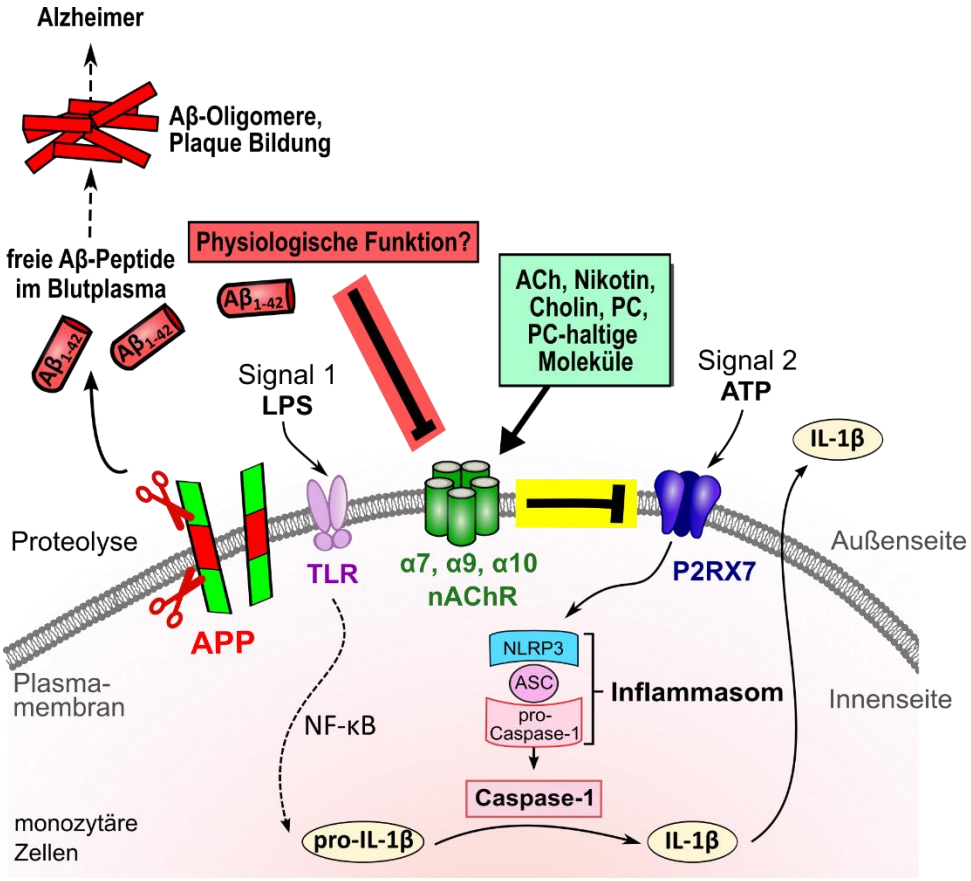

Abbildung 5. Das $Aβ_{1-42}$-Peptid ist ein negativer Regulator des cholinergen Kontrollmechanismus der ATP-induzierten Freisetzung von IL-1β aus mononukleären Phagozyten. Die Aktivierung von nAChRs mit den Untereinheiten α7, α9 und/oder α10 durch ACh, Nikotin, Cholin, PC oder PC-haltige Moleküle führt zur Inhibition des P2RX7 und somit der IL-1β-Freisetzung. Das Zellmembranprotein APP kann durch proteolytische Spaltung zu Aβ-Peptiden wie $Aβ_{1-42}$ prozessiert werden. Während in der Literatur für die $Aβ_{1-42}$-Petide eine pathophysiologische Rolle in der Alzheimer-Erkrankung beschrieben wurde, ist die biologische Funktion von APP und den Aβ-Peptiden weitgehend ungeklärt. Unsere Erkenntnisse deuten darauf hin, dass das $Aβ_{1-42}$-Peptid in mononukleären Phagozyten den cholinergen Kontrollmechanismus antagonisiert und die Freisetzung von IL-1β in Anwesenheit nikotinerger Agonisten erlaubt. Acetylcholin, ACh; *amyloid precursor protein*, APP; Amyloid-β, Aβ; Interleukin-1β, IL-1β; Lipopolysaccharid, LPS; nikotinische ACh-Rezeptoren, nAChRs; P2RX7, P2X7-Rezeptor; pentameres C-reaktives Protein, pCRP; Phosphocholin, PC; *Toll-like*-Rezeptoren, TLR. (**Hecker et al. 2015; Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Richter et al. 2020**)

Wir führten *in vitro*- und *ex vitro*-Experimente an LPS-stimulierten humanen monozytären Zellen durch und konnten dabei eine vollkommen neue Funktion des $Aβ_{1-42}$-Peptids aufzeigen (**Richter et al. 2020**): An monozytären nAChRs wirkt das $Aβ_{1-42}$-Peptid antagonistisch und ermöglicht so die ATP-induzierte Freisetzung von

IL-1β in Anwesenheit von nAChR-Agonisten (**Abb. 5**). Dabei wurde nicht nur der inhibitorische Effekt von klassischen nAChR-Agonisten wie ACh und Nikotin konzentrationsabhängig durch das $A\beta_{1-42}$-Peptid aufgehoben, sondern auch die Wirkung von PC und PC-haltigen Molekülen (**Richter et al. 2020**). Die von uns beobachteten Effekte des $A\beta_{1-42}$-Peptids scheinen auf einer antagonistischen Wirkung an den nAChRs zu beruhen (**Richter et al. 2020**). Dies wird bekräftigt durch die Tatsache, dass die $A\beta_{1-42}$-Peptid-induzierten Effekte auf cholinergen Regulationsmechanismus sehr denen der Conopeptide ArIB und RgIA4 ähnelten (**Richter et al. 2020**). Das $A\beta_{1-42}$-Peptid selbst induzierte hingegen keine IL-1β-Freisetzung aus LPS-stimulierten Monozyten und modulierte auch die ATP-induzierte IL-1β-Freisetzung nicht. Dementsprechend schien die Wirkung des $A\beta_{1-42}$-Peptids auf Interaktionen mit den nAChRs zu beruhen (**Richter et al. 2020**). Zudem konnten wir erste Hinweise darauf erbringen, dass das $A\beta_{1-42}$-Peptid auch *in vivo* wirkt: Bei chirurgischen Patienten war der Nachweis von IL-1β nur im Blut von Patienten möglich, in denen erhöhte $A\beta1_{-42}$-Peptid-Konzentrationen vorlagen (**Richter et al. 2020**).
Zusammenfassend konnten wir Hinweise darauf erbringen, dass das $A\beta_{1-42}$-Peptid ein endogener, negativer Regulator des cholinergen Kontrollmechanismus der ATP-induzierte Freisetzung von IL-1β in mononukleären Phagozyten ist (**Richter et al. 2020**). Diese Regulation scheint auf einer antagonistischen Wirkung an α9*-nAChRs zu beruhen (**Richter et al. 2020**).

3.6 "To channel or not to channel" – metabotrope Funktionen von unkonventionellen nAChRs in mononukleären Phagozyten

Wie zuvor beschrieben, konnte unsere Arbeitsgruppe zeigen, dass die Aktivierung von nAChRs mit den Untereinheiten α7, α9 und/oder α10 zu einer Inhibition des P2RX7 führt und somit die ATP-induzierte Freisetzung von IL-1β aus mononukleären Phagozyten wie Monozyten, Makrophagen inhibiert (**Hecker et al. 2015**; **Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Backhaus et al. 2017**). Um die nAChR-vermittelte Regulation des P2RX7 näher zu charakterisieren, führten wir elektrophysiologische Ganzzelllableitung mittels der *patch-clamp*-Methode (englisch: *patch* = Flicken; englisch: *to clamp* = festklemmen) (Hamill et al. 1981) an monozytären U937-Zellen durch. Im Einklang mit den Erkenntnissen aus den Messungen der ATP-induzierten IL-1β-Freisetzung, wurde die ATP-induzierte ionotrope Aktivität des

P2RX7 in Anwesenheit von nikotinergen Agonisten wie Nikotin (**Hecker et al. 2015**), Cholin, PC (**Richter et al. 2016**), DPPC (**Backhaus et al. 2017**) und pCRP (**Richter et al. 2018b**) gehemmt (siehe **Abb. 6**).

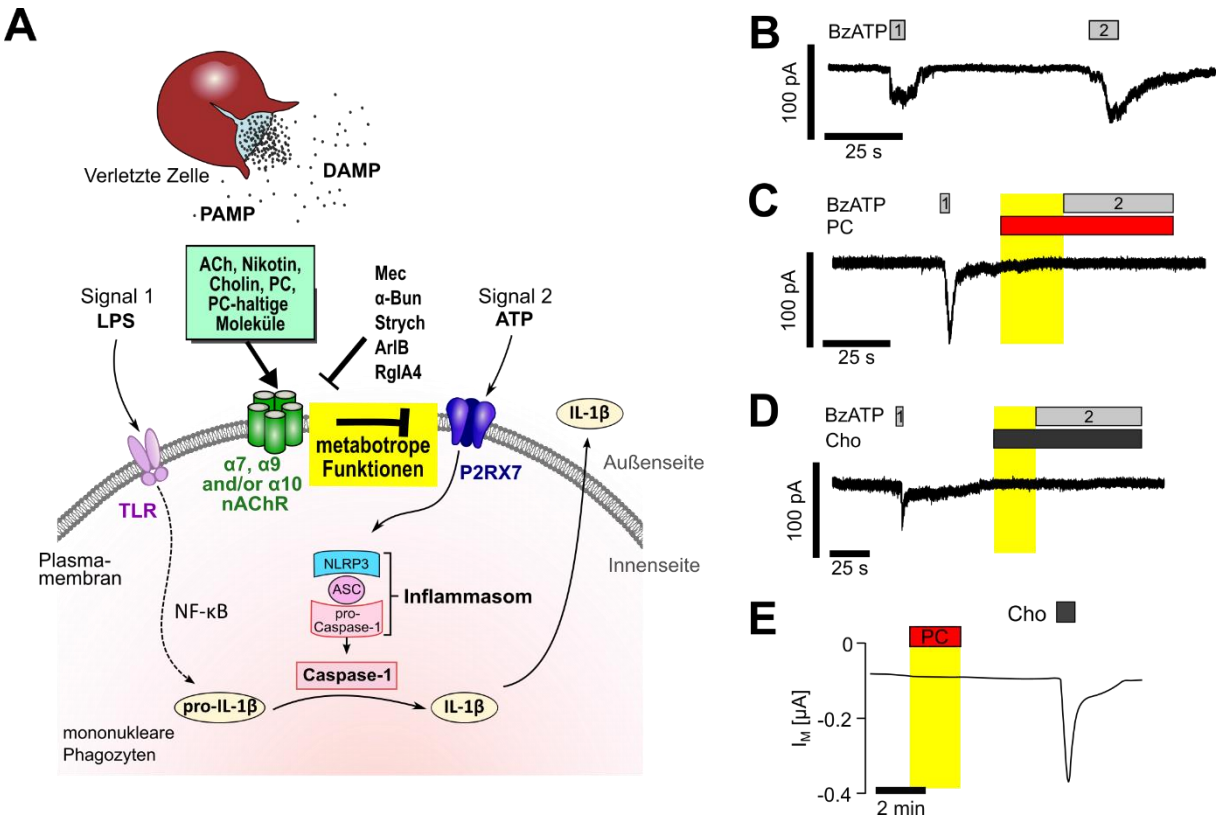

Abbildung 6. Unkonventionelle nAChRs modulieren die ATP-induzierte Freisetzung von IL-1β in mononukleären Phagozyten über metabotrope Funktionen. (A) Die Aktivierung von nAChRs mit den Untereinheiten α7, α9 und/oder α10 durch ACh, Nikotin, Cholin, PC oder PC-haltige Moleküle führt zur Inhibition des P2RX7 und somit der IL-1β-Freisetzung. **(B-D)** In elektrophysiologischen Ganzzellableitungen an monozytären U937-Zellen induzierten nikotinerge Agonisten wie PC (100 µM; **C**) und Cho (100 µM; **D**) selbst keinerlei Änderungen in den transmembranen Ionenströmen (Gelb hinterlegt), inhibierten aber effizient die BzATP-induzierte ionotrope Antwort des P2RX7. **E)** Exemplarische Stromkurve aus einer elektrophysiologischen Messung (Zwei-Elektroden Spannungsklemme) an einer *Xenopus laevis*-Oozyte, welche humane α9α10-nAChRs überexprimierte**.** PC (100 µM) induzierte im Gegensatz zu dem klassischen Agonisten Cholin (1 mM), keine Ionenströme (Gelb hinterlegt). 2'-3'-O-(4-Benzoylbenzoyl)Adenosin-5'-Triphosphat, BzATP; α-Bungarotoxin, α-Bun; Acetylcholin, ACh; Cholin, Cho; *damage-associated molecular patterns*, DAMP; Interleukin-1β, IL-1β; Lipopolysaccharid, LPS; Mecamylamin, Mec; nikotinische ACh-Rezeptoren, nAChRs; P2X7-Rezeptor, P2RX7; *pathogen-associated molecular patterns*, PAMP; Phosphocholin, PC; Strychnin, Strych; *Toll-like*-Rezeptoren, TLR. (**Hecker et al. 2015; Zakrzewicz & Richter et al. 2017; Richter et al. 2016**)

Dieser Effekt war zudem sensitiv gegenüber nAChR-Antagonisten wie Mecamylamin (**Hecker et al. 2015**; **Richter et al. 2016**) und RgIA4 (**Richter et al. 2016; Richter et al. 2018b**), wodurch die Beteiligung der nAChRs untermauert wird. Interessanterweise zeigte sich in den Experimenten jedoch auch, dass die Applikation der nikotinergen Agonisten wie PC (**Abb. 6C**) und Cholin (**Abb. 6D**) selbst keinerlei Änderungen in den transmembranen Ionenströmen der monozytären Zellen induzierte.

In weiteren Untersuchungen testeten wir die Wirkung von PC und PC-haltigen Molekülen auf die kanonische, ionotrope Funktion der neuronalen nAChRs mit den Untereinheiten α7, α9 und/oder α10. Dazu verwendeten wir das etablierte heterologe Expressionssystem der *Xenopus laevis* (*X. laevis*)-Oozyten (Wagner et al. 2000; Papke und Stokes 2010), um verschiedene Kombinationen (Homomere und Heteromere) der humanen nAChR-Untereinheiten α7, α9 und α10 in elektrophysiologischen Messungen mit der Zwei-Elektroden-Spannungsklemme (engl. *two-electrode voltage-clamp*, TEVC) auf ihre ionotrope Funktion hin zu untersuchen. Sowohl die schnell desensitisierenden α7-nAChRs als auch α9- und α9α10- nAChRs funktionieren in diesem Expressionssystem als Liganden-abhängige Ionenkanäle (Verbitsky et al. 2000; Papke und Porter Papke 2002; Papke et al. 2018). Die klassischen nAChR-Agonisten ACh und Cholin dienten in unseren Experimenten als Positivkontrolle zum Nachweis der Expression von nAChRs mit den Untereinheiten α7 und α9 (**Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Richter et al. 2018b**). Wir konnten zeigen, dass PC, G-PC, LPC und pCRP auch an den heterolog exprimierten nAChRs keine ionotropen Funktionen induzieren (**Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Richter et al. 2018b**), was uns zu der Hypothese führte, dass es sich um endogene nAChR *silent agonists* handelt. Laut Definition bringen *silent agonists* die Rezeptoren in ihren desensitisierten Zustand, ohne dabei deren Ionenkanal-Funktion zu aktivieren (Horenstein und Papke 2017). Die beobachteten Effekte von PC und PC-haltigen Molekülen könnten jedoch auch auf eine antagonisierende Wirkung zurückzuführen sein. Deshalb untersuchten wir in weiteren TEVC-Experimenten die Wirkung der Substanzen auf Cholin- und/oder ACh-induzierte Ionenströme von heterolog exprimierten nAChRs. Dabei zeigte sich, dass in Anwesenheit von PC, G-PC, LPC und pCRP die Cholin- bzw. ACh-induzierten Ionenströme inhibiert wurden (**Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Richter et al. 2018b**). Die dabei beobachtete langsame Kinetik der PC-, G-PC-, LPC

und pCRP-induzierten Inhibition sowie der langsame Anstieg der Cholin- und/oder ACh-induzierten Ionenströme nach dem Auswaschen der Substanzen, deutet jedoch auf eine Desensitisierung der Rezeptoren durch *silent agonists* hin (**Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Richter et al. 2018b**). Zudem sprechen die Ergebnisse aus den Arbeiten zur ATP-induzierten IL-1β-Freisetzung, die zeigen, dass die Effekte von PC und PC-haltigen Molekülen durch nAChR-Antagonisten aufgehoben werden können, für eine agonistische Wirkung.

Zusammenfassend konnten wir zeigen, dass unkonventionelle nAChRs in mononukleären Phagozyten einen metabotropen Signalweg induzieren, um die Aktivität des P2RX7 und somit die ATP-induzierte Freisetzung von IL-1β zu inhibieren. Zudem konnten Hinweise erbracht werden, dass PC und PC-haltige Moleküle Eigenschaften von *silent agonists* besitzen, da sie auch an kanonischen nAChRs keine Ionenströme induzieren.

3.7 Unkonventionelle nAChRs aktivieren die endotheliale NO-Synthase, um die Aktivität des ATP-sensitiven P2RX7 zu regulieren

Der Antwort auf die Frage, wie nAChRs in mononukleären Phagozyten über metabotrope Signale-vermittelt die Aktivität des P2RX7 modulieren können, konnten wir in einer weiteren Studie näherkommen. Wir stellten die Hypothese auf, dass NO-Synthasen (NOS) an dem cholinergen Kontrollmechanismus beteiligt sein könnten und durch Nitrosylierung die Aktivität des P2RX7 inhibieren (siehe **Abb. 7**). In Experimenten an diversen humanen mononukleären Phagozyten wurde die inhibierende Wirkung nikotinerger Agonisten auf die ATP-induzierte Freisetzung von IL-1β in Anwesenheit verschiedener NOS-Inhibitoren wie L-NIO (Boucher et al. 1999) und Nw-Nitro-L-Argininmethylesterhydrochlorid (Zhang et al. 1997) abgeschwächt (**Richter et al. 2023a**).

Es gibt drei Formen von NOS: die neuronale NOS (nNOS, *NOS1*), induzierbare NOS (iNOS, *NOS2*) und die endotheliale NOS (eNOS, *NOS3*) (Förstermann und Sessa 2012). Während die Funktion der nNOS und eNOS auf Proteinebene reguliert wird, ist die iNOS konstitutiv aktiv und wird auf mRNA-Ebene reguliert (Förstermann und Sessa 2012).

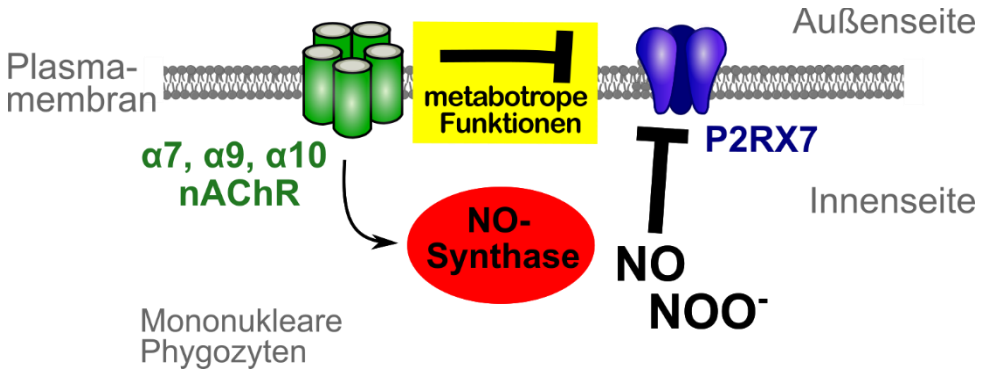

Abbildung 7. Schematische Übersicht des hypothetischen, metabotropen Signalmechanismus. Wir konnten Hinweise darauf erbringen, dass in mononukleären Phagozyten die Aktivierung nikotinischer Acetylcholinrezeptoren (nAChR) mit den Untereinheiten α7, α9 und/oder α10 einen metabotropen Signalweg induziert. Dieser beinhaltet die Aktivierung von NO-Synthasen, welche NO und/oder Peroxynitrit (NOO^-) produzieren. NO/NOO^- inhibieren die ionotrope Funktion des ATP-sensitiven P2RX7. (**Richter et al. 2023b [VIII]**)

Da der von uns identifizierte cholinerge Regulationsmechanismus sehr schnell abläuft und die nAChR-Agonisten innerhalb weniger Minuten die Inhibition der ATP-abhängigen IL-1β-Freisetzung induzieren, war die Beteiligung der iNOS unwahrscheinlich. Die Expression der nNOS konnte, im Gegensatz zur eNOS, von uns in real-time RT-PCR-Experimenten an U937-Zellen nicht detektiert werden (**Richter et al. 2023a**). In Endothelzellen wurde zudem bereits beschrieben, dass Nikotin die eNOS über Phosphorylierung aktivieren kann und die NO-Produktion anregt (Wu et al. 2009). Daher konzentrierten sich unsere weiteren Untersuchungen auf die eNOS. Mittels siRNA-Experimenten an monozytären U937-Zellen zum *silencing* der *NOS3* sowie Untersuchungen an PBMCs von Mäusen, die Gen-defizient für die eNOS (*Nos3*) waren, konnte die eNOS als wichtiges Element im Signalweg down-stream der nAChRs identifiziert werden (**Richter et al. 2023a**). Um die NOS-Aktivität zu imitieren, wurden die NO/Peroxynitrit-Donoren S-Nitroso-N-Acetyl-DL-Penicillamin (SNAP) und SIN-1 (Dasgupta et al. 2018) eingesetzt. In *in vitro*-Experimenten an monozytären U937-Zellen, THP-1-Zellen und THP-1-M1-Makrophagen, sowie *ex vivo*-Experimenten an primären humanen Monozyten, Maus-PBMCs und aus dem Maus-

Knochenmark-generierten Makrophagen wurde die ATP-induzierte IL-1β-Freisetzung durch SNAP und SIN-1 gehemmt (**Richter et al. 2023a**).

Um eine mögliche Modifikation der Ionenkanalfunktion des P2RX7 durch NO näher zu untersuchen, führten wir intrazelluläre Ca^{2+}-Messungen sowie elektrophysiologische Untersuchungen durch wie Ganzzellableitungen mit der *patch-clamp*-Methode an HEK293-Zellen, welche stabil den humanen P2RX7 überexprimieren (zur Verfügung gestellt von Prof. G. Schmalzing, RWTH Aachen). Übereinstimmend mit unserer Hypothese, wurde die ionotrope Aktivität des P2RX7 durch den NO-Donor SIN-1 gehemmt (**Richter et al. 2023a**). In Kooperation mit Prof. G. Schmalzing führten wir Mutagenese-Experimente am humanen P2RX7 durch und mutierten in der Cystein-reichen Unterdomäne der zytoplasmatischen C-terminalen Domäne die Cysteine C377 und C388 zu Alanin (Mutanten C377A, C388A). Anschließend wurden die C377A- oder die C388A-Mutante stabil in HEK293-Zelle exprimiert und elektrophysiologische Ganzzellableitungen durchgeführt (**Richter et al. 2023a**). Das C377 ist das letzte Cystein in der Cystein-reichen Domäne des P2RX7 (Becker et al. 2008; Costa-Junior et al. 2011), wohingegen C388 außerhalb dieser Domäne liegt, aber näher an der Grenzfläche der P2RX7-Kanalpore (**Richter et al. 2023a**). Keine der Mutationen an sich führte zu Einschränkungen der ionotropen Funktion des P2RX7 (**Richter et al. 2023a**). In den C377A-Mutanten verlor SIN-1 jedoch seine inhibierende Wirkung (**Richter et al. 2023a**). Gleiches zeigte sich teils in den C388A-Mutanten, wobei die Ionenströme in Anwesenheit von ATP und SIN-1 sehr variabel waren und von einer Verdopplung der Ionenströme bis zum vollständigen Ausbleiben der inhibitorischen Wirkung von SIN-1 variierten (**Richter et al. 2023a**). Für diese variablen Ergebnisse in den C388A-Mutanten konnte keine Erklärung gefunden werden.

Zusammenfassend konnten wir zeigen, dass die eNOS an dem cholinergen Regulationsmechanismus beteiligt ist. Zudem konnten wir zahlreiche Hinweise erbringen, dass das C377 in der C-terminalen Domäne (sowie ggf. C388) eine potenzielle Angriffsstelle für NO ist und NO-abhängige Modifikationen in dieser Cystein-reichen Domäne die ionotrope Funktion des P2RX7 zu inhibieren.

3.8 Liganden von α9-nAChRs: Werkzeuge der Grundlagenforschung und potenzielle Therapeutika*

Wie unter **Kapitel 3.6 und 3.7** beschrieben, konnten wir in unseren Forschungsarbeiten endogene nAChR-Agonisten identifizieren, die über metabotrope Funktionen unkonventioneller nAChRs die ATP-induzierte IL-1β-Freisetzung aus mononukleären Phagozyten inhibieren. Vielmehr noch rückte die nAChR-Untereinheit α9 durch unsere Arbeiten immer mehr in den Vordergrund (vgl. **Tabelle 1**). Zudem induzierten PC und PC-haltige Moleküle keine ionotropen Effekte an kanonischen α7* und α9* nAChRs (**Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Richter et al. 2018b**), was sie laut Definition der Wirkung von nAChR-*silent agonists* (Horenstein und Papke 2017) ähneln lässt.

Wir verglichen in einer Arbeit den endogenen nAChR-Agonisten PC mit dem *silent agonist p*CF3-N,N-Diethyl-N-Phenyl-Piperazin (*p*CF3-diEPP) (Quadri et al. 2018) bezüglich ihrer anti-inflammatorischen Wirkung sowie der Effekte auf kanonische nAChRs (**Richter et al. 2022**). In Experimenten an mononukleären Phagozyten zeigte sich *p*CF3-diEPP als potenter Inhibitor der LPS- und ATP-induzierten Freisetzung von pro-inflammatorischen Zytokinen wie IL-1β (**Richter et al. 2022**). So wurde die ATP-induzierte Freisetzung von IL-1β aus humanen PBMCs durch *p*CF3-diEPP mit einem IC_{50}-Wert von 64 fM inhibiert (**Richter et al. 2022**). Interessanterweise beruhte die inhibierende Wirkung von *p*CF3-diEPP wie von PC auf den nAChR-Untereinheiten α7 und α9*. Zudem zeigte sich in TEVC-Messungen an in *X. laevis*-Oozyten heterolog exprimierten humanen nAChRs erstmalig, dass der ursprünglich als α7-spezifische *silent agonist p*CF3-diEPP ein partieller Agonist von homomeren α9- und heteromeren α9α10-nAChRs ist (**Richter et al. 2022**). *p*CF3-diEPP hatte zudem bessere pharmakologische Eigenschaften, wohingegen PC schnell seine inhibierende Wirkung verlor und auch keinen Einfluss auf die LPS-induzierte Zytokin-Freisetzung von IL-6 und TNF-α hatte (**Richter et al. 2022**).

Wir untersuchten in einer weiteren Arbeit zwei spezifische α9*-nAChRs Liganden, den α9-nAChR-Agonist *p*CN-diEPP und den α9-nAChR-Antagonist *m*CN-diEPP (Papke et al. 2022a) auf ihre anti-inflammatorische sowie potenziell analgetische Funktion (**Richter et al. 2023b**). *p*CN-diEPP erwies sich dabei als potenter Inhibitor der ATP-induzierten Freisetzung von IL-1β aus monozytären Zellen. Eine Ko-Applikation von

*p*CN-diEPP zusammen mit PC oder ACh führte interessanterweise zu einer Abschwächung dieser inhibierenden Wirkung (**Richter et al. 2023b**), ein Phänomen, das bis jetzt nicht aufgeklärt werden konnte. *m*CN-diEPP antagonisierte die inhibierende Wirkung von PC, ACh und *p*CN-diEPP und zeigte allein appliziert keinen Effekt auf die ATP-induzierte IL-1β-Freisetzung (**Richter et al. 2023b**).

Ein etabliertes Tiermodell für Untersuchungen von inflammatorischem Schmerz ist die Injektion von *complete Freund's adjuvant* (CFA) in die Hinterpfoten von Tieren (Khan et al. 2013). Im CFA-Tiermodell für inflammatorischen Schmerz zeigte *p*CN-diEPP einen effektiven, analgetischen Effekt (**Richter et al. 2023b**). Dieser analgetische Effekt war zudem unabhängig von der α7-Untereinheit, wie in Experimenten an α7-nAChR Gen-defizienten Mäusen gezeigt werden konnte (**Richter et al. 2023b**). Der α9-nAChR-Antagonist *m*CN-diEPP wirkte im Tiermodell ebenfalls schmerzlindernd, wenn auch nur partiell und in hohen Konzentrationen (**Richter et al. 2023b**).

Zusammenfassend trugen unsere Forschungsarbeiten dazu bei, dass neue, spezifische Liganden von α9*-nAChRs identifiziert werden konnten, die in der Grundlagenforschung nun als Werkzeuge zur Verfügung stehen, um die Funktion und Bedeutung der α9*-nAChRs weiter aufzuklären. Zudem zeigen Agonisten der α9*-nAChRs eine vielversprechende anti-inflammatorische Wirkungen auf die ATP-induzierte Freisetzung von IL-1β und sowie effektive, analgetische Effekte im Schmerzmodel.

3.9 Unkonventionelle versus kanonische nAChRs

Wie in den vorherigen Kapiteln beschrieben, konnten starke Hinweise darauf erbracht werden, dass die nAChR-Untereinheiten α7, α9 und α10 für die cholinerge Regulation der ATP-induzierten IL-1β-Freisetzung durch Agonisten wie ACh, Cholin, Nikotin, PC und PC-haltige Moleküle benötigt werden, teils auch in unterschiedlichen Kombinationen der Untereinheiten (vgl. **Tabelle 1**). Wir versuchten das gleichzeitige Vorkommen der humanen nAChR-Untereinheiten α7, α9 und α10 im heterologen Expressionssystem der *X. laevis*-Oozyten zu imitieren (**Richter et al. 2023b**). Die Oozyten wurden dazu mit RNA injiziert für α7-Homomere, α9α10-Heteromere, oder α7 in Kombination mit unterschiedlichen RNA Verhältnissen für die α9- und α10-Untereinheiten (1:2, 1:3, 1:4). Dabei ergaben sich neue Mysterien bezüglich der Sensitivität der verschiedenen nAChR-Untereinheiten-Kombinationen auf

verschiedene Liganden (vgl. **Tabelle 2**). Wurde α7 zusammen mit einem hohen Anteil von α9α10 exprimiert ((α7)1:(α9α10)4), so ähnelten die ACh-induzierten Stromverläufe denen von heteromeren α9α10-nAChRs (**Richter et al. 2023b**). Durch die Ko-Expression in niedrigeren Verhältnissen ((α7)1:(α9α10)2 und (α7)1:(α9α10)3), kam es zu einer im Vergleich zu α7-Homomeren und heteromeren α9α10-nAChRs niedrigeren ACh-induzierten Stromantwort (**Richter et al. 2023b**).

Nikotin ist als Agonist an α7-Homomeren, sowie Antagonist an α9-Homomeren beschrieben (Verbitsky et al. 2000; Papke et al. 2007). Diese Ergebnisse konnten in unserer Arbeit bestätigt werden (**Richter et al. 2023b**). Interessanterweise war der IC_{50}-Wert in α9α10-nAChR exprimierenden Oozyten mit 437 ± 73 µM (**Richter et al. 2023b**) jedoch deutlich höher als bei homomeren α9-nAChRs, für die ein IC_{50}-Wert von 32 µM beschrieben wurde (Verbitsky et al. 2000). Diese Ergebnisse deuten darauf hin, dass die Anwesenheit der α10-Untereinheit die Nikotin-Sensitivität der α9-Untereinheit um ein Vielfaches reduziert. Durch die Ko-Expression aller drei Untereinheiten in niedrigeren Verhältnissen ((α7)1:(α9α10)2 und (α7)1:(α9α10)3), entstanden gemischte Populationen von Nikotin-sensitiven und -insensitiven nAChRs (**Richter et al. 2023b**). Bei einem höheren Verhältnis ((α7)1:(α9α10)4) waren keine Nikotin-induzierten Ionenströme detektierbar.

Der α9α10-nAChR-Agonist *p*CN-diEPP induzierte an α7-nAChRs nur geringe Ionenströme, welche mit steigendem Verhältnis der α9α10-Ko-Expression anstiegen und im hohen Verhältnis ((α7)1:(α9α10)4) identisch zu den Ionenströmen der heteromeren α9α10-nAChRs waren (**Richter et al. 2023b**). Während *m*CN-diEPP (partieller α7-Antagonist, sowie Antagonist von α9α10-nAChRs) die ACh-induzierten Ionenströme von α9α10-nAChR exprimierenden Oozyten vollständig inhibierte, wurde in allen α7-ko-exprimierenden Oozyten, unabhängig vom RNA-Injektions-Verhältnis, diese antagonistische Funktion deutlich reduziert (**Richter et al. 2023b**). Diese Ergebnisse deuten zum einen darauf hin, dass heteromere α7α9α10-nAChRs in den Oozyten exprimiert werden. Zum anderen scheint die Anwesenheit der α7-Untereinheit die α9α10-Untereinheinheiten vor dem Antagonisten *m*CN-diEPP zu schützen.

Tabelle 2: Liganden von nAChRs, deren Wirkung auf die ATP-induzierten Freisetzung von IL-1β aus mononukleären Phagozyten, sowie auf die ionotrope Funktion von heterolog exprimierte humanen nAChR-Untereinheiten (homomere α7, α9 sowie heteromere α9/α10).

Ligand	Effekt auf ATP-induzierte IL-1β Freisetzung	Effekt auf heterolog exprimierte nAChR-Untereinheiten	Effekt auf ACh- oder Cholin-induzierte Ionenströme	*Rebound*-Effekt
ACh	Inhibition [I],[II]	Agonist von α7, α9 und α9α10 [I].	-	-
Cholin	Inhibition [I],[II]	Agonist von α7, α9 und α9α10 [II].	-	-
Nikotin	Inhibition [I]	α7-Agonist; α9α10-Antagonist [VII].	Inhibiert reversibel ACh-induzierte Ionenströme von α9α10-nAChRs mit einer IC_{50} von 437 ± 73 µM [VII].	auf α9α10-nAChRs [VII]
PC	Inhibition [I],[II],[VI]	Keine ionotropen Effekte auf α7-, α7α9-, α7α9α10-, α9α10-nAChRs [I],[II],[VI].	Inhibiert reversibel Cholin-induzierte Ionenströme von α9α10-nAChRs [II]. Effekte auf ACh-induzierte Ionenströme von α9α10- deutlich geringer und kein Effekt auf α7-nAChRs [VI].	auf α9- und α9α10-nAChRs [VI]
G-PC	Inhibition [I]	Keine ionotropen Effekte auf α7-, α7α9-, α7α9α10-, α9α10-nAChRs [I].	Inhibiert reversibel ACh-induzierte Ionenströme von α7- und α9α10-nAChRs [I]	?
LPC	Inhibition [I]	Keine ionotropen Effekte auf α7-, α7α9-, α7α9α10-, α9α10-nAChRs [I].	Inhibiert reversibel ACh-induzierte Ionenströme von α7- und α9α10-nAChRs [I].	?
pCRP	Inhibition [III]	Keine ionotropen Effekte auf α7-, α7α9-, α7α9α10-, α9α10-nAChRs [III].	Inhibiert reversibel ACh-induzierte Ionenströme von α9α10-nAChRs [III].	?
***p*CF3-diEPP**	Inhibition [VI]	*Silent Agonist* für α7-nAChRs [VI]. Keine ionotropen Effekte auf α9- und α9α10-nAChRs [VI].	Inhibiert mit 30 µM reversibel ACh-induzierte Ionenströme von α7-, α9- und α9α10-nAChRs [VI]. Bei 1 µM kein Effekt auf α7 nAChRs [VI].	auf α9α10-nAChRs [VI]
***p*CN-diEPP**	Inhibition. Zudem Antagonisierung der Wirkung von ACh und PC [VII].	*Silent Agonist* für α7-, sowie Agonist an α9α10-nAChRs [VII].	?	?
***m*CN-diEPP**	Keine, aber antagonisiert die Wirkung von ACh, PC und *p*CN-diEPP [VII].	Partieller α7-Antagonist, sowie Antagonist von α9α10-nAChRs [VII].	Inhibiert reversibel ACh-induzierte Ionenströme von α7- und α9α10-nAChRs [VII].	?
MLA	?	selektiver Antagonist der α7 nAChRs [VII].	Inhibiert reversibel ACh-induzierte Ionenströme von α7, α9 und α9α10 nAChRs [VII].	auf α9α10 nAChRs [VII]

Acetylcholin, ACh; Dipalmitoylphosphatidylcholin, DPPC; Glycerophosphocholin, G-PC; Lysophosphatidylcholin, LPC; 4-(3-Cyanophenyl)-1,1-Diethylpiperazin-1-ium, *m*CN-diEPP; Methyllycaconitin, MLA; nikotinische ACh-Rezeptoren, nAChRs; Phosphocholin, PC; 4-(4-Cyanophenyl)-1,1-Diethylpiperazin-1-ium, *p*CN-diEPP; pentameres C-reaktives Protein, pCRP; nicht getestet, ?. [I] Zakrzewicz & Richter et al. 2017; [II] Richter et al. 2016; [III] Richter et al. 2018b; [VI] Richter et al. 2022; [VII] Richter et al. 2023b.

In den zuvor beschriebenen Untersuchungen trat noch ein weiteres Phänomen auf, der sogenannte *rebound*-Effekt (**Tabelle 2**). Wurde die Wirkung von Liganden wie *p*CF3-diEPP, PC (**Richter et al. 2022**), Nikotin (**Richter et al. 2023b**) oder Methyllycaconitin (MLA) auf die ACh-induzierten Ionenströme im heterologen Expressionssystem untersucht, so kam es nach dem Auswaschen der Liganden zu einer Potenzierung der ACh-induzierten Ionenströme (**Richter et al. 2022**; **Richter et al. 2023b**) (**Tabelle 2**). Dieser *rebound*-Effekt war am stärksten in α9α10-exprimierenden Oozyten detektierbar und schwach bis gar nicht in homomeren α7- und α9-nAChRs (**Tabelle 2**).

Zusammenfassend erbrachten wir Hinweise darauf, dass es auch im heterologen Expressionsmodell der *X. laevis*-Oozyten zur Formierung von α7α9α10-nAChRs kommt. Die An- und Abwesenheit einer oder mehrerer Untereinheiten in nAChR-Heteromeren kann zudem einen maßgeblichen Einfluss auf die Wirkung von Liganden haben.

4. Diskussion

4.1 Besonderheiten der cholinergen Regulation der ATP-vermittelten Freisetzung des pro-inflammatorischen Zytokins IL-1β

In der vorliegenden Arbeit werden die Grundzüge eines neuartigen cholinergen Kontrollmechanismus der ATP-vermittelten Freisetzung von IL-1β beschrieben, der über nAChRs vermittelt wird. Die Aktivierung der nAChRs mit den aus evolutionärer Sicht hoch-konservierten nAChR-Untereinheiten α7, α9 und/oder α10 führt über metabotrope Signalwege zur Inhibition der ionotropen Funktion des ATP-sensitiven P2RX7. Damit wird die Bildung des NLRP3-Inflammasoms, die Aktivierung der Caspase-1 sowie die Reifung und Ausschüttung von IL-1β deutlich reduziert. Zahlreiche Befunde der vorliegenden Arbeit stehen im deutlichen Gegensatz zum bisherigen wissenschaftlichen Bild, das man sich von anti-inflammatorischen cholinergen Mechanismen sowie von der Struktur, der Funktion und von den nAChR-Liganden gemacht hat. Schon allein die Kombination der nAChR-Untereinheiten α7, α9 und/oder α10 ist ungewöhnlich. Auch zur Regulation der ionotropen Funktion des P2RX7 erbrachte die vorliegende Arbeit unerwartete Erkenntnisse. Diese ungewöhnlichen Aspekte sollen im Folgenden diskutiert werden.

4.2 Die ATP-vermittelte IL-1β-Ausschüttung wird durch die Aktivierung von nAChRs reguliert.

Bereits im Jahre 2000 konnten Hinweise erbracht werden, dass nAChRs das Immunsystem modulieren und regulieren können (Borovikova et al. 2000). Der Fokus einer Vielzahl der Publikationen lag dabei auf nAChR-vermittelten Effekten auf der Expressionsebene von Zytokinen, der Zellproliferation oder Migration (Borovikova et al. 2000; Bruchfeld et al. 2010; Beckmann und Lips 2013; Hoover 2017). Erste Hinweise auf eine cholinerge Regulation der Schaden- (ATP-) vermittelten Effekte in Immunzellen konnten 2009 in einer Studie von Hecker et al. in der Sektion Experimentelle Chirurgie, JLU Gießen, erbracht werden (Hecker et al. 2009). Bei Untersuchungen zur non-neuronalen ACh-Synthese in Nierentransplantaten, konnte zum einen die Expression von nAChRs in intravaskulären Leukozyten nachgewiesen werden (Hecker et al. 2009). Außerdem zeigte sich, dass intravaskuläre Leukozyten

aus allogenen Transplantaten eine 6-fach höhere ACh-Konzentration aufwiesen als entsprechende Leukozyten aus Isotransplantaten (Hecker et al. 2009). Interessanterweise wurden auch Hinweise auf einen auto-/parakrinen cholinergen Regulationsmechanismus erbracht, der ATP-vermittelte Effekte während einer akuten Abstoßungsreaktion unterdrückt (Hecker et al. 2009).

In der vorliegenden Arbeit wurde klar gezeigt, dass die Stimulation von nAChRs mit den Untereinheiten α7, α9 und/oder α10 in mononukleären und epithelialen Zellen zu einer Inhibition der ATP-vermittelten IL-1β-Ausschüttung führt (**Hecker et al. 2015**; **Richter et al. 2016; Zakrzewicz & Richter et al. 2017;** vgl. **Kapitel 3.1**). Im Gegensatz zu den bisher bekannten Mechanismen der anti-inflammatorischen Wirkung von nAChR-Agonisten, handelt es sich hier um die Regulation der posttranslationalen Reifung von IL-1β und dessen Ausschüttung. Zudem werden die ATP-unabhängigen Mechanismen zur Zytokin-Freisetzung nicht beeinflusst (**Hecker et al. 2015**). Aus therapeutischer Sicht könnte somit bei Trauma-Patienten spezifisch die Schaden-vermittelte, sterile Inflammation gegenreguliert werden, ohne dabei die Infektabwehr von Pathogenen zu unterdrücken und das Entstehen einer Sepsis zu fördern.

4.3 Unkonventionelle nAChR-Liganden regulieren die ATP-vermittelte IL-1β-Ausschüttung.

Neben klassischen nAChR-Agonisten wurden in der vorliegenden Arbeit diverse unkonventionelle nAChR-Liganden identifiziert, welche den cholinergen Regulationsmechanismus der ATP-vermittelte IL-1β-Ausschüttung aus mononukleären Phagozyten modulieren können. Diese unkonventionellen nAChR-Liganden sollen im Folgenden diskutiert werden.

Metabolite von Phosphatidylcholinen (Lecithine) konnten in unseren Arbeiten als endogene Agonisten der nAChRs mit den Untereinheiten α7, α9 und/oder α10 identifiziert werden. Dazu gehören PC, G-PC, LPC, DPPC sowie Makromoleküle die kovalent oder nicht kovalent mit PC assoziiert sind (PC-LOS, pCRP) (**Hecker et al. 2015**; **Richter et al. 2016; Zakrzewicz & Richter et al. 2017; Backhaus et al. 2017; Richter et al. 2018a; Richter et al. 2018b**). Phosphatidylcholine bestehen aus einer PC-Kopfgruppe und zwei nicht-polaren Fettsäureschwänzen (Podo et al. 2007; Li und Vance 2008). Auch der *surfactant* (*surface active agent*), d.h. die lipid- und proteinhaltige oberflächenaktive Schicht in der menschlichen Lunge, besteht

hauptsächlich aus Phospholipiden wie Phosphatidylcholinen mit **DPPC** als einer der Hauptkomponenten (Li und Vance 2008).

Werden von Phosphatidylcholinen über Phospholipasen enzymatisch eine der beiden Fettsäureschwänze entfernt, führt dies zur Bildung von **LPC**, welches durch Lysophospholipasen zu **G-PC** weiter metabolisiert werden kann (Podo et al. 2007). **PC** ist ein Stoffwechselintermediat bei der Synthese von Phosphatidylcholin (Li und Vance 2008). Dabei wird über die Nahrung aufgenommenes Cholin über Cholin-Transporter in die Zellen aufgenommen und unter enzymatischer Beteiligung der Cholinkinase zunächst zu PC phosphoryliert (Li und Vance 2008). In einem zweiten Schritt kann PC dann über die PC:Cytidylyltransferase weiter zu Phosphatidylcholin synthetisiert (Li und Vance 2008) oder beispielsweise in Hepatozyten zu Betain oxidiert werden (Pritchard und Vance 1981).

Für Metabolite von Phosphatidylcholinen wurde bereits eine Vielzahl von immunregulatorischen Funktionen im angeborenen und erworbenen Immunsystem gezeigt (Kabarowski et al. 2002; Wang und Wu 2011; Carneiro et al. 2013; Liu et al. 2020). Fehlregulationen der Phosphatidylcholin-Synthesewege können hingegen zur Tumorpathogenese beitragen. So konnte in den letzten Jahrzehnten in einer Vielzahl von Studien ein Zusammenhang von ehrhöhten Konzentrationen von freiem Cholin, PC und G-PC mit Krebszellwachstum aufgezeigt werden (Saito et al. 2022). Zudem wird ein Zusammenhang mit den Phosphatidylcholin-Metaboliten und der Resistenz gegen Krebstherapien hypothetisiert, was u.a. auf deren immunregulatorischen Funktionen zurückgeführt wird (Saito et al. 2022). Wir sind jedoch die ersten, die einen cholinergen Mechanismus für PC, G-PC, LPC und DPPC beschreiben konnten (**Hecker et al. 2015**; **Richter et al. 2016; Zakrzewicz & Richter et al. 2017; Backhaus et al. 2017;** vgl. **Kapitel 3.2**). Die von uns eingesetzten Konzentrationen entsprechen physiologischen Konzentrationen, die im humanen Plasma vorkommen können (Ilcol et al. 2002; Drobnik et al. 2003; Ilcol et al. 2005; Quehenberger et al. 2010). Es zeigten sich zudem in Abhängigkeit des verwendeten PC-haltigen Moleküls Unterschiede im Bedarf der nAChR-Untereinheiten (vgl. **Tabelle 1**). Anhand der erbrachten Ergebnisse scheint die nAChR-Untereinheit α9 unerlässlich für die cholinerge Regulation der Freisetzung von IL-1β aus mononukleären Phagozyten zu sein (**Hecker et al. 2015**; **Richter et al. 2016; Zakrzewicz & Richter et al. 2017; Backhaus et al. 2017**).

Während PC eine essenzielle Rolle für eukaryotische Zellmembranen spielt, gibt es zudem diverse **PC-Modifikationen** von Oberflächenmolekülen (Proteine, Glykolipide) in Pflanzen (Bobenchik et al. 2011) und Pilzen (Park et al. 1997). Auch zahlreiche Bakterien wie *H. influenzae*, *Streptococcus pneumoniae* (*S. pneumoniae*) oder *Neisseria meningitidis* (*N. meningitidis*) und eukaryotische Parasiten wie Helminthen exprimieren PC-modifizierte Zellmembran-Komponenten und können aktiv PC-modifizierte Makromoleküle sezernieren (Fischer 2000; Grabitzki und Lochnit 2009; Clark und Weiser 2013; Young et al. 2013; Zhang et al. 2023a). Diese PC-modifizierten Makromoleküle modulieren das Immunsystem des Wirtes und ermöglichen dadurch die Besiedlung und das Überleben im Wirt (Zhang et al. 2023a). So wird beispielsweise die Produktion pro-inflammatorischer Zytokine von Makrophagen und Dendritischen Zellen (IL-6, IL-12, TNF-α) unterdrückt, die Aktivierung von T- und B-Zellen abgeschwächt, die Freisetzung von IgG2a reduziert und gleichzeitig die Immunreaktionen vom Th2-Typ gefördert (Grabitzki und Lochnit 2009). Bisher war unbekannt, über welche Rezeptoren die anti-inflammatorischen Wirkungen PC-modifizierter Makromoleküle wie PC-LOS vermittelt werden. Unsere Arbeiten haben gezeigt, dass diese Substanzen über nAChRs mit den Untereinheiten α7, α9 und/oder α10 wirken können und die ATP-vermittelte IL-1β-Ausschüttung effizient inhibieren (**Hecker et al. 2015**; **Richter et al. 2018a;** vgl. **Kapitel 3.2 und 3.3**). Wir können jedoch keine Aussage darüber treffen, ob PC-LOS noch weitere Mechanismen auslösen können. Da Pathogene diesen anti-inflammatorischen cholinergen Mechanismus zum Umgehen der Immunabwehr des Wirtes ausnützen könnten, ergeben sich aus diesen Erkenntnissen jedoch neue therapeutische Ansätze: In Patienten mit chronisch obstruktiver Lungenerkrankung (COPD, engl. *chronic obstructive pulmonary disease*) kommt es z.B. aufgrund der reduzierten *mucociliary clearance* häufig zu Exazerbationen, die in 50% der Fällen durch Pathogene wie *H. influenza* verursacht werden (Lugade et al. 2014; Simpson et al. 2016). Zur Behandlung der Exazerbationen werden dann neben Corticosteroiden und Bronchodilatatoren hauptsächlich Antibiotika verabreicht, wodurch Antibiotikaresistenzen von *H. influenzae* begünstigt werden. Die erste Antibiotikaresistenz gegen Ampicillin und Amoxicillin wurde bereits 1972 entdeckt (Lugade et al. 2014). Anhand der von uns gewonnenen Erkenntnisse könnte eine Kurzzeitbehandlung von COPD-Patienten mit nAChR-Antagonisten dazu führen, dass

das wirtseigene Immunsystem aktiviert und somit die Infektion unter Kontrolle gebracht wird.

In unseren Arbeiten ergaben sich zudem weitere Erkenntnisse bezüglich der Sensitivitäten von nAChRs gegenüber PC, PC-haltigen Molekülen und PC-modifizierten Makromolekülen. Im Vergleich zu freiem PC, war die IC_{50}-Konzentration von G-PC und LPC um eine Größenordnung niedriger (vgl. **Tabelle 1; Hecker et al. 2015; Zakrzewicz & Richter et al. 2017**). Die Modifizierung von PC mit einem großen Molekül wie BSA führte sogar zu einer Potenzierung des inhibierenden Effekts um den Faktor 10 (vgl. **Tabelle 1; Hecker et al. 2015**). Die niedrigsten IC_{50}-Konzentrationen zeigten sich mit ~25 nM in den Untersuchungen mit PC-LOS (vgl. **Tabelle 1**; **Hecker et al. 2015**). Es ist denkbar, dass gebundenes PC stabiler ist als freies PC, dass die zelluläre Aufnahme bzw. der Einbau in die Zellmembran so verhindert wird und dadurch die Potenzierung des inhibierenden Effekts zustande kommt. Sowohl bei PC-LOS als auch bei PC-BSA ist davon auszugehen, dass die Makromoleküle als Ganzes mit den nAChRs interagieren, da die PC-Kopfgruppen kovalent gebunden sind. Das ist aufgrund der Geometrie der Ligandenbindungsstelle an nAChRs ein erstaunlicher Befund, der im folgenden Abschnitt näher beleuchtet wird.

Wie in der Einleitung beschrieben ist IL-1β ein wichtiger Mediator der unspezifischen systemischen **Akute-Phase-Reaktion** des Körpers auf pathophysiologische Störungen seiner Homöostase (siehe **Kapitel 1.1**). Zusammen mit weiteren Mediatoren wie IL-6 und TNF-α induziert IL-1β dabei die Expression und Sekretion von sogenannten Akute-Phase-Proteinen wie CRP, α1-Antitrypsin (AAT) und dem *secretory leukocyte protease inhibitor* (SLPI) (Gabay und Kushner 1999; Douglas und Hannila 2022). Bei akuten und chronischen Entzündungsreaktionen im Organismus steigen die Konzentrationen der Akute-Phase-Proteine im Blut an und dienen als sensitive klinische Marker in der Diagnostik. So kann die Konzentration von CRP innerhalb von 48 – 72 h nach der pathophysiologischen Störung um das 1.000-fache ansteigen und Konzentrationen im Blut von über 300 µg/ml erreichen (Gabay und Kushner 1999; Pepys und Hirschfield 2003; Mantovani et al. 2008). Die Akute-Phase-Proteine können pleiotrope Effekte induzieren, von der Infektabwehr und Beseitigung der Erreger, über Schutzfunktionen gegen endogene Proteasen (welche von aktivierten Neutrophilen freigesetzt werden), bis hin zur Modulation der Entzündungsreaktionen (Richter et al. 2022).

Das Akute-Phase-Protein CRP bekam seinen Namen aufgrund seiner Fähigkeit, in Patienten im akuten Stadium der Lobärpneumonie das C-Polysaccharid der Kapsel von *S. pneumoniae* zu binden (Mold et al. 1981). Es ist zudem der erste PRR der identifiziert wurde (Garlanda et al. 2005; Mantovani et al. 2008). CRP ist ein Pentraxin, welches hauptsächlich in der Leber gebildet wird (Pepys und Hirschfield 2003; Mantovani et al. 2008). Aber auch Immunzellen wie Makrophagen scheinen die Fähigkeit zur Expression und Freisetzung von CRP zu besitzen (Kaplan et al. 2014). Im Blutstrom zirkulierendes natives CRP besteht aus 5 homomeren Untereinheiten, die sich zu einem Pentamer zusammenlagern (pCRP) (Mantovani et al. 2008). Jede Untereinheit besitzt eine Ca^{2+}-abhängige Bindungsstelle für PC oder PC-haltige Moleküle wie Phosphatidylcholine und LPC (Pepys und Hirschfield 2003; Eisenhardt et al. 2009). Auch PC-haltige Oberflächenmoleküle von *H. influenzae*, *S. pneumoniae*, und *N. meningitidis* können von CRP gebunden werden (Zhang et al. 2023a). Die Bindung von PC-haltigen Molekülen erfolgt dabei in einem 1:1 Verhältnis pro pCRP-Untereinheit (Pepys und Hirschfield 2003; Mantovani et al. 2008). Des Weiteren gibt es Ca^{2+}-abhängige Bindungen mit Chromatin, Histonen, Glykanen, Poly-L-Lysin und Poly-L-Arginin (Zhou et al. 2024). Neuere Studien zeigen, dass CRP auch als Multimer (Dekamer) und einer Fibrillen-ähnlichen Struktur vorliegen kann (Boncler et al. 2019; Noone et al. 2021; Zhou et al. 2024). Die Bildung dieser Dekamere scheint vom pH-Wert abhängig zu sein (Noone et al. 2021). Die physiologischen/pathophysiologischen Effekte dieser CRP-Multimere sind jedoch noch nicht vollständig geklärt (Zhang et al. 2023a; Zhou et al. 2024).

Es wurden bis heute zahlreiche pro- und anti-inflammatorische Funktionen von CRP beschrieben. Dabei gehen die pro-inflammatorischen Funktionen meist auf die aktivierte Form des CRP zurück, dem monomeren CRP (mCRP). mCRP entsteht, wenn pCRP an PC-Gruppen von aktivierten bzw. geschädigten Zellmembranen bindet (Potempa et al. 2021). Es kommt zu einer Konformationsänderung und Entstehung von Wasser-unlöslichem mCRP (Eisenhardt et al. 2009; Potempa et al. 2021). Das aktivierte mCRP ist dementsprechend häufig in geschädigten/entzündeten Geweben zu finden (Eisenhardt et al. 2009; Ahrens et al. 2011; Potempa et al. 2021). mCRP präsentiert die Bindungsstellen für Fc-Rezeptoren und das Komplementsystem, wodurch diverse pro-inflammatorische Effekte ausgelöst werden, wie eine erhöhte Expression pro-inflammatorischer Zytokine (Ahrens et al. 2011; Sproston und

Ashworth 2018; Potempa et al. 2021), Anlockung und Aktivierung von Monozyten sowie die Produktion von reaktiven Sauerstoffspezies (Eisenhardt et al. 2009). Für das pCRP wurden hingegen anti-inflammatorische Effekte beschrieben, wie die vermehrte Expression des anti-inflammatorischen IL-1-Rezeptor-Antagonisten sowie eine protektive Wirkung bei inflammatorischen Erkrankungen wie Sepsis (Mold et al. 2002), Arthritis (Jiang et al. 2006) und Atherosklerose (Torzewski et al. 2014; Potempa et al. 2021). CRP scheint zudem eine essenzielle, physiologische Funktion zu besitzen, da es bis heute keine Beschreibung von CRP-Gen-defizienten Säugetieren gibt (Pepys und Hirschfield 2003).

Unsere Ergebnisse zeigten deutlich, dass natives pCRP, das aus der Pleuraflüssigkeit von Patienten isoliert wurde, als nAChR-Agonist wirkt und die ATP-vermittelte Ausschüttung von IL-1β inhibiert (**Richter et al. 2018b;** vgl. **Kapitel 3.4**). pCRP-Plasmakonzentrationen von 3 – 10 µg/ml gelten aus klinischer Sicht als leicht erhöht (Kushner und Antonelli 2015). Der von uns detektierte IC_{50}-Wert von ~5 µg/ml (**Richter et al. 2018b**) würde dementsprechend einer sehr milden Entzündungsreaktion entsprechen. Hinweise darauf, dass der pCRP-induzierte cholinerge Regulationsmechanismus auch *in vivo* aktiv ist, konnten wir in einer kleinen prospektiven Studie an Polytrauma-Patienten erbringen. In Korrelationsanalysen der Plasmawerte für CRP und IL-1β zeigte sich eine negative Korrelation, was mit unserer Hypothese übereinstimmt, dass pCRP die IL-1β-Freisetzung inhibiert (**Richter et al. 2018b**). Eine solche negative Korrelation von hohen prä-operativen CRP-Werten mit post-operativem Fieber konnte auch in einer retrospektiven Studie an Lungenkrebspatientinnen aufgezeigt werden (Meyer et al. 2020). Bei männlichen Lungenkrebspatienten gab es eine solche Korrelation interessanterweise nicht (Meyer et al. 2020). Wir konnten eindeutig zeigen, dass die Wirksamkeit von pCRP von der Anwesenheit eines aktiven Liganden abhängt, der PC sein kann (**Richter et al. 2018b**). Dabei wird die Wirksamkeit von freiem PC durch die Anwesenheit von CRP potenziert. Wir wissen jedoch nicht, ob der gesamte pCRP-PC-Komplex am nAChR bindet, oder ob PC durch CRP auf den nAChR übertragen wird. In weiteren Arbeiten konnten wir neben pCRP auch die Akute-Phase-Proteine AAT und SLPI als Elemente dieser möglichen cholinergen negativen Rückkopplungsschleife identifizieren (Siebers et al. 2018; Zakrzewicz et al. 2019). Im Gegensatz zu pCRP aktivieren AAT (Siebers et al. 2018) und SLPI (Zakrzewicz et al. 2019) jedoch nicht direkt die nAChRs, sondern

induzieren die Freisetzung eines bisher nicht identifizierten nAChR-Liganden, welcher dann den cholinergen Mechanismus in mononukleären Phagozyten aktiviert. Auf Grund der Tatsache, dass CRP (Pepys und Hirschfield 2003) und die nAChR-Untereinheiten α7, α9 und α10 (Lipovsek et al. 2012; Boffi et al. 2017) evolutionär hoch-konserviert sind und zudem mononukleäre Phagozyten bereits in primitiven, vielzelligen Organismen als Immunzellen vorkommen (Buchmann 2014), könnte dieser von uns identifizierte Kontrollmechanismus demnach älter sein als das neuronale cholinerge System selbst.

Die Frage, wie eine Bindung bzw. Modifikation mit PC große Moleküle wie pCRP, PC-BSA und PC-LOS zu effektiveren nAChR-Agonisten machen kann, bleibt vorerst ungeklärt. Eine Möglichkeit wäre, dass PC in dieser Form nicht so schnell von den Zellen degradiert/metabolisiert oder in die Zellmembran integriert werden kann. Es bedarf weiterer Forschung, um diese Frage aufzuklären.

Als eine weitere besondere Gruppe wirksamer nAChR-Agonisten an mononukleären Phagozyten zeigten sich in der vorliegenden Arbeit die sogenannten ***silent agonists***, die an klassischen nAChRs keine ionotropen Funktionen auslösen, aber diese desensitisieren. Im cholinergen Forschungsbereich sind Untersuchungen zu *silent agonists* und dem damit einhergehenden desensitisierten Zustand von Rezeptoren hauptsächlich für α7-nAChRs bekannt. Es gibt eine Vielzahl von Forschungsarbeiten, die sich mit der Synthese und Suche nach neuen α7-nAChR *silent agonists* befassen (Thomsen und Mikkelsen 2012; Papke et al. 2015; Horenstein und Papke 2017; Papke et al. 2023). Die Ideen zur Synthese synthetischer nAChR-Agonisten geht dabei auf die chemischen Strukturen der klassischen Agonisten ACh und Nikotin zurück (Beers und Reich 1970). Man ging davon aus, dass effektive nikotinerge Agonisten eine geladene Stickstoffgruppe sowie eine Wasserstoffbindung-Gruppe mit einem Abstand von 5,9 Å benötigen (Beers und Reich 1970). Später zeigte sich, dass für einen effektiven Agonisten der neuronalen nAChRs nur die geladene Stickstoffgruppe in Form von Tetramethylammonium notwendig ist (Papke et al. 1996). Daraufhin wurden zahlreiche Varianten von quartären Ammoniumsalzen synthetisiert und deren Effekte auf den α7-nAChR charakterisiert (Papke et al. 2014; Papke et al. 2023). Effektive *silent agonists* können leicht mit nAChR-Antagonisten verwechselt werden, da sie bedingt durch die schnelle Desensitisierung der Rezeptoren in elektrophysiologischen Messungen keine Ionenströme induzieren (Papke et al. 2023). Um dieses Problem zu

umgehen, können bei Untersuchungen an homomeren α7-nAChRs jedoch α7-spezifische positive allosterische Modulatoren (PAMs) eingesetzt werden, wie die Typ-II PAMs PNU-120596 oder TQS (4-Naphthalen-1-yl-3a,4,5,9b-Tetrahydro-3-H-Cyclopenta[c]quinolin-8-Sulfonsäureamid) (Grønlien et al. 2007; Young et al. 2008). Diese PAMs binden innerhalb der Transmembrandomäne des α7-nAChR und ermöglichen den Übergang vom desensitisierten hin zum aktivierten, ionotropen Zustand des Rezeptors (Papke et al. 2023). Durch Ko-Applikation von α7-*silent agonists* mit PAMs können so ionotrope Funktionen detektierbar gemacht und *silent agonists* pharmakologisch charakterisiert werden (Papke 2014; Papke et al. 2023). Papke et al. konnten durch Modifikationen der quartären Ammoniumsalze, wie polare oder nicht-polare Kopfgruppen, diverse Analoge synthetisieren (Quadri et al. 2016; Quadri et al. 2017). Darunter fanden sich hoch effiziente α7-*silent agonists* wie *p*CF3-diEPP (Quadri et al. 2016; Quadri et al. 2018). Interessanterweise war in mononukleären Phagozyten der *silent agonist p*CF3-diEPP ein wirksamer nAChR-Agonist, der effizient die ATP-vermittelte Freisetzung von IL-1β inhibierte (**Richter et al. 2022;** vlg. **Kapitel 3.8**). Die inhibitorische Wirkung beruhte auf der Aktivität der nAChR-Untereinheiten α7, α9 und/oder α10 (**Richter et al. 2022**). Durch weiter Untersuchungen an humanen nAChR-Untereinheiten im heterologen Expressionssystem der *X. laevis* Oozyten konnten für *p*CF3-diEPP zudem erstmalig Funktionen als partieller α9*-nAChR-Agonist mit starker desensitisierender Wirkung auf die klassischen α9α10-nAChRs beschrieben werden (**Richter et al. 2022**). Diese Ergebnisse deuten darauf hin, dass der desensitisierte Zustand der nAChRs in mononukleären Phagozyten zur Inaktivierung des P2RX7 führen kann. Dies lässt bereits unkonventionelle Eigenschaften dieser nAChRs erahnen, was im Folgenden (siehe **Kapitel 4.4**) näher diskutiert wird.

In die Gruppe der unkonventionellen Liganden von nAChRs, die Funktionen im angeborenen Immunsystem übernehmen, reiht sich auch **$A\beta_{1-42}$** ein. Dieses wirkt jedoch im Gegensatz zu den oben beschriebenen Liganden als Antagonist der nAChRs mit den Untereinheiten α7, α9 und/oder α10. So wurde die inhibierende Wirkung von klassischen und unkonventionellen nAChR-Agonisten auf die ATP-induzierte IL-1β-Freisetzung in Anwesenheit von $A\beta_{1-42}$ aufgehoben (**Richter et al. 2020;** vgl. **Kapitel 3.5**).

Es gibt in der Literatur Hinweise darauf, dass das APP und dessen Spaltprodukte, die Aβ-Peptide, mit dem cholinergen System interagieren. So wurden zum Beispiel die α7-Untereinheiten der nAChRs als direkte Interaktionspartner der Aβ-Peptide $Aβ_{1-40}$ und $Aβ_{1-42}$ beschrieben (Nagele et al. 2002; Lee und Wang 2003; Parri und Dineley 2010; Sadigh-Eteghad et al. 2014; Lasala et al. 2019). APP ist aus evolutionärer Sicht wie die nAChRs ein hoch konserviertes Zellmembranprotein. Es wird von Geweben aller drei Keimblätter exprimiert wie dem Nervensystem, der Haut, dem Fettgewebe, der Skelettmuskulatur, dem Herz, der Niere, dem Gastrointestinaltrakt, dem Thymus, der Lunge und der Leber (Selkoe 1994; Mattson 1997; Sinha und Lieberburg 1999; Mattson 2004; Thinakaran und Koo 2008; Puig und Combs 2013). APP kann durch zwei verschiedene Wege der proteolytischen Spaltung des Membranproteins kanonisch zu seinen Spaltprodukten prozessiert werden. Dabei erfolgt der sogenannte nicht-amyloidogene Weg u.a. durch die Protease α-Sekretase, welche durch Proteolyse die Entstehung des wasserlöslichen Produkts “sAPPα“ (sezerniertes APPα) induziert (Chen et al. 2017). sAPPα werden neuroprotektive Funktionen und damit eine schützende Funktion gegen die Entstehung von Alzheimer zugesprochen (Mattson 1997). Der amyloidogene Weg ist hingegen durch die Spaltung des APP durch die Protease β-Sekretase und die γ-Sekretase Presenilin-2 (Gen: *PSEN2*) gekennzeichnet, was zur Entstehung der Aβ-Peptide wie $Aβ_{1-42}$ führt (Chen et al. 2017; Müller et al. 2017; Zhang et al. 2023b). Es gibt eine Vielzahl von Hinweisen, dass das $Aβ_{1-42}$-Peptid sich leicht zu Fibrillen formiert und dadurch zur Plaquebildung im Gehirn von Alzheimerpatienten beiträgt (Sinha und Lieberburg 1999; Thinakaran und Koo 2008; Heneka et al. 2015; Zhang et al. 2023b). Neben den nicht-amyloidogenen und amyloidogenen Wegen wurden noch weitere, unkonventionelle Mechanismen zur Prozessierung von APP identifiziert (Müller et al. 2017).

Im Gegensatz zu T-Lymphozyten, natürlichen Killerzellen (NK-Zellen) und Granulozyten, wird APP von Monozyten und Makrophagen exprimiert sowie zu Aβ-Peptiden prozessiert (Sondag und Combs 2004; Sondag und Combs 2006; Sondag und Combs 2010). Sondag et al. konnten zeigen, dass über einen Tyrosinkinase-abhängigen Signalweg die Expression von APP sowie die Prozessierung von Aβ-Peptiden hochreguliert wird (Sondag und Combs 2004; Sondag und Combs 2006; Sondag und Combs 2010). Es wurden zudem einige immunologische Effekte aufgezeigt. So kann das $Aβ_{1-42}$-Peptid sowohl die Freisetzung der Inflammasom-

abhängigen Zytokine IL-1β und IL-18 (Reale et al. 2012; François et al. 2015; Gold und El Khoury 2015) als auch der Inflammasom-unabhängigen Zytokine wie IL-6 und TNF-α (Kaplin et al. 2009) induzieren.

Eine Interaktion von α7-nAChRs mit $Aβ_{1-42}$-Peptiden wurde erstmals durch Wang et al. beschrieben (Wang et al. 2000a; Wang et al. 2000b). Später wurden α4β2, α4α5β2, und α2αβ nAChRs als Interaktionspartner für APP und Aβ-Peptide identifiziert (Wu et al. 2004; Lamb et al. 2005). Der Effekt dieser Interaktion auf die ionotrope Funktion der nAChRs wird jedoch in der Literatur kontrovers diskutiert. So wurden sowohl agonistische als auch antagonistische Effekte von Aβ-Peptiden beschrieben (Liu et al. 2001; Pettit et al. 2001; Dineley et al. 2002; Parri und Dineley 2010; Sadigh-Eteghad et al. 2014; Fabiani und Antollini 2019). Im cholinergen Regulationsmechanismus der ATP-induzierten Freisetzung von IL-1β scheint $Aβ_{1-42}$ antagonistisch an den unkonventionellen nAChRs in mononukleären Phagozyten zu wirken (**Richter et al. 2020**). Da neben den klassischen nAChR-Agonisten auch die inhibitorische Wirkung von G-PC und DPPC durch das $Aβ_{1-42}$-Peptid aufgehoben wurde, konnten wir zudem erstmals einen Hinweis zur Interaktion von $Aβ_{1-42}$-Peptiden mit α9*-nAChRs erbringen, die nicht notwendigerweise α7-Untereinheiten enthalten (**Richter et al. 2020; Tabelle 1**). In unseren Untersuchungen wurden 2,54 µM $Aβ_{1-42}$-Peptide benötigt, um einen halbmaximalen Effekt zu erzielen (**Richter et al. 2020**). Dies entspricht 10 µg/ml $Aβ_{1-42}$-Peptiden und somit aus physiologischer Sicht sehr hohen Konzentrationen. So wurden in einer Studie von Roher et al. an Alzheimerpatienten und gesunden Kontrollprobanden über einem Zeitraum von 12 Monaten fluktuierende $Aβ_{1-42}$-Peptid-Konzentrationen im Plasma detektiert mit einem Mittelwert 132 pg/ml (Roher et al. 2009). In Studien zur Ligandenbindung an α7-nAChRs wurden Interaktionen mit $Aβ_{1-42}$-Peptiden ebenfalls im picomolaren Bereich beschrieben (Wang et al. 2000a; Wang et al. 2000b). Wenn man bedenkt, dass Aβ-Peptide jedoch auch von Monozyten selbst prozessiert werden können (Sondag und Combs 2004; Sondag und Combs 2006; Sondag und Combs 2010) und somit Aβ-Peptide als parakrine und/oder autokrine Faktoren fungieren, wären lokale Konzentrationen im mikromolaren Bereich durchaus vorstellbar. Als endogener, negativer Regulator könnten $Aβ_{1-42}$-Peptide eine wichtige Rolle für die Aufrechterhaltung und/oder Wiederherstellung der Immunbalance bei traumainduzierter Immundysfunktion sein und präventiv gegen Infektionen und Sepsis sein (vgl. **Abb. 1**). Aus klinischer Sicht könnten APP und seine

Aβ-Peptide somit ein prognostischer Biomarker und Ansatzpunkt personalisierter Medizin sein: Bei großen chirurgischen Eingriffen würden hohe Plasmakonzentrationen von Aβ-Peptiden inflammatorische Komplikationen in Form von steriler Inflammation vorhersagen. Wenn sich diese Hypothese in weiteren Forschungsarbeiten bestätigt lässt, wären Prognosen zur postoperativen Immundysfunktion bereits präoperativ möglich. Aβ-zentrierte Interventionen könnten dann frühzeitig eingeleitet und inflammatorische Komplikationen entgegengewirkt werden.

Zusammenfassend konnten in der vorliegenden Arbeit diverse unkonventionelle Liganden der nAChRs mit den Untereinheiten α7, α9 und/oder α10 identifiziert werden, die in mononukleären Phagozyten effizient die Freisetzung von IL-1β modulieren können. Dabei ergaben sich zudem erste Hinweise darauf, dass es sich bei diesen nAChRs nicht um klassische, ionotrope Rezeptoren handelt, sondern um unkonventionelle nAChRs, was im Folgenden näher diskutiert wird.

4.4 Unkonventionelle nAChRs regulieren die ATP-vermittelte IL-1β-Ausschüttung.

nAChRs sind ursprünglich aus dem Nervensystem als Liganden-gesteuerte ionotrope Rezeptoren mit einer Permeabilität für Kationen wie Na^+, K^+ und Ca^{2+} bekannt, wo sie u.a. eine wichtige ionotrope Rolle bei der Reizweiterleitung spielen (Ullian et al. 1997; Katz et al. 2000; Verbitsky et al. 2000; Wessler und Kirkpatrick 2008; Changeux 2012; Stokes et al. 2015). Unsere Erkenntnisse aus den elektrophysiologischen *patch-clamp*-Messungen deuten hingegen darauf hin, dass nAChRs mit den Untereinheiten α7, α9 und/oder α10 in mononukleären Phagozyten einen unkonventionellen, metabotropen Signalweg induzieren, um den P2RX7 zu inhibieren (siehe **Abb. 6**).

In der Tat gibt es bis heute nur wenige Hinweise auf ionotrope Funktionen von nAChRs in mononukleären Phagozyten. Zudem beruhen die wenigen, zugrundeliegenden Hinweise meist auf indirekten Beobachtungen der ionotropen nAChR-Funktionen. So wurde in Mikrogliazellen von Ratten gezeigt, dass Cholin die Phagozytose-Kapazität nur in Anwesenheit von extrazellulärem Ca^{2+} induziert (Takata et al. 2010). Gleiches wurde für die Nikotin-induzierten anti-inflammatorischen Effekte in murinen Makrophagen gezeigt (Tsoyi et al. 2011). In elektrophysiologischen *patch-clamp*-Messungen an humanen aus PBMCs-differenzierten Makrophagen konnten ACh- und

Cholin-induzierte Einzelkanalströme abgeleitet werden (Báez-Pagán et al. 2015). Dabei zeigte sich eine erhöhte und verlängerte Offenwahrscheinlichkeit der Einzelkanäle in Anwesenheit des α7-nAChR PAM PNU-120596 (Báez-Pagán et al. 2015), was auf die Beteiligung von α7-nAChRs hindeutet. Ähnliche Ergebnisse zeigten sich in einer kleinen Subpopulation von aus PBMCs-differenzierten Makrophagen in elektrophysiologischen Ganzzellableitungen (Nurkhametova et al. 2020). Aus elektrophysiologischer und technischer Sicht stellt sich jedoch die Frage, wie Báez-Pagán et al. (Báez-Pagán et al. 2015) in primären Zellen Einzelkanalströme von nAChRs ableiten konnten, die von den Zellen nicht überexprimiert wurden. Offen bleibt auch die Frage, wieso in der Arbeit von Nurkhametova et al. (Nurkhametova et al. 2020) nur eine kleine Subpopulation der Makrophagen ionotrop auf nikotinerge Agonisten reagierten.

Im Einklang mit unseren Erkenntnissen zum metabotropen Signalweg von nAChRs in mononukleären Phagozyten (**Abb. 6**) stehen eine Vielzahl weiterer Publikationen, in denen ebenfalls keine Änderungen der transmembranen Ionenströme durch nikotinerge Agonisten in Immunzellen detektiert werden konnten (Peng et al. 2004; Razani-Boroujerdi et al. 2007; Hecker et al. 2009; Mikulski et al. 2010). Auch im Darmepithel konnten metabotrope Funktionen von nAChRs gezeigt werden, wobei die genaue Zusammensetzung der nAChR-Untereinheiten ungeklärt blieb (Lottig et al. 2019). Es gibt immer mehr Hinweise darauf, dass nAChRs in non-neuronalen Zellen beide Arten von Signalwegen, kanonische ionotrope und non-kanonische metabotrope, nutzen können, um ihre Effekte zu induzieren. Somit könnte jeder Signalweg auch spezifische, biologische Prozesse induzieren und/oder modulieren und zudem auch von den verschiedenen Zusammensetzungen der nAChR-Untereinheiten abhängig sein (Grando 2014; Grau et al. 2018; Kabbani und Nichols 2018; Lottig et al. 2019).

Der Antwort auf die Frage, wie nAChRs in mononukleären Phagozyten über metabotrope Signale-vermittelt die Aktivität des P2RX7 modulieren können, konnten wir in einer weiteren Studie näherkommen. Wir konnten starke Hinweise darauf erbringen, dass die cholinergen Signale die ionotrope Funktion des P2RX7 durch Proteinmodifikation inhibieren (**Richter et al. 2023a;** vgl. **Kapitel 3.7**). Aus der Literatur ist bekannt, das NO, welches durch NOS synthetisiert werden kann, die Funktion von Proteinen durch Nitrosylierung modulieren kann (Gonzalez et al. 2009; Althaus et al.

2011; Wang et al. 2021). So werden z.B. GABA-Rezeptoren und Ca^{2+}-sensitiven K^+-Ionenkanälen durch Nitrosylierung aktiviert, wohingegen NMDA-Rezeptoren und Na^+-Ionenkanälen durch Nitrosylierung inhibiert werden (Gonzalez et al. 2009; Althaus et al. 2011; Wang et al. 2021). Wir konnten zeigen, dass der von uns identifizierte cholinerge Regulationsmechanismus der ATP-induzierten IL-1β-Freisetzung die Aktivität von NOS bedarf (**Richter et al. 2023a**). Durch Experimente an eNOS Gen-defizienten Mäusen, konnte innerhalb der Familie der NOS die eNOS als wichtiges Element im Signalweg down-stream der nAChRs identifiziert werden (**Richter et al. 2023a**). Zudem konnten wir die Aktivität der NOS mittels verschiedener Peroxynitrat- und NO-Donoren imitieren und somit weitere Hinweise darauf erbringen, das nAChRs über NOS-vermittelt die ionotrope Funktion des P2RX7 modifizieren.

In der Familie der purinergen Rezeptoren zeichnet sich der P2RX7 durch eine charakteristische, große, zytoplasmatische C-terminale Domäne aus, deren Struktur erst kürzlich mittels Kryo-Elektronenmikroskopie aufgeklärt werden konnte (McCarthy et al. 2019). Diese besteht aus 3 funktionellen Unter-Domänen: 1. Eine Cystein-reiche Domäne, welche die Rezeptor-Desensitisierung verhindert; 2. Eine zytoplasmatische *cap*-Domäne, welche mit dem kurzen N-Terminus des P2RX7 interagiert und zur Bildung der Pore des Ionenkanals beiträgt; 3. eine sogenannte Ballast-Domäne, in welcher kürzlich eine Calmodulin (CaM)-Bindungsregion identifiziert wurde, die vermutlich zur intrazellulären Regulation und Stabilität der P2RX7-Ionenkanalfunktion beiträgt (McCarthy et al. 2019; Sander et al. 2022). Die C-terminale Domäne enthält mehrere Konsensussequenzen, welche die Interaktion mit anderen Proteinen ermöglichen (Becker et al. 2008; Costa-Junior et al. 2011). Zudem gibt es eine Vielzahl von Aminosäureresten, welche als Angriffsstelle für kovalente Proteinmodifikationen des P2RX7 wie Palmityelierungen dienen können (Becker et al. 2008; Costa-Junior et al. 2011; McCarthy et al. 2019). Durch Palmityelierungen bilden die Cystein-reichen Domänen des P2RX7 eine Art Anker in der Zellmembran, wodurch der Rezeptor nicht desensitisieren kann (McCarthy et al. 2019). Andere purinerge Rezeptoren wie der P2RX3 besitzen diese Cyteine-reiche Domäne hingegen nicht und zeichnen sich durch eine schnelle Rezeptor-Desensitisierung aus (McCarthy et al. 2019; Oken et al. 2022). Durch Mutageneseexperimente am humanen P2RX7, konnten wir nun erstmalig Hinweise darauf erbringen, dass das C377 in der Cystein-reichen Domäne durch NO-haltige Verbindungen modifiziert werden kann (**Richter et al. 2023a**). Da die C337A-

Mutanten vollkommen die Sensitivität gegenüber NO verloren, deutetet dies auf eine essenzielle Bedeutung dieses Cysteins für Proteinmodifikationen des P2RX7 hin (**Richter et al. 2023a**). Auch wenn es bei Experimenten an C388A-Mutanten zu gemischten Ergebnissen kam, scheinen NO-abhängige Modifikationen in der Cystein-reichen Domäne die ionotrope Funktion des P2RX7 zu inhibieren (**Richter et al. 2023a**) und somit ein essenzieller Bestandteil des metabotropen cholinergen Regulationsmechanismus zu sein. Der genaue molekulare Mechanismus, wie nAChRs in mononukleären Phagozyten über metabotrope Signale die Aktivität des P2RX7 modulieren können, ist jedoch noch nicht vollständig aufgeklärt und es bedarf weiterer Forschung.

Es gelang uns erstmalig eine spezifische eNOS-vermittelte Inhibition des P2RX7 aufzuzeigen, die somit spezifisch die ATP-induzierten IL-1β-Freisetzung hemmt, was von großem klinischem Interesse ist. Dieser metabotrope Mechanismus kann neben klassischen nAChR-Agonisten auch durch die von uns entdeckten unkonventionellen endogenen Agonisten wie PC und PC-haltige Moleküle induziert werden (**Hecker et al. 2015**; **Richter et al. 2016; Zakrzewicz & Richter et al. 2017; Backhaus et al. 2017; Richter et al. 2018b; Richter et al. 2023a**). Wir konnten in unseren Arbeiten zeigen, dass diese unkonventionellen nAChR-Agonisten zudem an den heterolog exprimierten, humanen nAChRs (homomere α7- und α9-nAChRs, heteromere α9α10-nAChRs) keine ionotropen Funktionen induzieren (**Richter et al. 2016; Zakrzewicz & Richter et al. 2017; Richter et al. 2018b;** vgl. **Kapitel 3.6**). Diese *silent agonist*-Eigenschaften lassen vermuten, dass die unkonventionellen nAChR-Agonisten keine Nebenwirkungen im neuronalen System induzieren würden. Dementsprechend ergeben sich aus unseren Erkenntnissen vielversprechende neue therapeutische Ansätze zur Prävention und Behandlung der sterilen Inflammation bei chirurgischen Patienten, ohne dabei die Infektabwehr der Patienten herabzusetzen.

4.5 Synthetische α9-nAChRs-Liganden sind Werkzeuge in der Grundlagenforschung und potenzielle anti-inflammatorische und analgetische Therapeutika.*

In der vorliegenden Arbeit konnten zahlreiche Hinweise erbracht werden, dass die nAChR-Untereinheit α9 unerlässlich für die cholinerge Regulation der Freisetzung von

IL-1β aus mononukleären Phagozyten ist (**Hecker et al. 2015**; **Richter et al. 2016; Zakrzewicz & Richter et al. 2017; Backhaus et al. 2017**). Untersuchungen an α9-nAChRs waren lange Zeit schwierig, da es kaum selektive Liganden gibt. Lediglich das Conopeptid RgIA4 ist als selektiver Antagonist der α9*-nAChRs bekannt (siehe **Kapitel 3.1**). Bis heute gibt es zudem keinen PAM, der es erlauben würde, einen möglichen desensitisierten Zustand von α9*-nAChRs "sichtbar" zu machen. Unsere Arbeiten (**Zakrzewicz & Richter et al. 2017; Richter et al. 2022**) führten dazu, dass zahlreiche weitere, zuvor als partielle α7-Agonisten oder *silent agonists* beschriebene Varianten von quartären Ammoniumsalzen im heterologen Expressionsmodel der *X. laevis*-Oozyten auf ihre möglichen Effekte auf α9*-nAChRs untersucht wurden. Dabei konnten einige selektive Agonisten und Antagonisten für die α9-Untereinheit identifiziert werden (Bavo et al. 2022; Papke et al. 2022a; Giraudo et al. 2023).

In einer früheren Studie wurde *p*CF3-diEPP bereits im Tiermodell effektiv gegen inflammatorischen Schmerz eingesetzt (Quadri et al. 2018). Unsere Erkenntnisse, dass *p*CF3-diEPP auch an α9*-nAChRs wirkt (vgl. **Kapitel 4.4**), deuteten darauf hin, dass *p*CF3-diEPP und andere α9*-nAChR-spezifische diEPP-Analoge eine therapeutische Option zur Behandlung von inflammatorischen Erkrankungen und Schmerz sein könnten. Diese Idee wurde schließlich durch ein Patent geschützt (*U.S. Provisional Patent Application Serial No. 63/216,327, Title: N,N-diethyl-N'-phenylpiperazines as Agonists of Alpha9-containing nAChR and Use as Treatments for Neuropathic and Inflammatory Pain. Inventor(s): R.L. Papke, N.A. Horenstein, M. Quadri, V. Grau, K. Richter, A.R. Simard; Ref No.: T18479US001 (222107-8866)*).

Wir konnten weiterhin zeigen, dass der α9-nAChR-Agonist *p*CN-diEPP (Papke et al. 2022a) eine potente inhibitorische Wirkung auf die ATP-induzierten Freisetzung von IL-1β aus monozytären Zellen hat (**Richter et al. 2023b;** vgl. **Kapitel 3.8**). Neben den anti-inflammatorischen Funktionen zeigte *p*CN-diEPP auch eine effektive, analgetische Wirkung im Schmerzmodel (**Richter et al. 2023b**). Da *p*CN-diEPP ein Agonist der α9*-nAChRs ist, scheint dementsprechend die Aktivierung von α9*-nAChRs für den analgetischen Effekt verantwortlich zu sein. Im Widerspruch dazu steht, dass der α9α10-nAChR-Antagonisten *m*CN-diEPP im Tiermodell ebenfalls schmerzlindernd wirkte, wenn auch nur partiell und beim Einsatz hoher Konzentrationen (**Richter et al. 2023b**). Die Ergebnisse mit *m*CN-diEPP stimmen jedoch mit früheren Arbeiten überein, in denen analgetische Effekte durch α9α10-

nAChR-Antagonisten wie RgIA4 im Tiermodell für inflammatorischen Schmerz gezeigt wurden (Christensen et al. 2017; Romero et al. 2017; Toma et al. 2020). Eine mögliche Erklärung für die unterschiedlichen und teils kontroversen Ergebnisse der α9*-nAChR Agonisten und Antagonisten könnte sein, dass es, wie oben beschrieben, in non-neuronalen Zellen sowohl kanonische als auch non-kanonische nAChR-Signalwege geben kann, die Zelltyp-abhängig, aber auch abhängig von der Kombination der nAChR-Untereinheiten unterschiedliche Funktionen induzieren könnten (Grando 2014; Grau et al. 2018; Kabbani und Nichols 2018; Lottig et al. 2019). Zudem sind beim inflammatorischen Schmerz neben den Zellen des angeborenen Immunsystems auch diverse andere Immunzellen und sensorische Neurone beteiligt (Baral et al. 2019), ein komplexes Netzwerk, das bis heute nicht vollständig verstanden ist. Zur Schmerzbehandlung werden vor allem in den Vereinigten Staaten seit Jahrzehnten Opioide-Schmerzmittel eingesetzt. Durch den Missbrauch von Opioid-Schmerzmittel befinden sich die Vereinigten Staaten in einer seit 1970 anhaltenden Opioid-Krise, die mittlerweile jährlich mehrere Zehntausend Tote durch Überdosis fordert (Paul et al. 2023). Unsere Erkenntnisse zur analgetischen Wirkung von α9*-nAChR-Liganden bieten daher einen vielversprechenden Ansatz zur Entwicklung von neuen, nicht süchtig machenden Schmerzmitteln.

Auch wenn unsere Arbeiten Hinweise erbrachten, dass eine selektive Aktivierung des cholinergen anti-inflammatorischen Systems mittels α9*-nAChR-Liganden inflammatorischen Schmerz reduzieren kann (**Richter et al. 2023b**), so scheinen dabei insbesondere die unkonventionellen nAChRs mit ihren nicht-kanonischen (metabotropen) Funktionen, eine essenzielle Rolle spielen. Es stellt sich somit die Frage, inwieweit die Charakterisierung der Wirkung von Liganden auf die kanonische Ionenkanalfunktion von nAChRs dazu genutzt werden kann, eine Vorhersage zu deren Effekt auf die unkonventionellen nAChRs zu treffen. Immerhin gehören α7-nAChR *silent agonists* mit zu den effektivsten Modulatoren von Entzündungsreaktionen (Horenstein und Papke 2017). Wir haben starke Hinweise darauf, dass die nAChR-Untereinheiten α7, α9 und α10 für die cholinerge Regulation der ATP-induzierten IL-1β-Freisetzung benötigt werden, teils auch in unterschiedlichen Kombinationen der Untereinheiten (vgl. **Tabelle 1** und **Kapitel 4.3**). Wie diese unkonventionelle nAChRs jedoch strukturell aussehen, ist völlig unklar. Ob es sich bei den unkonventionellen nAChRs um Heteromere aus α7- und α9-nAChR-Untereinheiten handelt, und/oder α7-

Homomere und α9α10-Heteromere gleichzeitige in den mononukleären Phagozyten vorkommen und ob sich die nAChR-Untereinheiten klassisch als Pentamere zusammenlagern, ist bis heute unklar. Prinzipiell könnte die α9-Untereinheit sich auch zu nicht-pentameren Oligomeren mit der α7- und/oder α10-Untereinheit zusammenlagern, die dementsprechend keine Ionenkanalpore bilden, aber dennoch metabotrope Signalwege induzieren können.

Wir imitierten das gleichzeitige Vorkommen der humanen nAChR-Untereinheiten α7, α9 und α10 im heterologen Expressionssystem der *X. laevis*-Oozyten für kanonische nAChRs (**Richter et al. 2023b**). Die Expression von nAChR-Untereinheiten in *X. laevis*-Oozyten ist im Allgemeinen nur sehr gering und instabil. Dieses Phänomen ist auch aus der Synaptogenese bekannt (Huebsch und Maimone 2003). Während der Synaptogenese an motorischen Endplatten bedarf es nAChR-assoziierter Proteine wie Rapsyn (Gen: *RAPSN*), um die nAChRs in Clustern mit hoher Dichte in den Membranen zu organisieren und eine effiziente Reizweiterleitung zu ermöglichen (Huebsch und Maimone 2003). Deshalb wird im heterologen Expressionssystem der *X. laevis*-Oozyte häufig RNA für Rapsyn (Froehner et al. 1990; Ramarao und Cohen 1998) oder RIC3 (*resistance to inhibitors of cholinesterase* 3; Gen: *RIC*3) (Halevi et al. 2003; Noonan und Beech 2023) ko-injiziert, um eine stabile nAChR-Expression zu erhalten (**Richter et al. 2016; Richter et al. 2017; Richter et al. 2022; Richter et al. 2023b**), ohne dabei die pharmakologischen Eigenschaften der nAChRs zu verändern (Halevi et al. 2003). Wir konnten zum ersten Mal Hinweise darauf erbringen, dass es auch im heterologen Expressionsmodell der *X. laevis-Oozyten* zur Formierung von heteromeren α7α9α10-nAChRs kommt (**Richter et al. 2023b;** vgl. **Kapitel 3.9**). Vielmehr noch konnten wir zeigen, dass die An- und Abwesenheit einer oder mehrerer Untereinheiten in diesen nAChR-Heteromeren einen maßgeblichen Einfluss auf die Wirkung von Liganden haben kann (**Richter et al. 2023b**). So führte z.B. die Ko-Expression der α10-Untereinheit dazu, dass die Nikotin-Sensitivität der α9-Untereinheit um ein Vielfaches reduziert wurde (**Richter et al. 2023b**).

In einer Vielzahl von Publikationen zu elektrophysiologischen Messungen von Ionenkanälen/Rezeptoren wurden nach dem Auswaschen der Liganden eine Potenzierung der Stromantwort detektiert, die sogenannten “*hump*” oder “*rebound*“ Effekte (Adams 1975; Ebihara und Akaike 1992; Wu et al. 1994; Liu et al. 2008). Diese *rebound*-Effekte wurden von uns für eine Vielzahl von nAChR-Liganden detektiert und

waren interessanterweise am stärksten in α9α10-exprimierenden Oozyten finden (**Tabelle 2**). In homomeren α7- und α9-nAChRs zeigten sich hingegen keine bis nur sehr schwache *rebound*-Effekte, was auf eine Beteiligung der α10-Untereinheit hindeutete. Die nAChR-Untereinheit α10 galt lange Zeit als nicht-funktionelle, stille Rezeptoruntereinheit (Yu et al. 2013; Zouridakis et al. 2019; Hone und McIntosh 2022). Man ging ferner davon aus, dass α9*-nAChRs die α10-Untereinheit benötigen, um einen funktionellen Ionenkanal zu bilden (Yu et al. 2013; Zouridakis et al. 2019; Hone und McIntosh 2022). Interessanterweise konnte kürzlich gezeigt werden, dass die Behandlung von α10-nAChR exprimierenden Oozyten mit Alkaloiden wie MLA oder Strychnin zur Bildung funktioneller, ionotroper α10-nAChRs führt (Hone und McIntosh 2022). Ohne diese Vorbehandlung konnten keine ionotropen Funktionen der homomeren α10-nAChRs nachgewiesen werden (Hone und McIntosh 2022). Wir stellten uns die Frage, ob der von uns detektierte *rebound*-Effekt möglicherweise ebenfalls auf einem ähnlichen/demselben Mechanismus beruht. Um dies zu überprüfen, führten wir TEVC-Messungen an heterolog exprimierten α7- und α9-Homomeren, sowie heteromeren α9α10-nAChRs durch und detektierten die ACh-induzierte Ionenströme in Ab- und Anwesenheit von MLA (**Richter et al. 2023b**). MLA wird oft als selektiver Antagonist der α7-nAChRs, aber auch inverser Agonist beschrieben (Williams et al. 2011; Papke et al. 2018). Diese Selektivität konnte von uns nicht bestätigt werden, da MLA die ACh-induzierten Ionenströme der α7- und α9-Homomeren, sowie heteromeren α9α10-nAChRs inhibierte (**Richter et al. 2023b**). Ein MLA-induzierter *rebound*-Effekt zeigte sich an α9α10-nAChRs, was bei homomeren α7- und α9-nAChRs nicht der Fall war (**Tabelle 2**). Diese Ergebnisse deuten auf eine Aktivierung von vorher nicht-funktionellen, stillen α10-nAChRs hin (**Richter et al. 2023b**). Da der *rebound*-Effekt aber auch bei Agonisten wie PC und synthetischen *silent agonists* wie *p*CF3-diEPP auftrat (**Tabelle 2**), stellt sich die Frage, ob es sich um agonistische oder antagonistische Effekte der Liganden handelt. Vielmehr noch ergibt sich die Frage, ob Nikotin und MLA keine Antagonisten, sondern *silent agonists* der nAChR-Untereinheit α9 und/oder α10 sind.

Auch nach über 20 Jahren der Forschung zur Bedeutung von nAChRs für die Regulation des Immunsystems, ist unser Wissen über unkonventionelle aber auch über kanonische nAChRs immer noch unvollständig. Es ist aber zu erwarten, dass auch in mononukleären Phagozyten die Effekte von nikotinergen Liganden maßgeblich

von der Identität und Quantität der exprimierten nAChR-Untereinheiten abhängig ist. Die mRNA-Expression von nAChRs ist in diesen Zellen normalerweise sehr gering, variiert von Individuum zu Individuum und lässt zugleich keinen Rückschluss auf die Expression funktioneller Rezeptoren zu (Kawashima et al. 2007; Fujii et al. 2017a; Zoli et al. 2018). Zudem können zahlreiche endogene und exogene Faktoren die Expression und Funktion von nAChRs beeinflussen. So wurden zum Beispiel endogene, Toxin-ähnliche Prototoxine wie die *secreted ly6/uPAR-related proteins* (SLURPs) beschrieben, die die Expression und Funktion von nAChRs maßgeblich modulieren können (Miwa et al. 2019). Des Weiteren ist im humanen Genom das Gen *CHRFAM7A* vorhanden, welches das sogenannte dupα7-Protein kodiert (Gault et al. 1998; Di Lascio et al. 2022). Dieses dupα7-Protein kann mit der α7-nAChR-Untereinheit interagieren und dabei als negativer Modulator die Funktion von α7*-nAChRs inhibieren (Di Lascio et al. 2022). Da das dupα7-Protein auch in einer Vielzahl von Zelllinien sowie in heterologen Expressionssystemen zu finden ist, die in der cholinergen Forschung zum Einsatz kommen (Di Lascio et al. 2022), könnten dadurch ebenfalls Untersuchungen zu unkonventionellen und kanonischen nAChRs erschwert werden und zu widersprüchlichen Ergebnissen führen. In unseren Arbeiten (**Kapitel 3**) wurde versucht, bei den humanen Probanden und in den Tierexperimenten immer ein ausgewogenes Geschlechterverhältnis von 50:50 einzuhalten. Der Aspekt von geschlechtsspezifischen Unterschieden im cholinergen System ist bisher in der Forschung jedoch unterrepräsentiert. Wie kürzlich in einem Review von Moen und Lee publiziert, gibt es aber zahlreiche Hinweise darauf, dass das biologische Geschlecht und Geschlechtshormone die Expression von nAChRs beeinflusst (Moen und Lee 2021). Auch die Aufnahme von Genuss- und Suchtmitteln wie Kaffee und Zigaretten (Papke et al. 2022b) und die Ernährung (Shariff et al. 2016; Souza et al. 2019; Costa et al. 2020) können einen Einfluss auf das cholinerge System haben und ggf. die Expression von nAChRs modulieren. Dies gilt auch für Phosphatidylcholine und deren Metabolite, welche ebenfalls über die Ernährung aufgenommen werden können (van Parys et al. 2021) und die von uns als endogene Agonisten der nAChRs mit den Untereinheiten α7, α9 und/oder α10 identifiziert wurden (vgl. **Kapitel 4.3**). Untersuchungen zu funktionellen nAChRs werden zudem dadurch erschwert, dass es bis heute keine geeigneten Werkzeuge wie spezifische Antikörper gegen nAChR-Untereinheiten gibt (Herber et al. 2004; Moser et al. 2007; Richter und Grau 2023), die

eine spezifische Aussage über die Proteinexpression erlauben würden. Um die von uns entdeckten, neuen Aspekte bezüglich der Regulierung und Assemblierung der nAChR Untereinheiten α7, α9 und α10 vollständig aufzuklären, sind demnach weitere Untersuchungen nötig, mit einem breiten Spektrum von verschiedenen Methoden und gut charakterisierten selektiven Werkzeugen, in heterologen Expressionssystemen bis hin zu Untersuchungen *in vivo* nach wissenschaftlich hoch-standardisierten Protokollen.

Zusammenfassend konnten in der vorliegenden Arbeit neue Erkenntnisse zu unkonventionellen nAChR-Liganden sowie kanonischen und unkonventionellen nAChRs erbracht werden. Gleichzeitig wurden neue spannende Mysterien zu unkonventionellen non-neuronalen und neuronalen nAChRs und ihren Liganden aufgezeigt, deren Aufklärung weitere Forschung bedarf. Dank synthetischer α9*-nAChR-Liganden stehen in der Grundlagenforschung nun weitere “Werkzeuge“ zur Verfügung, die zur Entwicklung zukünftiger therapeutischer Anwendung, von der Behandlung inflammatorischer Erkrankungen wie der Trauma-induzierten sterilen Inflammation bis hin zu inflammatorischem Schmerz, beitragen können.

5. Zusammenfassung

Trotz intensiver Forschung führen Polytraumata und schwere Operationen auch heute noch zu lebensbedrohlichen systemischen Entzündungen, Sepsis und Multiorganversagen, mit einer nicht akzeptablen hohen Gesamtletalität (Westphal und Kampmeier 2015). Eine bedeutende Rolle in der Pathogenese dieser traumainduzierten Immundysfunktion spielt das hochpotente pro-inflammatorische Zytokin IL-1β, welches von Monozyten, Makrophagen sowie von Epithelzellen synthetisiert wird und unerlässlich für die Immunabwehr gegen Infektionen ist. Die Synthese und Freisetzung von IL-1β muss strikt reguliert werden (Dinarello 2018). Nach Verletzungen wird die Reifung und Ausschüttung von IL-1β durch extrazelluläres ATP, das aus dem Zytoplasma geschädigter Zellen stammt, stimuliert und aktives IL-1β sezerniert. Unter normalen Bedingungen bleiben diese pro-inflammatorischen Reaktionen lokal begrenzt. Bei schweren Traumata und großen chirurgischen Eingriffen kommt es hingegen durch massive Gewebeschädigung zu einer überschießenden Zytokin-Ausschüttung und folglich zur traumainduzierten Immundysfunktion. Bis heute gibt es neben entzündungshemmenden Therapien, welche die Infektanfälligkeit aber maßgeblich erhöhen können, keine geeigneten Behandlungen für die traumainduzierte Immundysfunktion.

In dieser kumulativen Habilitationsschrift wurden verschiedene Forschungsprojekte zusammengefasst, die einen neuen anti-inflammatorischen cholinergen Kontrollmechanismus der ATP-vermittelten Freisetzung von IL-1β aus mononukleären Phagozyten identifizieren und charakterisieren (**Hecker et al. 2015**; **Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Backhaus et al. 2017 Richter et al. 2018a**). Diese cholinerge Regulation beruht auf der Aktivierung von unkonventionellen nAChRs mit den evolutionär hoch konservierten Untereinheiten α7, α9 und/oder α10 durch klassische Agonisten wie ACh, Cholin und Nikotin. Es konnten aber auch mehrere bisher unbekannte, endogene nAChR-Agonisten identifiziert werden: Phosphatidylcholine sowie deren Metabolite wie PC und PC-haltige Moleküle. Gleichzeitig konnte eine vollkommen neue biologische Funktion des Akute-Phase-Proteins CRP, ein sensitiver Entzündungsmarker und Standardparameter in der klinischen Blutanalyse, aufgeklärt werden: In seiner endogenen, PC-bindenden, pentameren Form inhibiert CRP effizient und über

nAChR-vermittelt die monozytäre ATP-induzierte Freisetzung von IL-1β (**Richter et al. 2018b**). Interessanterweise hängt dieser inhibitorische Effekt nicht nur von dem CRP-gebundenen Liganden ab, vielmehr noch potenziert CRP die cholinerge Wirkung von freiem PC um ein Vielfaches (**Richter et al. 2018b**). Eine prospektive klinische Studie legt zudem nahe, dass erhöhte CRP-Konzentrationen vor einer traumainduzierten Immundysfunktion schützen (**Richter et al. 2018b**). Demnach scheint CRP Teil einer negativen Rückkopplungsschleife zu sein, welche die IL-1β-Freisetzung bei systemischer Entzündung limitiert.

Als einen potenziellen Gegenspieler des cholinergen Regulationsmechanismus, konnte das $A\beta_{1\text{-}42}$-Peptid identifiziert werden (**Richter et al. 2020**), welches bisher hauptsächlich im Zusammenhang mit der Alzheimer-Krankheit untersucht wurde. An unkonventionellen, monozytären nAChRs wirkt $A\beta_{1\text{-}42}$ *in vitro* antagonistisch und ermöglicht so die ATP-induzierte IL-1β-Freisetzung in Anwesenheit von nAChR-Agonisten. Da wir zudem Hinweise erbringen konnten, dass das $A\beta_{1\text{-}42}$-Peptid auch *in vivo* wirkt (**Richter et al. 2020**), könnte das $A\beta_{1\text{-}42}$-Peptid somit als endogener, pro-inflammatorischer Regulator präventiv gegen Infektionen und Sepsis sein.

Die hier zusammengefassten Arbeiten haben zudem wesentlich zu einem Paradigmenwechsel im wissenschaftlichen Blick auf die nAChRs beigetragen. Während nAChRs kanonisch als ionotrope Rezeptoren eine unerlässliche Rolle für die Reizweiterleitung im neuronalen System spielen, sind es metabotrope Funktionen der unkonventionellen nAChRs in mononukleären Phagozyten, die zur Inhibition des ATP-sensitiven P2X7 Rezeptors führen und somit die IL-1β-Freisetzung inhibieren (**Hecker et al. 2015; Zakrzewicz & Richter et al. 2017; Richter et al. 2016; Richter et al. 2023a**). Diese Erkenntnisse sind von großer Bedeutung für die Rezeptorforschung und gehen weit über die cholinerge Kontrolle der IL-1β-Freisetzung hinaus. Durch unsere Erkenntnisse rückte auch die α9-nAChR-Untereinheit immer mehr in den Fokus. In weiteren Arbeiten konnten neben synthetischen nAChR-*silent agonists* auch spezifische α9-Agonisten charakterisiert werden (**Richter et al. 2022; Richter et al. 2023b**). Dabei zeigten sich neben der anti-inflammatorischen Wirkung auch eine potente analgetischen Wirkung von α9-nAChR-Liganden, die somit eine vielversprechende therapeutische Option zur Behandlung von inflammatorischen Erkrankungen und Schmerz sein könnten.

Ein großer Vorteil des hier identifizierten cholinergen Kontrollmechanismus ist, dass gezielt die traumainduzierte (ATP-abhängige) Freisetzung von IL-1β inhibiert, gleichzeitig die IL-1β-abhängige Pathogenabwehr jedoch nicht völlig unterbunden wird. Zudem erwarten wir von den endogenen nAChR-Agonisten wie Phosphatidylcholine und seine Metabolie, sowie von den synthetischen *silent agonists*, dass sie nicht mit den kanonischen nAChR-Funktionen im Nervensystem interferieren. Somit ist dieser Mechanismus ein vielversprechender Ansatzpunkt für die dringend benötigten präventiven Therapien einer traumainduzierten Immundysfunktion.

6. Literaturverzeichnis

Abualrous, Esam T.; Sticht, Jana; Freund, Christian (2021): Major histocompatibility complex (MHC) class I and class II proteins: impact of polymorphism on antigen presentation. *Current opinion in immunology* 70, S. 95–104. DOI: 10.1016/j.coi.2021.04.009.

Adams, P. R. (1975): A study of desensitization using voltage clamp. *European journal of physiology* 360 (2), S. 135–144. DOI: 10.1007/BF00580536.

Ahrens, I.; Domeij, H.; Eisenhardt, S. U.; Topcic, D.; Albrecht, M.; Leitner, E. et al. (2011): Opposing effects of monomeric and pentameric C-reactive protein on endothelial progenitor cells. *Basic research in cardiology* 106 (5), S. 879–895. DOI: 10.1007/s00395-011-0191-y.

Althaus, Mike; Pichl, Alexandra; Clauss, Wolfgang G.; Seeger, Werner; Fronius, Martin; Morty, Rory E. (2011): Nitric oxide inhibits highly selective sodium channels and the Na^+/K^+-ATPase in H441 cells. *American journal of respiratory cell and molecular biology* 44 (1), S. 53–65. DOI: 10.1165/2009-0335OC.

Báez-Pagán, Carlos A.; Delgado-Vélez, Manuel; Lasalde-Dominicci, José A. (2015): Activation of the macrophage α7 nicotinic acetylcholine receptor and control of inflammation. *Journal of neuroimmune pharmacology* 10 (3), S. 468–476. DOI: 10.1007/s11481-015-9601-5.

Baral, Pankaj; Udit, Swalpa; Chiu, Isaac M. (2019): Pain and immunity: implications for host defence. *Nature immunology* 19 (7), S. 433–447. DOI: 10.1038/s41577-019-0147-2.

Bauernfeind, Franz; Ablasser, Andrea; Bartok, Eva; Kim, Sarah; Schmid-Burgk, Jonathan; Cavlar, Taner; Hornung, Veit (2011): Inflammasomes: current understanding and open questions. C*ellular and molecular life sciences* 68 (5), S. 765–783. DOI: 10.1007/s00018-010-0567-4.

Bavo, Francesco; Pallavicini, Marco; Pucci, Susanna; Appiani, Rebecca; Giraudo, Alessandro; Eaton, Brek et al. (2022): From 2-triethylammonium ethyl ether of 4-stilbenol (MG624) to selective small-molecule antagonists of human α9α10 nicotinic receptor by modifications at the ammonium ethyl residue. *Journal of medicinal chemistry* 65 (14), S. 10079–10097. DOI: 10.1021/acs.jmedchem.2c00746.

Becker, Daniel; Woltersdorf, Ronja; Boldt, Wolfgang; Schmitz, Stephan; Braam, Ursula; Schmalzing, Günther; Markwardt, Fritz (2008): The P2X7 carboxyl tail is a regulatory module of P2X7 receptor channel activity. *Journal of biological chemistry* 283 (37), S. 25725–25734. DOI: 10.1074/jbc.M803855200.

Beckmann, Janet; Lips, Katrin Susanne (2013): The non-neuronal cholinergic system in health and disease. *Pharmacology* 92 (5-6), S. 286–302. DOI: 10.1159/000355835.

Beers, W. H.; Reich, E. (1970): Structure and activity of acetylcholine. *Nature* 228 (5275), S. 917–922. DOI: 10.1038/228917a0.

Bobenchik, April M.; Augagneur, Yoann; Hao, Bing; Hoch, Jeffrey C.; Ben Mamoun, Choukri (2011): Phosphoethanolamine methyltransferases in phosphocholine biosynthesis: functions and potential for

antiparasite therapy. *FEMS microbiology reviews* 35 (4), S. 609–619. DOI: 10.1111/j.1574-6976.2011.00267.x.

Boffi, Juan Carlos; Marcovich, Irina; Gill-Thind, JasKiran K.; Corradi, Jeremías; Collins, Toby; Lipovsek, María Marcela et al. (2017): Differential contribution of subunit interfaces to α9α10 nicotinic acetylcholine receptor function. *Molecular pharmacology* 91 (3), S. 250–262. DOI: 10.1124/mol.116.107482.

Boncler, Magdalena; Wu, Yi; Watala, Cezary (2019): The multiple faces of C-reactive protein-physiological and pathophysiological implications in cardiovascular disease. *Molecules* 24 (11). DOI: 10.3390/molecules24112062.

Borovikova, L. V.; Ivanova, S.; Zhang, M.; Yang, H.; Botchkina, G. I.; Watkins, L. R. et al. (2000): Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 405 (6785), S. 458–462. DOI: 10.1038/35013070.

Bortolotti, Perrine; Faure, Emmanuel; Kipnis, Eric (2018): Inflammasomes in tissue damages and immune disorders after trauma. *Frontiers in immunology* 9, S. 1900. DOI: 10.3389/fimmu.2018.01900.

Boucher, J. L.; Moali, C.; Tenu, J. P. (1999): Nitric oxide biosynthesis, nitric oxide synthase inhibitors and arginase competition for L-arginine utilization. *Cellular and molecular life sciences* 55 (8-9), S. 1015–1028. DOI: 10.1007/s000180050352.

Bouzat, Cecilia; Lasala, Matías; Nielsen, Beatriz Elizabeth; Corradi, Jeremías; Del Esandi, María Carmen (2018): Molecular function of α7 nicotinic receptors as drug targets. *Journal of physiology* 596 (10), S. 1847–1861. DOI: 10.1113/JP275101.

Briggs, Clark A.; McKenna, David G. (1996): Effect of MK-801 at the human α7 nicotinic acetylcholine receptor. *Neuropharmacology* 35 (4), S. 407–414. DOI: 10.1016/0028-3908(96)00006-8.

Broz, Petr; Dixit, Vishva M. (2016): Inflammasomes: mechanism of assembly, regulation and signalling. *Nature reviews immunology* 16 (7), S. 407–420. DOI: 10.1038/nri.2016.58.

Bruchfeld, A.; Goldstein, R. S.; Chavan, S.; Patel, N. B.; Rosas-Ballina, M.; Kohn, N. et al. (2010): Whole blood cytokine attenuation by cholinergic agonists *ex vivo* and relationship to vagus nerve activity in rheumatoid arthritis. *Journal of internal medicine* 268 (1), S. 94–101. DOI: 10.1111/j.1365-2796.2010.02226.x.

Buchmann, Kurt (2014): Evolution of innate immunity: clues from invertebrates via fish to mammals. In: *Frontiers in immunology* 5, S. 459. DOI: 10.3389/fimmu.2014.00459.

Carneiro, Alan Brito; Iaciura, Bruna Maria Ferreira; Nohara, Lilian Lie; Lopes, Carla Duque; Veas, Esteban Mauricio Cordero; Mariano, Vania Sammartino et al. (2013): Lysophosphatidylcholine triggers TLR2- and TLR4-mediated signaling pathways but counteracts LPS-induced NO synthesis in peritoneal macrophages by inhibiting NF-κB translocation and MAPK/ERK phosphorylation. *PloS one* 8 (9), e76233. DOI: 10.1371/journal.pone.0076233.

Cetin, Hakan; Beeson, David; Vincent, Angela; Webster, Richard (2020): The structure, function, and physiology of the fetal and adult acetylcholine receptor in muscle. *Frontiers in molecular neuroscience* 13, S. 581097. DOI: 10.3389/fnmol.2020.581097.

Changeux, Jean-Pierre (2012): The nicotinic acetylcholine receptor: the founding father of the pentameric ligand-gated ion channel superfamily. *Journal of biological chemistry* 287 (48), S. 40207–40215. DOI: 10.1074/jbc.R112.407668.

Chen, Guo-Fang; Xu, Ting-Hai; Yan, Yan; Zhou, Yu-Ren; Jiang, Yi; Melcher, Karsten; Xu, H. Eric (2017): Amyloid beta: structure, biology and structure-based therapeutic development. *Acta pharmacologica Sinica* 38 (9), S. 1205–1235. DOI: 10.1038/aps.2017.28.

Chernyavsky, Alexander I.; Arredondo, Juan; Skok, Maryna; Grando, Sergei A. (2010): Auto/paracrine control of inflammatory cytokines by acetylcholine in macrophage-like U937 cells through nicotinic receptors. *International immunopharmacology* 10 (3), S. 308–315. DOI: 10.1016/j.intimp.2009.12.001.

Christensen, Sean B.; Hone, Arik J.; Roux, Isabelle; Kniazeff, Julie; Pin, Jean-Philippe; Upert, Grégory et al. (2017): RgIA4 potently blocks mouse α9α10 nAChRs and provides long lasting protection against oxaliplatin-induced cold allodynia. *Frontiers in cellular neuroscience* 11, S. 219. DOI: 10.3389/fncel.2017.00219.

Clark, Sarah E.; Weiser, Jeffrey N. (2013): Microbial modulation of host immunity with the small molecule phosphorylcholine. *Infection and immunity* 81 (2), S. 392–401. DOI: 10.1128/IAI.01168-12.

Costa, S. O.; Souza, C. M.; Lanza, P. G.; Sartori, J. O.; Ignacio-Souza, L. M.; Candreva, T. et al. (2020): Maternal high fat diet consumption reduces liver alpha7 nicotinic cholinergic receptor expression and impairs insulin signalling in the offspring. *Scientific reports* 10 (1), S. 48. DOI: 10.1038/s41598-019-56880-3.

Costa-Junior, Helio Miranda; Sarmento Vieira, Flávia; Coutinho-Silva, Robson (2011): C terminus of the P2X7 receptor: treasure hunting. *Purinergic signalling* 7 (1), S. 7–19. DOI: 10.1007/s11302-011-9215-1.

Dasgupta, Subhajit; Gomez, Jenny-Jaramillo; Singh, Inderjit; Khan, Mushfiquddin (2018): S-Nitrosylation in regulation of Inflammation and cell damage. *Current drug targets* 19 (15), S. 1831–1838. DOI: 10.2174/1389450119666180213094747.

Di Lascio, Simona; Fornasari, Diego; Benfante, Roberta (2022): The human-restricted isoform of the α7 nAChR, CHRFAM7A: a double-edged sword in neurological and inflammatory disorders. *International journal of molecular sciences* 23 (7). DOI: 10.3390/ijms23073463.

Dinarello, C. A. (1988): Interleukin-1. *Digestive diseases and sciences* 33 (3 Suppl), 25S-35S. DOI: 10.1007/BF01538128.

Dinarello, C. A.; Renfer, L.; Wolff, S. M. (1977): Human leukocytic pyrogen: purification and development of a radioimmunoassay. *Proceedings of the National Academy of Sciences* 74 (10), S. 4624–4627. DOI: 10.1073/pnas.74.10.4624.

Dinarello, Charles A. (2013): Overview of the interleukin-1 family of ligands and receptors. *Seminars in immunology* 25 (6), S. 389–393. DOI: 10.1016/j.smim.2013.10.001.

Dinarello, Charles A. (2018): Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunological reviews* 281 (1), S. 8–27. DOI: 10.1111/imr.12621.

Dineley, Kelly T.; Bell, Karen A.; Bui, Duy; Sweatt, J. David (2002): beta-Amyloid peptide activates alpha 7 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. *Journal of biological chemistry* 277 (28), S. 25056–25061. DOI: 10.1074/jbc.M200066200.

Douglas, Tinsley Claire; Hannila, Sari S. (2022): Working from within: how secretory leukocyte protease inhibitor regulates the expression of pro-inflammatory genes. *Biochemistry and cell biology* 100 (1), S. 1–8. DOI: 10.1139/bcb-2021-0284.

Drobnik, Wolfgang; Liebisch, Gerhard; Audebert, Franz-Xaver; Frohlich, Dieter; Gluck, Thomas; Vogel, Peter et al. (2003): Plasma ceramide and lysophosphatidylcholine inversely correlate with mortality in sepsis patients. *Journal of lipid research* 44 (4), S. 754–761. DOI: 10.1194/jlr.m200401-jlr200.

Ebihara, S.; Akaike, N. (1992): Strychnine-induced potassium current in CA1 pyramidal neurones of the rat hippocampus. *British journal of pharmacology* 106 (4), S. 823–827. DOI: 10.1111/j.1476-5381.1992.tb14419.x.

Eisenhardt, Steffen U.; Habersberger, Jonathon; Murphy, Andrew; Chen, Yung-Chih; Woollard, Kevin J.; Bassler, Nicole et al. (2009): Dissociation of pentameric to monomeric C-reactive protein on activated platelets localizes inflammation to atherosclerotic plaques. *Circulation research* 105 (2), S. 128–137. DOI: 10.1161/CIRCRESAHA.108.190611.

Elgoyhen, A. B.; Vetter, D. E.; Katz, E.; Rothlin, C. V.; Heinemann, S. F.; Boulter, J. (2001): alpha10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proceedings of the National Academy of Sciences* 98 (6), S. 3501–3506. DOI: 10.1073/pnas.051622798.

Fabiani, Camila; Antollini, Silvia S. (2019): Alzheimer's disease as a membrane disorder: spatial cross-talk among beta-amyloid peptides, nicotinic acetylcholine receptors and lipid rafts. *Frontiers in cellular neuroscience* 13, S. 309. DOI: 10.3389/fncel.2019.00309.

Fischer, W. (2000): Phosphocholine of pneumococcal teichoic acids: role in bacterial physiology and pneumococcal infection. *Research in microbiology* 151 (6), S. 421–427. DOI: 10.1016/s0923-2508(00)00174-1.

Förstermann, Ulrich; Sessa, William C. (2012): Nitric oxide synthases: regulation and function. *European heart journal* 33 (7), 829-37, 837a-837d. DOI: 10.1093/eurheartj/ehr304.

François, Arnaud; Julian, Adrien; Ragot, Stéphanie; Dugast, Emilie; Blanchard, Ludovic; Brishoual, Sonia et al. (2015): Inflammatory stress on autophagy in peripheral blood mononuclear cells from patients with Alzheimer's disease during 24 months of follow-up. *PloS one* 10 (9), e0138326. DOI: 10.1371/journal.pone.0138326.

Froehner, S. C.; Luetje, C. W.; Scotland, P. B.; Patrick, J. (1990): The postsynaptic 43K protein clusters muscle nicotinic acetylcholine receptors in *Xenopus* oocytes. *Neuron* 5 (4), S. 403–410. DOI: 10.1016/0896-6273(90)90079-u.

Fujii, Takeshi; Mashimo, Masato; Moriwaki, Yasuhiro; Misawa, Hidemi; Ono, Shiro; Horiguchi, Kazuhide; Kawashima, Koichiro (2017a): Expression and function of the cholinergic system in immune cells. *Frontiers in immunology* 8, S. 1085. DOI: 10.3389/fimmu.2017.01085.

Fujii, Takeshi; Mashimo, Masato; Moriwaki, Yasuhiro; Misawa, Hidemi; Ono, Shiro; Horiguchi, Kazuhide; Kawashima, Koichiro (2017b): Physiological functions of the cholinergic system in immune cells. *Journal of pharmacological sciences* 134 (1), S. 1–21. DOI: 10.1016/j.jphs.2017.05.002.

Gabay, C.; Kushner, I. (1999): Acute-phase proteins and other systemic responses to inflammation. *New England journal of medicine* 340 (6), S. 448–454. DOI: 10.1056/NEJM199902113400607.

Garlanda, Cecilia; Bottazzi, Barbara; Bastone, Antonio; Mantovani, Alberto (2005): Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. *Annual review of immunology* 23, S. 337–366. DOI: 10.1146/annurev.immunol.23.021704.115756.

Gault, J.; Robinson, M.; Berger, R.; Drebing, C.; Logel, J.; Hopkins, J. et al. (1998): Genomic organization and partial duplication of the human alpha7 neuronal nicotinic acetylcholine receptor gene (CHRNA7). *Genomics* 52 (2), S. 173–185. DOI: 10.1006/geno.1998.5363.

Gharpure, Anant; Noviello, Colleen M.; Hibbs, Ryan E. (2020): Progress in nicotinic receptor structural biology. *Neuropharmacology* 171, S. 108086. DOI: 10.1016/j.neuropharm.2020.108086.

Giard, D. J.; Aaronson, S. A.; Todaro, G. J.; Arnstein, P.; Kersey, J. H.; Dosik, H.; Parks, W. P. (1973): *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *Journal of the National Cancer Institute* 51 (5), S. 1417–1423. DOI: 10.1093/jnci/51.5.1417.

Giraudo, Alessandro; Pallavicini, Marco; Bolchi, Cristiano (2023): Small molecule ligands for α9 * and α7 nicotinic receptors: A survey and an update, respectively. *Pharmacological research* 193, S. 106801. DOI: 10.1016/j.phrs.2023.106801.

Gold, Maike; El Khoury, Joseph (2015): β-amyloid, microglia, and the inflammasome in Alzheimer's disease. *Seminars in immunopathology* 37 (6), S. 607–611. DOI: 10.1007/s00281-015-0518-0.

Gonzalez, Daniel R.; Treuer, Adriana; Sun, Qi-An; Stamler, Jonathan S.; Hare, Joshua M. (2009): S-Nitrosylation of cardiac ion channels. *Journal of cardiovascular pharmacology* 54 (3), S. 188–195. DOI: 10.1097/FJC.0b013e3181b72c9f.

Grabitzki, Julia; Lochnit, Günter (2009): Immunomodulation by phosphocholine-biosynthesis, structures and immunological implications of parasitic PC-epitopes. *Molecular immunology* 47 (2-3), S. 149–163. DOI: 10.1016/j.molimm.2009.09.035.

Grando, Sergei A. (2014): Connections of nicotine to cancer. *Nature reviews cancer* 14 (6), S. 419–429. DOI: 10.1038/nrc3725.

Grando, Sergei A.; Kawashima, Koichiro; Kirkpatrick, Charles J.; Wessler, Ignaz (2007): Recent progress in understanding the non-neuronal cholinergic system in humans. *Life sciences* 80 (24-25), S. 2181–2185. DOI: 10.1016/j.lfs.2007.03.015.

Grau, Veronika; Richter, Katrin; Hone, Arik J.; McIntosh, J. Michael (2018): Conopeptides V11L;V16DArIB and RgIA4: powerful tools for the identification of novel nicotinic acetylcholine receptors in monocytes. *Frontiers in pharmacology* 9, S. 1499. DOI: 10.3389/fphar.2018.01499.

Graven, S. N.; Estrada-O, S.; Lardy, H. A. (1966): Alkali metal cation release and respiratory inhibition induced by nigericin in rat liver mitochondria. *Proceedings of the National Academy of Sciences* 56 (2), S. 654–658. DOI: 10.1073/pnas.56.2.654.

Grønlien, Jens Halvard; Håkerud, Monika; Ween, Hilde; Thorin-Hagene, Kirsten; Briggs, Clark A.; Gopalakrishnan, Murali; Malysz, John (2007): Distinct profiles of alpha7 nAChR positive allosteric modulation revealed by structurally diverse chemotypes. *Molecular pharmacology* 72 (3), S. 715–724. DOI: 10.1124/mol.107.035410.

Gross, Olaf; Thomas, Christina J.; Guarda, Greta; Tschopp, Jurg (2011): The inflammasome: an integrated view. *Immunological reviews* 243 (1), S. 136–151. DOI: 10.1111/j.1600-065X.2011.01046.x.

Halder, Namrita; Lal, Girdhari (2021): Cholinergic system and its therapeutic importance in inflammation and autoimmunity. *Frontiers in immunology* 12, S. 660342. DOI: 10.3389/fimmu.2021.660342.

Halevi, Sarah; Yassin, Lina; Eshel, Margalit; Sala, Francisco; Sala, Salvador; Criado, Manuel; Treinin, Millet (2003): Conservation within the RIC-3 gene family. Effectors of mammalian nicotinic acetylcholine receptor expression. *Journal of biological chemistry* 278 (36), S. 34411–34417. DOI: 10.1074/jbc.m300170200.

Hamill, O. P.; Marty, A.; Neher, E.; Sakmann, B.; Sigworth, F. J. (1981): Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *European journal of physiology* 391 (2), S. 85–100. DOI: 10.1007/BF00656997.

Hecker, Andreas; Küllmar, Mira; Wilker, Sigrid; Richter, Katrin; Zakrzewicz, Anna; Atanasova, Srebrena et al. (2015): Phosphocholine-modified macromolecules and canonical nicotinic agonists inhibit ATP-induced IL-1β release. *Journal of immunology* 195 (5), S. 2325–2334. DOI: 10.4049/jimmunol.1400974.

Hecker, Andreas; Mikulski, Zbigniew; Lips, Katrin S.; Pfeil, Uwe; Zakrzewicz, Anna; Wilker, Sigrid et al. (2009): Pivotal advance: up-regulation of acetylcholine synthesis and paracrine cholinergic signaling in intravascular transplant leukocytes during rejection of rat renal allografts. *Journal of leukocyte biology* 86 (1), S. 13–22. DOI: 10.1189/jlb.1107722.

Heneka, Michael T.; Carson, Monica J.; El Khoury, Joseph; Landreth, Gary E.; Brosseron, Frederic; Feinstein, Douglas L. et al. (2015): Neuroinflammation in Alzheimer's disease. *Lancet* 14 (4), S. 388–405. DOI: 10.1016/S1474-4422(15)70016-5.

Herber, Donna L.; Severance, Emily G.; Cuevas, Javier; Morgan, Dave; Gordon, Marcia N. (2004): Biochemical and histochemical evidence of nonspecific binding of alpha7nAChR antibodies to mouse brain tissue. *Journal of histochemistry and cytochemistry* 52 (10), S. 1367–1376. DOI: 10.1177/002215540405201013.

Hone, Arik J.; McIntosh, J. Michael (2022): Alkaloid ligands enable function of homomeric human α10 nicotinic acetylcholine receptors. *Frontiers in pharmacology* 13, S. 981760. DOI: 10.3389/fphar.2022.981760.

Hone, Arik J.; Whiteaker, Paul; Christensen, Sean; Xiao, Yingxian; Meyer, Erin L.; McIntosh, J. Michael (2009): A novel fluorescent alpha-conotoxin for the study of alpha7 nicotinic acetylcholine receptors. *Journal of neurochemistry* 111 (1), S. 80–89. DOI: 10.1111/j.1471-4159.2009.06299.x.

Hone, Arik J.; Whiteaker, Paul; Mohn, Jesse L.; Jacob, Michele H.; McIntosh, J. Michael (2010): Alexa fluor 546-ArIBV11L;V16A is a potent ligand for selectively labeling alpha 7 nicotinic acetylcholine receptors. *Journal of neurochemistry* 114 (4), S. 994–1006. DOI: 10.1111/j.1471-4159.2010.06819.x.

Hoover, Donald B. (2017): Cholinergic modulation of the immune system presents new approaches for treating inflammation. *Pharmacology & therapeutics* 179, S. 1–16. DOI: 10.1016/j.pharmthera.2017.05.002.

Horenstein, Nicole A.; Papke, Roger L. (2017): Anti-inflammatory silent agonists. *ACS medicinal chemistry letters* 8 (10), S. 989–991. DOI: 10.1021/acsmedchemlett.7b00368.

Horiuchi, Yoko; Kimura, Reika; Kato, Noriko; Fujii, Takeshi; Seki, Masako; Endo, Toyoshige et al. (2003): Evolutional study on acetylcholine expression. *Life sciences* 72 (15), S. 1745–1756. DOI: 10.1016/s0024-3205(02)02478-5.

Huebsch, Kimberly A.; Maimone, Margaret M. (2003): Rapsyn-mediated clustering of acetylcholine receptor subunits requires the major cytoplasmic loop of the receptor subunits. *Journal of neurobiology* 54 (3), S. 486–501. DOI: 10.1002/neu.10177.

Ilcol, Yesim Ozarda; Dilek, Kamil; Yurtkuran, Mustafa; Ulus, Ismail H. (2002): Changes of plasma free choline and choline-containing compounds' concentrations and choline loss during hemodialysis in ESRD patients. *Clinical biochemistry* 35 (3), S. 233–239. DOI: 10.1016/s0009-9120(02)00298-9.

Ilcol, Yeşim Ozarda; Ozbek, Resul; Hamurtekin, Emre; Ulus, Ismail H. (2005): Choline status in newborns, infants, children, breast-feeding women, breast-fed infants and human breast milk. *Journal of nutritional biochemistry* 16 (8), S. 489–499. DOI: 10.1016/j.jnutbio.2005.01.011.

Innocent, Neal; Livingstone, Phil D.; Hone, Arik; Kimura, Atsuko; Young, Tracey; Whiteaker, Paul et al. (2008): Alpha-conotoxin Arenatus IB[V11L,V16D] corrected is a potent and selective antagonist at rat and human native alpha7 nicotinic acetylcholine receptors. *Journal of pharmacology and experimental therapeutics* 327 (2), S. 529–537. DOI: 10.1124/jpet.108.142943.

Iwasaki, Akiko; Medzhitov, Ruslan (2015): Control of adaptive immunity by the innate immune system. *Nature immunology* 16 (4), S. 343–353. DOI: 10.1038/ni.3123.

Jiang, S.; Xia, D.; Samols, D. (2006): Expression of rabbit C-reactive protein in transgenic mice inhibits development of antigen-induced arthritis. *Scandinavian journal of rheumatology* 35 (5), S. 351–355. DOI: 10.1080/03009740600757963.

Johnson, D. S.; Martinez, J.; Elgoyhen, A. B.; Heinemann, S. F.; McIntosh, J. M. (1995): alpha-Conotoxin ImI exhibits subtype-specific nicotinic acetylcholine receptor blockade: preferential inhibition of homomeric alpha7 and alpha9 receptors. *Molecular pharmacology* 48 (2), S. 194–199.

Jonge, W. J. de; Ulloa, L. (2007): The alpha7 nicotinic acetylcholine receptor as a pharmacological target for inflammation. *British journal of pharmacology* 151 (7), S. 915–929. DOI: 10.1038/sj.bjp.0707264.

Jordens, J. Z.; Slack, M. P. (1995): *Haemophilus influenzae*: then and now. *European journal of clinical microbiology & infectious diseases* 14 (11), S. 935–948. DOI: 10.1007/BF01691374.

Kabarowski, Janusz H. S.; Xu, Yan; Witte, Owen N. (2002): Lysophosphatidylcholine as a ligand for immunoregulation. *Biochemical pharmacology* 64 (2), S. 161–167. DOI: 10.1016/s0006-2952(02)01179-6.

Kabbani, Nadine; Nichols, Robert A. (2018): Beyond the channel: metabotropic signaling by nicotinic receptors. *Trends in pharmacological sciences* 39 (4), S. 354–366. DOI: 10.1016/j.tips.2018.01.002.

Kabbani, Nadine; Nordman, Jacob C.; Corgiat, Brian A.; Veltri, Daniel P.; Shehu, Amarda; Seymour, Victoria A.; Adams, David J. (2013): Are nicotinic acetylcholine receptors coupled to G proteins? *BioEssays* 35 (12), S. 1025–1034. DOI: 10.1002/bies.201300082.

Kaplan, Marielle; Hamoud, Shadi; Tendler, Yevgeny; Meilin, Edna; Lazarovitch, Aviva; Nitecki, Samy; Hayek, Tony (2014): A significant correlation between C-reactive protein levels in blood monocytes derived macrophages versus content in carotid atherosclerotic lesions. *Journal of inflammation* 11 (1), S. 7. DOI: 10.1186/1476-9255-11-7.

Kaplin, Adam; Carroll, Katherine A. L.; Cheng, Jenn; Allie, Rameeza; Lyketsos, Constantine G.; Calabresi, Peter; Rosenberg, Paul B. (2009): IL-6 release by LPS-stimulated peripheral blood

mononuclear cells as a potential biomarker in Alzheimer's disease. *International psychogeriatrics* 21 (2), S. 413–414. DOI: 10.1017/S1041610208008107.

Katz, Eleonora; Verbitsky, Miguel; Rothlin, Carla V.; Vetter, Douglas E.; Heinemann, Stephen F.; Belén Elgoyhen, A. (2000): High calcium permeability and calcium block of the α9 nicotinic acetylcholine receptor. *Hearing research* 141 (1-2), S. 117–128. DOI: 10.1016/S0378-5955(99)00214-2.

Kaufmann, Stefan H. E. (2009): Mononukleäre Phagozyten und antigenpräsentierende Zellen. Medizinische Mikrobiologie und Infektiologie. Berlin, Heidelberg: Springer Berlin Heidelberg (Springer-Lehrbuch), S. 91–98.

Kawashima, K.; Fujii, T. (2000): Extraneuronal cholinergic system in lymphocytes. *Pharmacology & therapeutics* 86 (1), S. 29–48. DOI: 10.1016/S0163-7258(99)00071-6.

Kawashima, Koichiro; Yoshikawa, Ken; Fujii, Yoshihito X.; Moriwaki, Yasuhiro; Misawa, Hidemi (2007): Expression and function of genes encoding cholinergic components in murine immune cells. *Life sciences* 80 (24-25), S. 2314–2319. DOI: 10.1016/j.lfs.2007.02.036.

Khan, Salman; Shehzad, Omer; Chun, Jaemoo; Kim, Yeong Shik (2013): Mechanism underlying anti-hyperalgesic and anti-allodynic properties of anomalin in both acute and chronic inflammatory pain models in mice through inhibition of NF-κB, MAPKs and CREB signaling cascades. *European journal of pharmacology* 718 (1-3), S. 448–458. DOI: 10.1016/j.ejphar.2013.07.039.

Kushner, Irving; Antonelli, Maria J. (2015): What should we regard as an "elevated" C-reactive protein level? *Annals of internal medicine* 163 (4), S. 326. DOI: 10.7326/L15-5126.

Lamb, Patricia W.; Melton, Mark A.; Yakel, Jerrel L. (2005): Inhibition of neuronal nicotinic acetylcholine receptor channels expressed in *Xenopus* oocytes by beta-amyloid1-42 peptide. *Journal of molecular neuroscience* 27 (1), S. 13–21. DOI: 10.1385/JMN:27:1:013.

Lamkanfi, Mohamed; Sarkar, Anasuya; Vande Walle, Lieselotte; Vitari, Alberto C.; Amer, Amal O.; Wewers, Mark D. et al. (2010): Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia. *Journal of immunology* 185 (7), S. 4385–4392. DOI: 10.4049/jimmunol.1000803.

Lasala, Matías; Fabiani, Camila; Corradi, Jeremías; Antollini, Silvia; Bouzat, Cecilia (2019): Molecular modulation of human α7 nicotinic receptor by amyloid-β peptides. *Frontiers in cellular neurosciences.* 13, S. 37. DOI: 10.3389/fncel.2019.00037.

Lee, Daniel H. S.; Wang, Hoau-Yan (2003): Differential physiologic responses of alpha7 nicotinic acetylcholine receptors to beta-amyloid1-40 and beta-amyloid1-42. *Journal of neurobiology* 55 (1), S. 25–30. DOI: 10.1002/neu.10203.

Lei, Wei; Duan, Zhijun (2021): Advances in the treatment of cholinergic anti-inflammatory pathways in gastrointestinal diseases by electrical stimulation of vagus nerve. *Digestion* 102 (2), S. 128–138. DOI: 10.1159/000504474.

Li, Zhaoyu; Vance, Dennis E. (2008): Phosphatidylcholine and choline homeostasis. *Journal of lipid research* 49 (6), S. 1187–1194. DOI: 10.1194/jlr.r700019-jlr200.

Lipovsek, Marcela; Im, Gi Jung; Franchini, Lucía F.; Pisciottano, Francisco; Katz, Eleonora; Fuchs, Paul Albert; Elgoyhen, Ana Belén (2012): Phylogenetic differences in calcium permeability of the auditory hair cell cholinergic nicotinic receptor. *Proceedings of the National Academy of Sciences* 109 (11), S. 4308–4313. DOI: 10.1073/pnas.1115488109.

Liu, Panpan; Zhu, Wu; Chen, Chao; Yan, Bei; Zhu, Lei; Chen, Xiang; Peng, Cong (2020): The mechanisms of lysophosphatidylcholine in the development of diseases. *Life sciences* 247, S. 117443. DOI: 10.1016/j.lfs.2020.117443.

Liu, Q.; Kawai, H.; Berg, D. K. (2001): beta -Amyloid peptide blocks the response of alpha 7-containing nicotinic receptors on hippocampal neurons. *Proceedings of the National Academy of Sciences* 98 (8), S. 4734–4739. DOI: 10.1073/pnas.081553598.

Liu, Qiang; Whiteaker, Paul; Morley, Barbara J.; Shi, Fu-Dong; Lukas, Ronald J. (2017): Distinctive roles for α7*- and α9*-nicotinic acetylcholine receptors in inflammatory and autoimmune responses in the murine experimental autoimmune encephalomyelitis model of multiple sclerosis. *Frontiers in cellular neuroscience* 11, S. 287. DOI: 10.3389/fncel.2017.00287.

Liu, Qiang; Yu, Ke-wei; Chang, Yong-chang; Lukas, Ronald J.; Wu, Jie (2008): Agonist-induced hump current production in heterologously-expressed human alpha4beta2-nicotinic acetylcholine receptors. *Acta pharmacologica* 29 (3), S. 305–319. DOI: 10.1111/j.1745-7254.2008.00760.x.

López-Castejón, Gloria; Pelegrín, Pablo (2012): Current status of inflammasome blockers as anti-inflammatory drugs. *Expert opinion on investigational drugs* 21 (7), S. 995–1007. DOI: 10.1517/13543784.2012.690032.

Lottig, Lena; Bader, Sandra; Jimenez, Marcel; Diener, Martin (2019): Evidence for metabotropic function of epithelial nicotinic cholinergic receptors in rat colon. *British journal of pharmacology* 176 (9), S. 1328–1340. DOI: 10.1111/bph.14638.

Lugade, Amit A.; Bogner, Paul N.; Thatcher, Thomas H.; Sime, Patricia J.; Phipps, Richard P.; Thanavala, Yasmin (2014): Cigarette smoke exposure exacerbates lung inflammation and compromises immunity to bacterial infection. *Journal of immunology* 192 (11), S. 5226–5235. DOI: 10.4049/jimmunol.1302584.

Månsson, Martin; Hood, Derek W.; Moxon, E. Richard; Schweda, Elke K. H. (2003): Structural diversity in lipopolysaccharide expression in nontypeable *Haemophilus influenzae*. Identification of L-glycerol-D-manno-heptose in the outer-core region in three clinical isolates. *European journal of biochemistry* 270 (4), S. 610–624. DOI: 10.1046/j.1432-1033.2003.03399.x.

Mantovani, Alberto; Dinarello, Charles A.; Molgora, Martina; Garlanda, Cecilia (2019): Interleukin-1 and related cytokines in the regulation of inflammation and immunity. *Immunity* 50 (4), S. 778–795. DOI: 10.1016/j.immuni.2019.03.012.

Mantovani, Alberto; Garlanda, Cecilia; Doni, Andrea; Bottazzi, Barbara (2008): Pentraxins in innate immunity: from C-reactive protein to the long pentraxin PTX3. *Journal of clinical immunology* 28 (1), S. 1–13. DOI: 10.1007/s10875-007-9126-7.

Marchetti, Carlo; Swartzwelter, Benjamin; Gamboni, Fabia; Neff, Charles P.; Richter, Katrin; Azam, Tania et al. (2018): OLT1177, a β-sulfonyl nitrile compound, safe in humans, inhibits the NLRP3 inflammasome and reverses the metabolic cost of inflammation. *Proceedings of the National Academy of Sciences* 115 (7), E1530-E1539. DOI: 10.1073/pnas.1716095115.

Martelli, D.; McKinley, M. J.; McAllen, R. M. (2014): The cholinergic anti-inflammatory pathway: a critical review. *Autonomic neuroscience* 182, S. 65–69. DOI: 10.1016/j.autneu.2013.12.007.

Mattson, M. P. (1997): Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives. *Physiological reviews* 77 (4), S. 1081–1132. DOI: 10.1152/physrev.1997.77.4.1081.

Mattson, Mark P. (2004): Pathways towards and away from Alzheimer's disease. *Nature* 430 (7000), S. 631–639. DOI: 10.1038/nature02621.

McCarthy, Alanna E.; Yoshioka, Craig; Mansoor, Steven E. (2019): Full-length P2X7 structures reveal how palmitoylation prevents channel desensitization. *Cell* 179 (3), 659-670.e13. DOI: 10.1016/j.cell.2019.09.017.

Message, Simon D.; Johnston, Sebastian L. (2004): Host defense function of the airway epithelium in health and disease: clinical background. *Journal of leukocyte biology* 75 (1), S. 5–17. DOI: 10.1189/jlb.0703315.

Meyer, Moritz Mecki; Brandenburg, Leon; Hudel, Helge; Agné, Alisa; Padberg, Winfried; Erdogan, Ali et al. (2020): Who is afraid of CRP? Elevated preoperative CRP levels might attenuate the increase in inflammatory parameters in response to lung cancer surgery. *Journal of clinical medicine* 9 (10). DOI: 10.3390/jcm9103340.

Mikulski, Zbigniew; Hartmann, Petra; Jositsch, Gitte; Zasłona, Zbigniew; Lips, Katrin S.; Pfeil, Uwe et al. (2010): Nicotinic receptors on rat alveolar macrophages dampen ATP-induced increase in cytosolic calcium concentration. *Respiratory research* 11, S. 133. DOI: 10.1186/1465-9921-11-133.

Miwa, Julie M.; Anderson, Kristin R.; Hoffman, Katie M. (2019): Lynx prototoxins: roles of endogenous mammalian neurotoxin-like proteins in modulating nicotinic acetylcholine receptor function to influence complex biological processes. *Frontiers in pharmacology* 10, S. 343. DOI: 10.3389/fphar.2019.00343.

Moen, Janna K.; Lee, Anna M. (2021): Sex differences in the nicotinic acetylcholine receptor system of rodents: impacts on nicotine and alcohol reward behaviors. *Frontiers in neuroscience* 15, S. 745783. DOI: 10.3389/fnins.2021.745783.

Mold, C.; Nakayama, S.; Holzer, T. J.; Gewurz, H.; Du Clos, T. W. (1981): C-reactive protein is protective against *Streptococcus pneumoniae* infection in mice. *Journal of experimental medicine* 154 (5), S. 1703–1708. DOI: 10.1084/jem.154.5.1703.

Mold, Carolyn; Rodriguez, Wilfredo; Rodic-Polic, Bojana; Du Clos, Terry W. (2002): C-reactive protein mediates protection from lipopolysaccharide through interactions with Fc gamma R. *Journal of immunology* 169 (12), S. 7019–7025. DOI: 10.4049/jimmunol.169.12.7019.

Moser, N.; Mechawar, N.; Jones, I.; Gochberg-Sarver, A.; Orr-Urtreger, A.; Plomann, M. et al. (2007): Evaluating the suitability of nicotinic acetylcholine receptor antibodies for standard immunodetection procedures. *Journal of neurochemistry* 102 (2), S. 479–492. DOI: 10.1111/j.1471-4159.2007.04498.x.

Müller, Ulrike C.; Deller, Thomas; Korte, Martin (2017): Not just amyloid: physiological functions of the amyloid precursor protein family. *Nature reviews neuroscience* 18 (5), S. 281–298. DOI: 10.1038/nrn.2017.29.

Nagele, R. G.; D'Andrea, M. R.; Anderson, W. J.; Wang, H-Y (2002): Intracellular accumulation of beta-amyloid(1-42) in neurons is facilitated by the alpha 7 nicotinic acetylcholine receptor in Alzheimer's disease. *Neuroscience* 110 (2), S. 199–211. DOI: 10.1016/S0306-4522(01)00460-2.

Noonan, Jennifer D.; Beech, Robin N. (2023): Two residues determine nicotinic acetylcholine receptor requirement for RIC-3. *Protein science* 32 (9), e4718. DOI: 10.1002/pro.4718.

Noone, Dylan P.; van der Velden, Tijn T.; Sharp, Thomas H. (2021): Cryo-electron microscopy and biochemical analysis offer insights into the effects of acidic pH, such as occur during acidosis, on the complement binding properties of C-reactive protein. *Frontiers in immunology* 12, S. 757633. DOI: 10.3389/fimmu.2021.757633.

Nurkhametova, Dilyara; Siniavin, Andrei; Streltsova, Maria; Kudryavtsev, Denis; Kudryavtsev, Igor; Giniatullina, Raisa et al. (2020): Does cholinergic stimulation affect the P2X7 receptor-mediated dye uptake in mast cells and macrophages? *Frontiers in cellular neuroscience* 14, S. 548376. DOI: 10.3389/fncel.2020.548376.

Oken, Adam C.; Krishnamurthy, Ipsita; Savage, Jonathan C.; Lisi, Nicolas E.; Godsey, Michael H.; Mansoor, Steven E. (2022): Molecular pharmacology of P2X receptors: exploring druggable domains revealed by structural biology. *Frontiers in pharmacology* 13, S. 925880. DOI: 10.3389/fphar.2022.925880.

Olofsson, Peder S.; Rosas-Ballina, Mauricio; Levine, Yaakov A.; Tracey, Kevin J. (2012): Rethinking inflammation: neural circuits in the regulation of immunity. *Immunological reviews* 248 (1), S. 188–204. DOI: 10.1111/j.1600-065X.2012.01138.x.

Pang, Bing; Winn, Dana; Johnson, Ryan; Hong, Wenzhou; West-Barnette, Shayla; Kock, Nancy; Swords, W. Edward (2008): Lipooligosaccharides containing phosphorylcholine delay pulmonary clearance of nontypeable *Haemophilus influenzae*. *Infection and immunity* 76 (5), S. 2037–2043. DOI: 10.1128/IAI.01716-07.

Papke, R. L.; Bencherif, M.; Lippiello, P. (1996): An evaluation of neuronal nicotinic acetylcholine receptor activation by quaternary nitrogen compounds indicates that choline is selective for the alpha7 subtype. *Neuroscience letters* 213 (3), S. 201–204. DOI: 10.1016/0304-3940(96)12889-5.

Papke, Roger L. (2014): Merging old and new perspectives on nicotinic acetylcholine receptors. *Biochemical pharmacology* 89 (1), S. 1–11. DOI: 10.1016/j.bcp.2014.01.029.

Papke, Roger L.; Andleeb, Hina; Stokes, Clare; Quadri, Marta; Horenstein, Nicole A. (2022a): Selective agonists and antagonists of α9 versus α7 nicotinic acetylcholine receptors. *ACS chemical neuroscience* 13 (5), S. 624–637. DOI: 10.1021/acschemneuro.1c00747.

Papke, Roger L.; Bagdas, Deniz; Kulkarni, Abhijit R.; Gould, Timothy; AlSharari, Shakir D.; Thakur, Ganesh A.; Damaj, M. Imad (2015): The analgesic-like properties of the alpha7 nAChR silent agonist NS6740 is associated with non-conducting conformations of the receptor. *Neuropharmacology* 91, S. 34–42. DOI: 10.1016/j.neuropharm.2014.12.002.

Papke, Roger L.; Chojnacka, Kinga; Horenstein, Nicole A. (2014): The minimal pharmacophore for silent agonism of the α7 nicotinic acetylcholine receptor. *Journal of pharmacology and experimental therapeutics* 350 (3), S. 665–680. DOI: 10.1124/jpet.114.215236.

Papke, Roger L.; Dwoskin, Linda P.; Crooks, Peter A. (2007): The pharmacological activity of nicotine and nornicotine on nAChRs subtypes: relevance to nicotine dependence and drug discovery. *Journal of neurochemistry* 101 (1), S. 160–167. DOI: 10.1111/j.1471-4159.2006.04355.x.

Papke, Roger L.; Karaffa, Madison; Horenstein, Nicole A.; Stokes, Clare (2022b): Coffee and cigarettes: Modulation of high and low sensitivity α4β2 nicotinic acetylcholine receptors by n-MP, a biomarker of coffee consumption. *Neuropharmacology* 216, S. 109173. DOI: 10.1016/j.neuropharm.2022.109173.

Papke, Roger L.; Porter Papke, Julia K. (2002): Comparative pharmacology of rat and human alpha7 nAChR conducted with net charge analysis. *British journal of pharmacology* 137 (1), S. 49–61. DOI: 10.1038/sj.bjp.0704833.

Papke, Roger L.; Quadri, Marta; Gulsevin, Alican (2023): Silent agonists for α7 nicotinic acetylcholine receptors. *Pharmacological research* 190, S. 106736. DOI: 10.1016/j.phrs.2023.106736.

Papke, Roger L.; Stokes, Clare (2010): Working with OpusXpress: methods for high volume oocyte experiments. *Methods* 51 (1), S. 121–133. DOI: 10.1016/j.ymeth.2010.01.012.

Papke, Roger L.; Stokes, Clare; Damaj, M. Imad; Thakur, Ganesh A.; Manther, Khan; Treinin, Millet et al. (2018): Persistent activation of α7 nicotinic ACh receptors associated with stable induction of different desensitized states. *British journal of pharmacology* 175 (11), S. 1838–1854. DOI: 10.1111/bph.13851.

Park, Y. I.; Buszko, M. L.; Gander, J. E. (1997): Utilization of phosphocholine from extracellular complex polysaccharide as a source of cytoplasmic choline derivatives in *Penicillium fellutanum*. *Journal of bacteriology* 179 (4), S. 1186–1192. DOI: 10.1128/jb.179.4.1186-1192.1997.

Parri, R. H.; Dineley, T. K. (2010): Nicotinic acetylcholine receptor interaction with beta-amyloid: molecular, cellular, and physiological consequences. *Current Alzheimer research* 7 (1), S. 27–39. DOI: 10.2174/156720510790274464.

Paul, Barnali; Sribhashyam, Sashrik; Majumdar, Susruta (2023): Opioid signaling and design of analgesics. *Progress in molecular biology and translational science* 195, S. 153–176. DOI: 10.1016/bs.pmbts.2022.06.017.

Peng, Huashan; Ferris, Robert L.; Matthews, Tonya; Hiel, Hakim; Lopez-Albaitero, Andres; Lustig, Lawrence R. (2004): Characterization of the human nicotinic acetylcholine receptor subunit alpha (alpha)9 (CHRNA9) and alpha (alpha)10 (CHRNA10) in lymphocytes. *Life sciences* 76 (3), S. 263–280. DOI: 10.1016/j.lfs.2004.05.031.

Pepys, Mark B.; Hirschfield, Gideon M. (2003): C-reactive protein: a critical update. *Journal of clinical investigation* 111 (12), S. 1805–1812. DOI: 10.1172/JCI18921.

Pereira, Edna F. R.; Hilmas, Corey; Santos, Mariton D.; Alkondon, Manickavasagom; Maelicke, Alfred; Albuquerque, Edson X. (2002): Unconventional ligands and modulators of nicotinic receptors. *Journal of neurobiology* 53 (4), S. 479–500. DOI: 10.1002/neu.10146.

Pettit, D. L.; Shao, Z.; Yakel, J. L. (2001): beta-Amyloid(1-42) peptide directly modulates nicotinic receptors in the rat hippocampal slice. *Journal of neuroscience* 21 (1), RC120. DOI: 10.1523/JNEUROSCI.21-01-j0003.2001.

Piccioli, Patrizia; Rubartelli, Anna (2013): The secretion of IL-1β and options for release. *Seminars in immunology* 25 (6), S. 425–429. DOI: 10.1016/j.smim.2013.10.007.

Pinkerton, James W.; Kim, Richard Y.; Robertson, Avril A. B.; Hirota, Jeremy A.; Wood, Lisa G.; Knight, Darryl A. et al. (2017): Inflammasomes in the lung. *Molecular immunology* 86, S. 44–55. DOI: 10.1016/j.molimm.2017.01.014.

Podo, Franca; Sardanelli, Francesco; Iorio, Egidio; Canese, Rossella; Carpinelli, Giulia; Fausto, Alfonso; Canevari, Silvana (2007): Abnormal choline phospholipid metabolism in breast and ovary cancer:molecular bses for nninvasive imging aproaches. *Current medical imaging* 3 (2), S. 123–137. DOI: 10.2174/157340507780619160.

Potempa, Lawrence A.; Rajab, Ibraheem M.; Olson, Margaret E.; Hart, Peter C. (2021): C-reactive protein and cancer: interpreting the differential bioactivities of its pentameric and monomeric, modified isoforms. *Frontiers in immunology* 12, S. 744129. DOI: 10.3389/fimmu.2021.744129.

Pritchard, P. H.; Vance, D. E. (1981): Choline metabolism and phosphatidylcholine biosynthesis in cultured rat hepatocytes. *The Biochemical journal* 196 (1), S. 261–267. DOI: 10.1042/bj1960261.

Puig, Kendra L.; Combs, Colin K. (2013): Expression and function of APP and its metabolites outside the central nervous system. *Experimental gerontology* 48 (7), S. 608–611. DOI: 10.1016/j.exger.2012.07.009.

Quadri, Marta; Bagdas, Deniz; Toma, Wisam; Stokes, Clare; Horenstein, Nicole A.; Damaj, M. Imad; Papke, Roger L. (2018): The antinociceptive and anti-inflammatory properties of the α7 nAChR weak

partial agonist p-CF3N,N-diethyl-N'-phenylpiperazine. *Journal of pharmacology and experimental therapeutics* 367 (2), S. 203–214. DOI: 10.1124/jpet.118.249904.

Quadri, Marta; Matera, Carlo; Silnović, Almin; Pismataro, Maria Chiara; Horenstein, Nicole A.; Stokes, Clare et al. (2017): Identification of α7 nicotinic acetylcholine receptor silent agonists based on the spirocyclic quinuclidine-Δ2 -isoxazoline scaffold: synthesis and electrophysiological evaluation. *ChemMedChem* 12 (16), S. 1335–1348. DOI: 10.1002/cmdc.201700162.

Quadri, Marta; Papke, Roger L.; Horenstein, Nicole A. (2016): Dissection of N,N-diethyl-N'-phenylpiperazines as α7 nicotinic receptor silent agonists. *Bioorganic & medicinal chemistry* 24 (2), S. 286–293. DOI: 10.1016/j.bmc.2015.12.017.

Quehenberger, Oswald; Armando, Aaron M.; Brown, Alex H.; Milne, Stephen B.; Myers, David S.; Merrill, Alfred H. et al. (2010): Lipidomics reveals a remarkable diversity of lipids in human plasma. *Journal of lipid research* 51 (11), S. 3299–3305. DOI: 10.1194/jlr.M009449.

Ramarao, M. K.; Cohen, J. B. (1998): Mechanism of nicotinic acetylcholine receptor cluster formation by rapsyn. *Proceedings of the National Academy of Sciences* 95 (7), S. 4007–4012. DOI: 10.1073/pnas.95.7.4007.

Razani-Boroujerdi, S.; Boyd, R. T.; Davila-Garcia, M. I.; Nandi, J. S.; Mishra, N. C.; Singh, S. P. et al. (2007): T cells express 7-nicotinic acetylcholine receptor subunits that require a functional TCR and leukocyte-specific protein tyrosine kinase for nicotine-induced Ca^{2+} response. *Journal of immunology* 179 (5), S. 2889–2898. DOI: 10.4049/jimmunol.179.5.2889.

Reale, M.; Kamal, M. A.; Velluto, L.; Gambi, D.; Di Nicola, M.; Greig, N. H. (2012): Relationship between inflammatory mediators, Aβ levels and ApoE genotype in Alzheimer disease. *Current Alzheimer research* 9 (4), S. 447–457. DOI: 10.2174/156720512800492549.

Richter, Katrin; Amati, Anca-Laura; Padberg, Winfried; Grau, Veronika (2022): Negative regulation of ATP-induced inflammasome activation and cytokine secretion by acute-phase proteins: A mini review. *Frontiers in pharmacology* 13, S. 981276. DOI: 10.3389/fphar.2022.981276.

Richter, Katrin; Grau, Veronika (2023): Signaling of nicotinic acetylcholine receptors in mononuclear phagocytes. *Pharmacological research* 191, S. 106727. DOI: 10.1016/j.phrs.2023.106727.

Risberg, A.; Masoud, H.; Martin, A.; Richards, J. C.; Moxon, E. R.; Schweda, E. K. (1999): Structural analysis of the lipopolysaccharide oligosaccharide epitopes expressed by a capsule-deficient strain of *Haemophilus influenzae* Rd. *European journal of biochemistry* 261 (1), S. 171–180. DOI: 10.1046/j.1432-1327.1999.00248.x.

Roher, Alex E.; Esh, Chera L.; Kokjohn, Tyler A.; Castaño, Eduardo M.; van Vickle, Gregory D.; Kalback, Walter M. et al. (2009): Amyloid beta peptides in human plasma and tissues and their significance for Alzheimer's disease. *Alzheimer's & dementia* 5 (1), S. 18–29. DOI: 10.1016/j.jalz.2008.10.004.

Romero, Haylie K.; Christensen, Sean B.; Di Cesare Mannelli, Lorenzo; Gajewiak, Joanna; Ramachandra, Renuka; Elmslie, Keith S. et al. (2017): Inhibition of α9α10 nicotinic acetylcholine receptors prevents chemotherapy-induced neuropathic pain. *Proceedings of the National Academy of Sciences* 114 (10), E1825-E1832. DOI: 10.1073/pnas.1621433114.

Rosas-Ballina, Mauricio; Olofsson, Peder S.; Ochani, Mahendar; Valdés-Ferrer, Sergio I.; Levine, Yaakov A.; Reardon, Colin et al. (2011): Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science* 334 (6052), S. 98–101. DOI: 10.1126/science.1209985.

Sadigh-Eteghad, Saeed; Talebi, Mahnaz; Farhoudi, Mehdi; Golzari, Samad E.J.; Sabermarouf, Babak; Mahmoudi, Javad (2014): Beta-amyloid exhibits antagonistic effects on alpha 7 nicotinic acetylcholine receptors in orchestrated manner. *Journal of medical hypotheses and ideas* 8 (2), S. 49–52. DOI: 10.1016/j.jmhi.2014.01.001.

Saito, Renata de Freitas; Andrade, Luciana Nogueira de Sousa; Bustos, Silvina Odete; Chammas, Roger (2022): Phosphatidylcholine-derived lipid mediators: the crosstalk between cancer cells and immune cells. *Frontiers in immunology* 13, S. 768606. DOI: 10.3389/fimmu.2022.768606.

Sander, Simon; Müller, Isabel; Garcia-Alai, Maria M.; Nicke, Annette; Tidow, Henning (2022): New insights into P2X7 receptor regulation: Ca2+-calmodulin and GDP bind to the soluble P2X7 ballast domain. *Journal of biological chemistry* 298 (10), S. 102495. DOI: 10.1016/j.jbc.2022.102495.

Santana, Patricia T.; Martel, Jan; Lai, Hsin-Chih; Perfettini, Jean-Luc; Kanellopoulos, Jean M.; Young, John D. et al. (2016): Is the inflammasome relevant for epithelial cell function? *Microbes and infection* 18 (2), S. 93–101. DOI: 10.1016/j.micinf.2015.10.007.

Schäcke, Heike; Döcke, Wolf Dietrich; Asadullah, Khusru (2002): Mechanisms involved in the side effects of glucocorticoids. *Pharmacology & therapeutics* 96 (1), S. 23–43. DOI: 10.1016/s0163-7258(02)00297-8.

Schweda, E. K.; Brisson, J. R.; Alvelius, G.; Martin, A.; Weiser, J. N.; Hood, D. W. et al. (2000): Characterization of the phosphocholine-substituted oligosaccharide in lipopolysaccharides of type b *Haemophilus influenzae*. *European journal of biochemistry* 267 (12), S. 3902–3913. DOI: 10.1046/j.1432-1327.2000.01426.x.

Selkoe, D. J. (1994): Normal and abnormal biology of the beta-amyloid precursor protein. *Annual review of neuroscience* 17, S. 489–517. DOI: 10.1146/annurev.ne.17.030194.002421.

Shariff, Masroor; Quik, Maryka; Holgate, Joan; Morgan, Michael; Patkar, Omkar L.; Tam, Vincent et al. (2016): Neuronal nicotinic acetylcholine receptor modulators reduce sugar intake. *PloS one* 11 (3), e0150270. DOI: 10.1371/journal.pone.0150270.

Shen, B. Q.; Finkbeiner, W. E.; Wine, J. J.; Mrsny, R. J.; Widdicombe, J. H. (1994): Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl^- secretion. *American journal of physiology* 266 (5 Pt 1), L493-501. DOI: 10.1152/ajplung.1994.266.5.L493.

Siebers, Kathrin; Fink, Bijan; Zakrzewicz, Anna; Agné, Alisa; Richter, Katrin; Konzok, Sebastian et al. (2018): Alpha-1 antitrypsin inhibits ATP-mediated release of interleukin-1β via CD36 and nicotinic acetylcholine receptors. *Frontiers in immunology* 9, S. 877. DOI: 10.3389/fimmu.2018.00877.

Simard, Alain R.; Gan, Yan; St-Pierre, Stéphanie; Kousari, Ariana; Patel, Varun; Whiteaker, Paul et al. (2013): Differential modulation of EAE by α9*- and β2*-nicotinic acetylcholine receptors. *Immunology and cell biology* 91 (3), S. 195–200. DOI: 10.1038/icb.2013.1.

Simpson, Jodie L.; Baines, Katherine J.; Horvat, Jay C.; Essilfie, Ama-Tawiah; Brown, Alexandra C.; Tooze, Melinda et al. (2016): COPD is characterized by increased detection of Haemophilus influenzae, Streptococcus pneumoniae and a deficiency of Bacillus species. *Respirology* 21 (4), S. 697–704. DOI: 10.1111/resp.12734.

Sinha, S.; Lieberburg, I. (1999): Cellular mechanisms of beta-amyloid production and secretion. *Proceedings of the National Academy of Sciences* 6 (20), S. 11049–11053. DOI: 10.1073/pnas.96.20.11049.

Sondag, C. M.; Combs, C. K. (2006): Amyloid precursor protein cross-linking stimulates beta amyloid production and pro-inflammatory cytokine release in monocytic lineage cells. *Journal of neurochemistry* 97 (2), S. 449–461. DOI: 10.1111/j.1471-4159.2006.03759.x.

Sondag, Cindy M.; Combs, Colin K. (2004): Amyloid precursor protein mediates proinflammatory activation of monocytic lineage cells. *Journal of biological chemistry* 279 (14), S. 14456–14463. DOI: 10.1074/jbc.M313747200.

Sondag, Cindy M.; Combs, Colin K. (2010): Adhesion of monocytes to type I collagen stimulates an APP-dependent proinflammatory signaling response and release of Abeta1-40. *Journal of neuroinflammation* 7, S. 22. DOI: 10.1186/1742-2094-7-22.

Souza, Anelise Cristina Parras; Souza, Camilla Mendes; Amaral, Camila Libardi; Lemes, Simone Ferreira; Santucci, Leticia Foglia; Milanski, Marciane et al. (2019): Short-term high-fat diet consumption reduces hypothalamic expression of the nicotinic acetylcholine receptor α7 subunit (α7nAChR) and affects the anti-inflammatory response in a mouse model of sepsis. *Frontiers in immunology* 10, S. 565. DOI: 10.3389/fimmu.2019.00565.

Sproston, Nicola R.; Ashworth, Jason J. (2018): Role of C-reactive protein at sites of inflammation and infection. *Frontiers in immunology* 9, S. 754. DOI: 10.3389/fimmu.2018.00754.

Stegemann, Agatha; Böhm, Markus (2020): Targeting the α7 nicotinic acetylcholine receptor-A novel road towards the future treatment of skin diseases. *Experimental dermatology* 29 (9), S. 924–931. DOI: 10.1111/exd.14173.

Stokes, Clare; Treinin, Millet; Papke, Roger L. (2015): Looking below the surface of nicotinic acetylcholine receptors. *Trends in pharmacological sciences* 36 (8), S. 514–523. DOI: 10.1016/j.tips.2015.05.002.

St-Pierre, Stéphanie; Jiang, Wei; Roy, Patrick; Champigny, Camille; LeBlanc, Éric; Morley, Barbara J. et al. (2016): Nicotinic acetylcholine receptors modulate bone marrow-derived pro-inflammatory monocyte production and survival. *PloS one* 11 (2), e0150230. DOI: 10.1371/journal.pone.0150230.

Takata, Kazuyuki; Kitamura, Yoshihisa; Saeki, Mana; Terada, Maki; Kagitani, Sachiko; Kitamura, Risa et al. (2010): Galantamine-induced amyloid-{beta} clearance mediated via stimulation of microglial nicotinic acetylcholine receptors. *Journal of biological chemistry* 285 (51), S. 40180–40191. DOI: 10.1074/jbc.M110.142356.

Tan, Yixin; Chen, Quanzhu; Li, Xiaoling; Zeng, Zhaoyang; Xiong, Wei; Li, Guiyuan et al. (2021): Pyroptosis: a new paradigm of cell death for fighting against cancer. *Journal of experimental & clinical cancer research : CR* 40 (1), S. 153. DOI: 10.1186/s13046-021-01959-x.

Thinakaran, Gopal; Koo, Edward H. (2008): Amyloid precursor protein trafficking, processing, and function. *Journal of biological chemistry* 283 (44), S. 29615–29619. DOI: 10.1074/jbc.R800019200.

Thomsen, Morten S.; Mikkelsen, Jens D. (2012): The α7 nicotinic acetylcholine receptor ligands methyllycaconitine, NS6740 and GTS-21 reduce lipopolysaccharide-induced TNF-α release from microglia. *Journal of neuroimmunology* 251 (1-2), S. 65–72. DOI: 10.1016/j.jneuroim.2012.07.006.

Tian, Xiaoli; Sun, He; Casbon, Amy-Jo; Lim, Edward; Francis, Kevin P.; Hellman, Judith; Prakash, Arun (2017): NLRP3 inflammasome mediates dormant neutrophil recruitment following sterile lung injury and protects against subsequent bacterial pneumonia in mice. *Frontiers in immunology* 8, S. 1337. DOI: 10.3389/fimmu.2017.01337.

Toma, Wisam; Ulker, Esad; Alqasem, Mashael; AlSharari, Shakir D.; McIntosh, J. Michael; Damaj, M. Imad (2020): Behavioral and molecular basis of cholinergic modulation of pain: focus on nicotinic acetylcholine receptors. *Current topics in behavioral neurosciences* 45, S. 153–166. DOI: 10.1007/7854_2020_135.

Torzewski, Michael; Waqar, Ahmed Bilal; Fan, Jianglin (2014): Animal models of C-reactive protein. *Mediators of inflammation* 2014, S. 683598. DOI: 10.1155/2014/683598.

Tsoyi, Konstantin; Jang, Hwa Jin; Kim, Jong Woo; Chang, Hong Kyung; Lee, Young Soo; Pae, Hyun-Ock et al. (2011): Stimulation of alpha7 nicotinic acetylcholine receptor by nicotine attenuates inflammatory response in macrophages and improves survival in experimental model of sepsis through heme oxygenase-1 induction. *Antioxidants & redox signaling* 14 (11), S. 2057–2070. DOI: 10.1089/ars.2010.3555.

Ullian, E. M.; McIntosh, J. M.; Sargent, P. B. (1997): Rapid synaptic transmission in the avian ciliary ganglion is mediated by two distinct classes of nicotinic receptors. *Journal of neuroscience* 17 (19), S. 7210–7219. DOI: 10.1523/JNEUROSCI.17-19-07210.1997.

van Parys, Anthea; Karlsson, Therese; Vinknes, Kathrine J.; Olsen, Thomas; Øyen, Jannike; Dierkes, Jutta et al. (2021): Food sources contributing to intake of choline and individual choline forms in a

Norwegian cohort of patients with stable angina pectoris. *Frontiers in nutrition* 8, S. 676026. DOI: 10.3389/fnut.2021.676026.

Verbitsky, M.; Rothlin, C. V.; Katz, E.; Elgoyhen, A. B. (2000): Mixed nicotinic-muscarinic properties of the alpha9 nicotinic cholinergic receptor. *Neuropharmacology* 39 (13), S. 2515–2524. DOI: 10.1016/s0028-3908(00)00124-6.

Vladimer, Gregory I.; Marty-Roix, Robyn; Ghosh, Shubhendu; Weng, Dan; Lien, Egil (2013): Inflammasomes and host defenses against bacterial infections. *Current opinion in microbiology* 16 (1), S. 23–31. DOI: 10.1016/j.mib.2012.11.008.

Wagner, C. A.; Friedrich, B.; Setiawan, I.; Lang, F.; Bröer, S. (2000): The use of *Xenopus laevis* oocytes for the functional characterization of heterologously expressed membrane proteins. *Cellular physiology and biochemistry* 10 (1-2), S. 1–12. DOI: 10.1159/000016341.

Wang, H. Y.; Lee, D. H.; D'Andrea, M. R.; Peterson, P. A.; Shank, R. P.; Reitz, A. B. (2000a): beta-Amyloid(1-42) binds to alpha7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology. *Journal of biological chemistry* 275 (8), S. 5626–5632. DOI: 10.1074/jbc.275.8.5626.

Wang, H. Y.; Lee, D. H.; Davis, C. B.; Shank, R. P. (2000b): Amyloid peptide Abeta(1-42) binds selectively and with picomolar affinity to alpha7 nicotinic acetylcholine receptors. *Journal of neurochemistry* 75 (3), S. 1155–1161. DOI: 10.1046/j.1471-4159.2000.0751155.x.

Wang, Pu; Wei, Mengyan; Zhu, Xiufang; Liu, Yangong; Yoshimura, Kenshi; Zheng, Mingqi et al. (2021): Nitric oxide down-regulates voltage-gated Na+ channel in cardiomyocytes possibly through S-nitrosylation-mediated signaling. *Scientific reports* 11 (1), S. 11273. DOI: 10.1038/s41598-021-90840-0.

Wang, Qi; Wu, Yi-Jun (2011): Lysophosphatidylcholine induces Ca(2+) mobilization in Jurkat human T lymphocytes and CTLL-2 mouse T lymphocytes by different pathways. *European journal of pharmaceutical sciences* 44 (5), S. 602–609. DOI: 10.1016/j.ejps.2011.10.007.

Wessler, I.; Kilbinger, H.; Bittinger, F.; Kirkpatrick, C. J. (2001): The biological role of non-neuronal acetylcholine in plants and humans. *Japanese journal of pharmacology* 85 (1), S. 2–10. DOI: 10.1254/jjp.85.2.

Wessler, I.; Kirkpatrick, C. J. (2008): Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. *British journal of pharmacology* 154 (8), S. 1558–1571. DOI: 10.1038/bjp.2008.185.

Wessler, I.; Kirkpatrick, C. J.; Racké, K. (1999): The cholinergic 'pitfall': acetylcholine, a universal cell molecule in biological systems, including humans. *Clinical and experimental pharmacology & physiology* 26 (3), S. 198–205. DOI: 10.1046/j.1440-1681.1999.03016.x.

Westphal, Martin; Kampmeier, Tim (2015): The race against the "septic shark". *Critical care* 19 Suppl 3 (Suppl 3), S11. DOI: 10.1186/cc14729.

Whiteaker, Paul; Marks, Michael J.; Christensen, Sean; Dowell, Cheryl; Collins, Allan C.; McIntosh, J. Michael (2008): Synthesis and characterization of 125I-alpha-conotoxin ArIBV11L;V16A, a selective alpha7 nicotinic acetylcholine receptor antagonist. *Journal of pharmacology and experimental therapeutics* 325 (3), S. 910–919. DOI: 10.1124/jpet.108.136895.

Williams, Dustin K.; Wang, Jingyi; Papke, Roger L. (2011): Investigation of the molecular mechanism of the α7 nicotinic acetylcholine receptor positive allosteric modulator PNU-120596 provides evidence for two distinct desensitized states. *Molecular pharmacology* 80 (6), S. 1013–1032. DOI: 10.1124/mol.111.074302.

Wu, J.; Harata, N.; Akaike, N. (1994): Sevoflurane-induced ionic current in acutely dissociated CA1 pyramidal neurons of the rat hippocampus. *Brain research* 645 (1-2), S. 303–308. DOI: 10.1016/0006-8993(94)91664-0.

Wu, Jenny C. F.; Chruscinski, Andrzej; Jesus Perez, Vinicio A. de; Singh, Harvir; Pitsiouni, Maria; Rabinovitch, Marlene et al. (2009): Cholinergic modulation of angiogenesis: role of the 7 nicotinic acetylcholine receptor. *Journal of cellular biochemistry* 108 (2), S. 433–446. DOI: 10.1002/jcb.22270.

Wu, Jie; Kuo, Yen-Ping; George, Andrew A.; Xu, Lin; Hu, Jun; Lukas, Ronald J. (2004): beta-Amyloid directly inhibits human alpha4beta2-nicotinic acetylcholine receptors heterologously expressed in human SH-EP1 cells. *Journal of biological chemistry* 279 (36), S. 37842–37851. DOI: 10.1074/jbc.M400335200.

Young, Gareth T.; Zwart, Ruud; Walker, Alison S.; Sher, Emanuele; Millar, Neil S. (2008): Potentiation of alpha7 nicotinic acetylcholine receptors via an allosteric transmembrane site. *Proceedings of the National Academy of Sciences* 105 (38), S. 14686–14691. DOI: 10.1073/pnas.0804372105.

Young, N. Martin; Foote, Simon J.; Wakarchuk, Warren W. (2013): Review of phosphocholine substituents on bacterial pathogen glycans: synthesis, structures and interactions with host proteins. *Molecular immunology* 56 (4), S. 563–573. DOI: 10.1016/j.molimm.2013.05.237.

Yu, Rilei; Kompella, Shiva N.; Adams, David J.; Craik, David J.; Kaas, Quentin (2013): Determination of the α-conotoxin Vc1.1 binding site on the α9α10 nicotinic acetylcholine receptor. *Journal of medicinal chemistry* 56 (9), S. 3557–3567. DOI: 10.1021/jm400041h.

Zakrzewicz, Anna; Richter, Katrin; Zakrzewicz, Dariusz; Siebers, Kathrin; Damm, Jelena; Agné, Alisa et al. (2019): SLPI inhibits ATP-mediated maturation of IL-1β in human monocytic leukocytes: a novel function of an old player. *Frontiers in immunology* 10, S. 664. DOI: 10.3389/fimmu.2019.00664.

Zhang, H. Q.; Fast, W.; Marletta, M. A.; Martasek, P.; Silverman, R. B. (1997): Potent and selective inhibition of neuronal nitric oxide synthase by N omega-propyl-L-arginine. *Journal of medicinal chemistry* 40 (24), S. 3869–3870. DOI: 10.1021/jm970550g.

Zhang, Wenji; Lin, Hui; Zou, Mingmin; Yuan, Qinghua; Huang, Zhenrui; Pan, Xiaoying; Zhang, Wenjuan (2022): Nicotine in inflammatory diseases: anti-inflammatory and pro-inflammatory effects. *Frontiers in immunology* 13, S. 826889. DOI: 10.3389/fimmu.2022.826889.

Zhang, Yuan; Jen, Freda E-C; Fox, Kate L.; Edwards, Jennifer L.; Jennings, Michael P. (2023a): The biosynthesis and role of phosphorylcholine in pathogenic and nonpathogenic bacteria. *Trends in microbiology* 31 (7), S. 692–706. DOI: 10.1016/j.tim.2023.01.006.

Zhang, Yun; Chen, Huaqiu; Li, Ran; Sterling, Keenan; Song, Weihong (2023b): Amyloid β-based therapy for Alzheimer's disease: challenges, successes and future. *Signal transduction and targeted therapy* 8 (1), S. 248. DOI: 10.1038/s41392-023-01484-7.

Zhou, Hai-Hong; Tang, Yu-Long; Xu, Tian-Hao; Cheng, Bin (2024): C-reactive protein: structure, function, regulation, and role in clinical diseases. *Frontiers in immunology* 15, S. 1425168. DOI: 10.3389/fimmu.2024.1425168.

Zoli, Michele; Pucci, Susanna; Vilella, Antonietta; Gotti, Cecilia (2018): Neuronal and extraneuronal nicotinic acetylcholine receptors. *Current neuropharmacology* 16 (4), S. 338–349. DOI: 10.2174/1570159X15666170912110450.

Zouridakis, Marios; Papakyriakou, Athanasios; Ivanov, Igor A.; Kasheverov, Igor E.; Tsetlin, Victor; Tzartos, Socrates; Giastas, Petros (2019): Crystal structure of the monomeric extracellular domain of α9 nicotinic receptor subunit in complex with α-conotoxin RgIA: molecular dynamics insights into RgIA binding to α9α10 nicotinic receptors. *Frontiers in pharmacology* 10, S. 474. DOI: 10.3389/fphar.2019.00474.

7. Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Habilitationsschrift über „**Cholinerge Regulation der ATP-vermittelten Freisetzung des pro-inflammatorischen Zytokins Interleukin-1β“** selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe, insbesondere, dass alle Entlehnungen aus anderen Schriften mit Angabe der betreffenden Schrift gekennzeichnet sind.

Ich versichere, nicht die Hilfe einer kommerziellen Habilitations- oder Promotionsvermittlung in Anspruch genommen zu haben.

Gießen, den 31.10.2024

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Katrin Richter

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9. Anhang - Publikationen

1. Hecker A., Küllmar M., Wilker S., **Richter K.**, Atanasova S., Timm T., Zakrzewicz A., Klein J., Kaufmann A., Bauer S., Padberg W., Kummer W., Jaciauskiene S., Fronius M., Schweda E.K.H., Lochnit G., Grau V. (2015). Phosphocholine-modified macromolecules and canonical nicotinic agonists inhibit ATP-induced IL-1β release. *Journal of immunology* 195 (5), S. 2325-34. DOI: 10.4049/jimmunol.1400974.
2. Zakrzewicz A., **Richter K.**, Agné A., Wilker S., Siebers K., Fink B., Krasteva-Christ G., Althaus M., Padberg W., Hone A.J., McIntosh J.M., Grau V. (2017). Canonical and novel non-canonical cholinergic agonists inhibit ATP-induced release of monocytic interleukin-1β via different combinations of nicotinic acetylcholine receptor subunits α7, α9 and α10. *Frontiers in cellular neuroscience* 11, S. 189. DOI: 10.3389/fncel.2017.00189.
3. **Richter K.**, Mathes V., Fronius M., Althaus M., Hecker A., Krasteva-Christ G., Padberg W., Hone A.J., McIntosh J.M., Zakrzewicz A., Grau V. (2016). Phosphocholine – an agonist of metabotropic but not of ionotropic functions of α9-containing nicotinic acetylcholine receptors. *Scientific reports* 6, S. 28660. DOI:10.1038/srep28660.
4. Backhaus S., Zakrzewicz A., **Richter K.**, Damm J., Wilker S., Fuchs-Moll G., Küllmar M., Hecker A., Manzini I., Ruppert C., McIntosh J.M., Padberg W., Grau V. (2017). Surfactant inhibits ATP-induced release of interleukin-1β via nicotinic acetylcholine receptors. *Journal of lipid research* 58 (6), S. 1055-1066. DOI: 10.1194/jlr.M071506.
5. **Richter K.,** Koch C., Perniss A., Wolf P.M., Schweda E.K., Wichmann S., Wilker S., Magel I., Sander M., McIntosh J.M., Padberg M., Grau V. (2018a). Phosphocholine-modified lipooligosaccharides of *Haemophilus influenzae* inhibit ATP-induced IL-1β release by pulmonary epithelial cells. *Molecules* 23 (8), S. 1979. DOI: 10.3390/molecules23081979.
6. **Richter K.**, Sagawe S., Hecker A., Küllmar M., Askevold I., Damm J., Heldmann S., Pöhlmann M., Ruhrmann S., Sander M., Schlüter K.-D., Wilker S., König I.R., Kummer W., Padberg W., Hone A.J., McIntosh J.M., Zakrzewicz A., Koch C., Grau V. (2018b). C-reactive protein stimulates nicotinic acetylcholine receptors to control ATP-mediated monocytic inflammasome activation. *Frontiers in immunology* 9, S. 1604. DOI: 10.3389/fimmu.2018.01604.
7. **Richter K.**, Ogiemwonyi-Schaefer R.*, Wilker S., Chaveiro A.I., Agné A., Hecker M., Reichert M., Amati A.-L., Schlüter K.-D., Manzini I., Schmalzing G., McIntosh J.M., Padberg W., Grau V., Hecker A. (2020). Amyloid beta peptide ($A\beta_{1-42}$) reverses the cholinergic control of monocytic IL-1β release. *Journal of clinical medicine* 9 (9), S. 2887. DOI: 10.3390/jcm9092887.
8. **Richter K.**, Asci N., Singh V.K., Yakoob S.H., Meixner M., Zakrzewicz A., Liese J., Hecker A., Wilker S., Stumpf S., Schlüter K.-D., Rohde M., Gödecke A., Padberg W., Manzini I., Schmalzing G., Grau V. (2023a). Activation of endothelial NO synthase and P2X7 receptor modification mediates the cholinergic control of ATP-induced interleukin-1β release by mononuclear phagocytes. *Frontiers in immunology* 14, S. 1140592. DOI: 10.3389/fimmu.2023.1140592.

9. **Richter K.***, Papke R.L.*, Stokes C., Roy D.C., Espinosa E.S., Wolf P.M.K., Hecker A., Liese J., Singh V.K., Padberg W., Schlüter K.-D., Rohde M., McIntosh J.M., Morley B.J., Horenstein N.A., Grau V., Simard A.R. (2022). Comparison of the anti-inflammatory properties of two nicotinic acetylcholine receptor ligands, phosphocholine and *p*CF3-diEPP. *Frontiers in cellular neuroscience* 16, S. 779081. DOI: 10.3389/fncel.2022.779081.
10. **Richter K.**, Herz S.M., Stokes C., Damaj M.I., Grau V., Papke R.L. (2023b). Pharmacological profiles and anti-inflammatory activity of *p*CN-diEPP and *m*CN-diEPP, new alpha9alpha10 nicotinic receptor ligands. *Neuropharmacology* 240, S. 109717. DOI: 10.1016/j.neuropharm.2023.109717.

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Phosphocholine-Modified Macromolecules and Canonical Nicotinic Agonists Inhibit ATP-Induced IL-1β Release

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IL-1β is a potent proinflammatory cytokine of the innate immune system that is involved in host defense against infection. However, increased production of IL-1β plays a pathogenic role in various inflammatory diseases, such as rheumatoid arthritis, gout, sepsis, stroke, and transplant rejection. To prevent detrimental collateral damage, IL-1β release is tightly controlled and typically requires two consecutive danger signals. LPS from Gram-negative bacteria is a prototypical first signal inducing pro–IL-1β synthesis, whereas extracellular ATP is a typical second signal sensed by the ATP receptor P2X7 that triggers activation of the NLRP3-containing inflammasome, proteolytic cleavage of pro–IL-1β by caspase-1, and release of mature IL-1β. Mechanisms controlling IL-1β release, even in the presence of both danger signals, are needed to protect from collateral damage and are of therapeutic interest. In this article, we show that acetylcholine, choline, phosphocholine, phosphocholine-modified LPS from *Haemophilus influenzae*, and phosphocholine-modified protein efficiently inhibit ATP-mediated IL-1β release in human and rat monocytes via nicotinic acetylcholine receptors containing subunits α7, α9, and/or α10. Of note, we identify receptors for phosphocholine-modified macromolecules that are synthesized by microbes and eukaryotic parasites and are well-known modulators of the immune system. Our data suggest that an endogenous anti-inflammatory cholinergic control mechanism effectively controls ATP-mediated release of IL-1β and that the same mechanism is used by symbionts and misused by parasites to evade innate immune responses of the host. *The Journal of Immunology*, 2015, 195: 000–000.

Interleukin-1β is a multifunctional proinflammatory cytokine of the innate immune system that plays an important role in host defense (1). However, excessive release is hazardous because high systemic levels of IL-1β cause fever, shock, and organ damage. Thus, increased production and release of IL-1β play a pathogenic role in various inflammatory diseases, such as rheumatoid arthritis, sepsis, stroke, and transplant rejection (2). To prevent detrimental collateral damage, IL-1β release typically requires two consecutive danger signals (3, 4). LPS from Gram-negative bacteria is a prototypical first signal inducing pro–IL-1β synthesis. Extracellular ATP is a typical second signal sensed by the ATP-receptor P2X7 that triggers activation of the NLRP3-containing inflammasome, proteolytic cleavage of pro–IL-1β by caspase-1, and release of mature IL-1β (3–5). Mechanisms controlling IL-1β release are needed to protect the host from excessive inflammation but are largely unexplored.

Acetylcholine (ACh) was the first neurotransmitter to be identified, but it is also a regulator of the immune system with pro- and anti-inflammatory potential (6, 7). The first data pointing to an anti-inflammatory role of ACh date back to the year 2000, when Borovikova et al. (8) demonstrated that vagus nerve stimulation attenuates the systemic release of TNF in response to LPS. Furthermore, these investigators described the α-conotoxin–sensitive inhibition of the secretion of TNF, IL-1β, IL-6, and IL-18 from LPS-primed human macrophages in vitro (8). The current notion prevails that vagal stimulation triggers neuronal release of norepinephrine from the spleen that stimulates the release of ACh from splenic memory T cells. The released ACh is sensed by splenic macrophages via nicotinic receptors for ACh containing the α7 subunit (CHRNA7) (7, 9), leading to changes in cytokine expression via the Jak2-STAT3 signaling pathway (7, 10). This pathway is suggested to downmodulate LPS-induced expression of pro–IL-1β but not its cleavage or release.

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Abbreviations used in this article: ACh, acetylcholine; BzATP, 2′(3′)-*O*-(4-benzoylbenzoyl)ATP trieethylammonium salt; CHRNA7, nicotinic receptor for ACh containing the α7 subunit; CHRNA9, nicotinic receptor for ACh containing the α9 subunit; CHRNA10, nicotinic receptor for ACh containing the α10 subunit; LDH, lactate dehydrogenase; PC, phosphocholine; PC-BSA, PC-modified BSA; PC-LPS, PC-modified LPS; siRNA, small interfering RNA.

We demonstrated that activated monocytes accumulating in blood vessels of rat renal grafts produce ACh during acute rejection (11, 12). Because monocytes express nicotinic receptors for ACh, endogenously produced ACh might act as an autocrine or paracrine regulator of innate immunity. Interestingly, nicotinic ACh receptors do not function as ligand gated ion channels in primary rat monocytes and alveolar macrophages but rather as metabotropic receptors reducing ATP-induced intracellular calcium signals (11, 13). This led us to speculate that endogenous ACh also inhibits ATP-mediated inflammasome activation.

Strikingly, numerous bacteria and eukaryotic parasites colonizing mammals express cell wall components and secretory macromolecules modified with phosphocholine (PC), a molecule with structural similarity to ACh. PC-modified macromolecules exert strong anti-inflammatory effects on innate and adaptive immunity and help to evade the immune system of the host (14, 15). For example, extracellular bacteria chronically colonizing the human upper respiratory tract, such as the pathogen *Haemophilus influenzae*, carry PC-modifications on their LPS (16). PC-modified LPS (PC-LPS) is a virulence factor that depends on genes encoded in the *lic1* operon (15); however, the exact molecular mechanism of its anti-inflammatory action is still elusive.

In this study, we tested the hypotheses that cholinergic agonists inhibit ATP signaling in monocytes and limit the release of mature IL-1β into the circulation and that PC-modified macromolecules produced by pathogens also inhibit IL-1β release by triggering the suggested anti-inflammatory mechanism. We propose that this powerful endogenous pathway that protects the host from IL-1β–induced damage can be used by pathogens to evade host immunity.

Materials and Methods

Mononuclear blood leukocytes from experimental rat renal allografts

Experimental animals received humane care according to the National Institute's of Health's *Guide for the Care and Use of Laboratory Animals*. Animal experiments were approved by the local committee at the Regierungspräsidium Giessen, Hesse, Germany (permit number GI20/10 Nr. 23/2008). Orthotopic transplantation of allogeneic rat Dark Agouti kidneys to Lewis recipients, perfusion of graft blood vessels to isolate intravascular graft leukocytes, and Percoll gradient centrifugation to enrich for mononuclear leukocytes were performed 2 d after transplantation, as described previously (17). A total of 5×10^5 mononuclear cells was incubated for 3 h in 24-well plates at 37°C, 5% CO_2, in RPMI 1640 supplemented with 10% FCS (FCS gold) and 2 mM L-glutamine (all from PAA Laboratories, Cölbe, Germany). Thereafter, 2′(3′)-*O*-(4-benzoylbenzoyl)ATP trieethylammonium salt (BzATP; 100 μM; Sigma-Aldrich, Taufkirchen, Germany) was added in the presence or absence of ACh chloride (10 or 100 μM), nicotine (100 μM), and cholinesterase from *Electrophorus electricus* (1 U/ml) (all from Sigma-Aldrich). Supernatants were collected and stored at −20°C before cytokines and lactate dehydrogenase (LDH) were measured.

U937 cells

The human histiocytic lymphoma cell line U937 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and maintained in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine. Cells in the log-phase of growth were transferred to 24-well plates (1×10^6 cells/ml and per well) and primed with 1 μg/ml LPS from *Escherichia coli* (L2654; Sigma-Aldrich) for 5 h. Thereafter, BzATP (100 μM) or nigericin (50 μM; Sigma-Aldrich) was added for 30 min in the presence or absence of different concentrations of cholinergic agonists and antagonists. ACh chloride, choline chloride, PC chloride calcium salt tetrahydrate, mecamylamine hydrochloride, nicotine, and strychnine hydrochloride were obtained from Sigma-Aldrich, and α-bungarotoxin was obtained from Tocris Bioscience (Bristol, U.K.). Unless otherwise stated, the following concentrations were used: ACh 100 μM, mecamylamine 100 μM, nicotine 100 μM, strychnine 10 μM, and α-bungarotoxin 1 μM. Supernatants were collected 30 min after the addition of BzATP or nigericin and stored at −20°C to measure IL-1β and LDH. IL-1β, IL-6, and TNF-α were measured in the supernatants by Quantikine Immunoassays (R&D Systems, Minneapolis, MN).

Human PBMCs

Studies on primary human cells were approved by the local ethics committee of the University of Giessen (No. 81/13). PBMCs were freshly isolated from heparinized blood of healthy nonsmoking male volunteers by LeucoSep gradients (Greiner Bio-One, Frickenhausen, Germany). A total of 5×10^5 PBMC/0.5 ml/well was incubated for 3 h in 24-well plates in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine. Thereafter, nonadherent cells were removed, cell culture medium was replaced, and BzATP (100 μM) was added in the presence or absence of nicotine (100 μM) and ACh (10 μM), and incubated for 30 min. Supernatants were collected and stored at −20°C before measurement of IL-1β and LDH.

Human monocytes

Blood was obtained from healthy donors and either separated directly by gradient centrifugation using LeucoSep gradients or pulsed with 0.5 ng LPS/ml before separation. Thereafter, monocytes were purified by positive selection using Dynabeads CD14 (Invitrogen, Karlsruhe, Germany), according to the supplier's instructions. Isolated monocytes were cultivated as described for PBMCs, with the exception that the medium was not replaced after 3 h of culture. Thereafter BzATP, nicotine, ACh, choline, and PC were added for 30 min, as described,

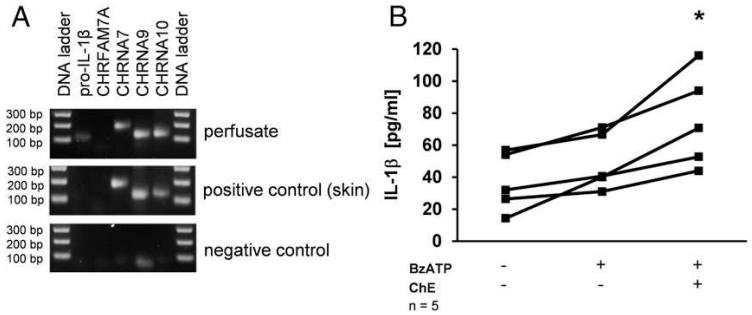

FIGURE 1. Endogenous ACh inhibits BzATP-induced release of IL-1β. Mononuclear leukocytes were isolated by intensive single-organ perfusion and Percoll density gradient centrifugation from the blood vessels of rat renal allografts on day 2 after transplantation in the Dark Agouti to Lewis rat strain combination. (**A**) Expression of pro–IL-1β and ACh receptor mRNA was investigated by real-time RT-PCR, and products were separated in agarose gels. The mRNA of pro–IL-1β, CHRNA7, CHRNA9, and CHRNA10 was detected, whereas the nonfunctional receptor CHRFAM7A was absent from mononuclear leukocytes and skin. In negative controls, in which cDNA was replaced by water, no product was obtained. Signals seen in the lower part of the gel are primer dimers. (**B**) BzATP only induced a modest increase in IL-1β release. When BzATP was combined with cholinesterase (ChE, 1 U/ml), release of IL-1β increased significantly. Data are presented as individual data points. *$p \leq 0.05$ versus BzATP alone, Wilcoxon signed-rank test.

and IL-1β, IL-6, and TNF-α were measured in the supernatant. The purity of the isolated monocytes was evaluated by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA) using FITC-labeled mAb M5E2 to CD14 (BioLegend, San Diego, CA); monocyte purity was >75%.

Cell viability

To test for cell viability, LDH released from dead cells was measured at the end of the experiments in cell culture supernatants by a Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI), according to the supplier's instructions, and compared with the total content of LDH in lysed cells. Irrespective of the reagents applied, LDH values typically remained <6% for U937 cells and <10% when primary cells were used.

RNA isolation and real-time RT-PCR

Total RNA was extracted using the QIAGEN RNeasy Miniprep Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. RNA samples were reverse transcribed using M-MLV Reverse Transcriptase (Promega, Mannheim, Germany). Real-time PCR was performed in an ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using Platinum SYBR Green qPCR Super Mix-UDG (Invitrogen). Signals were normalized to porphobilinogen deaminase. Primer pairs are shown in Supplemental Table I. Negative controls were included in each experiment, where the cDNA was replaced by water. The specificity of the PCR was confirmed by sequencing (SeqLab, Göttingen, Germany) and by separation in a 1.5% agarose gel. Gene expression was normalized to the housekeeping gene

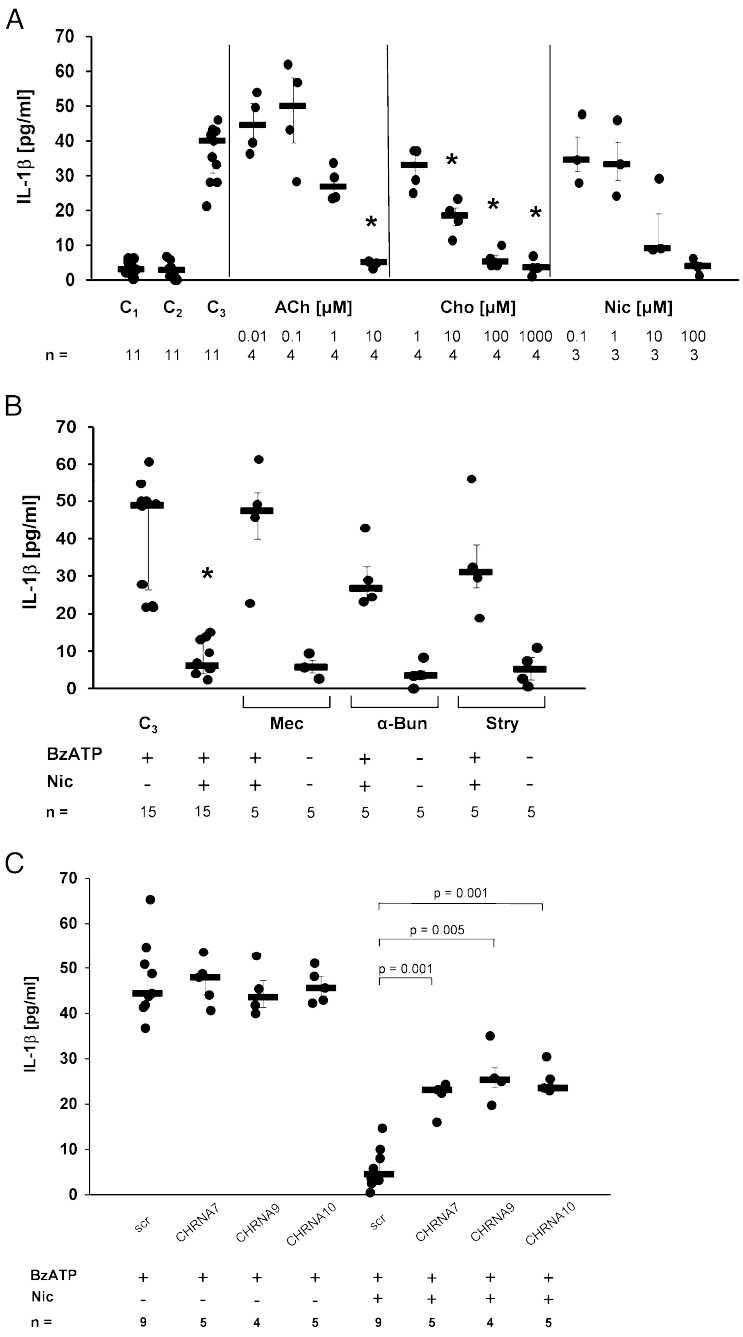

FIGURE 2. BzATP-mediated release of IL-1β from U937 cells is inhibited by activation of CHRNA7, CHRNA9, and/or CHRNA10. U937 cells were primed with LPS from *E. coli* for 5 h, and BzATP was added for another 30 min to trigger IL-1β release, which was measured by ELISA. Controls: supernatants from untreated cells (C1), from cells primed with LPS (C2), and from LPS-primed cells stimulated with BzATP (C3). (**A**) Addition of ACh, choline (Cho), and nicotine (Nic) dose dependently inhibited BzATP-induced IL-1β release. (**B**) The effect of nicotine was antagonized by mecamylamine (Mec), α-bungarotoxin (α-Bun), and strychnine (Stry). (**C**) Transfection of siRNA targeting CHRNA7, CHRNA9, and CHRNA10, but not of control siRNA (scr), blunted the effect of nicotine. *$p \leq 0.05$ versus C3, Kruskal–Wallis test, followed by the Mann–Whitney rank-sum test.

Table I. Concentrations of compounds causing an ~50% inhibition (IC_{50}) of BzATP-mediated release of IL-1β from LPS-primed U937 cells

Compound	IC_{50}
ACh	1 μM
Nicotine	10 μM
Choline	10 μM
PC	10 μM
PC-LPS RM118	25 nM
PC-LPS NTHi123323	25 nM
PC-BSA	140 nM

porphobilinogen deaminase and analyzed using the $2^{-\Delta Ct}$ method, and the mean values of the controls were set to one arbitrary unit.

Transfection of siRNA

The expression of CHRNA7, the nicotinic receptor for ACh containing the α9 subunit (CHRNA9), and the nicotinic receptor for ACh containing the α10 subunit (CHRNA10) in U937 cells was reduced using small interfering RNA (siRNA) technology. Cells were transfected with ON-TARGETplus human CHRNA7, CHRNA9, or CHRNA10 siRNA SMARTpool or with negative control ON-TARGETplus Non-targeting Control Pool (Thermo Fisher Scientific, Schwerte, Germany) to control for nonspecific gene inhibition. Cells were transfected with 30 pmol siRNA/1 × 10^6 cells using Amaxa Cell Line Nucleofector Kit C and Nucleofector II Device (both from Lonza Cologne, Cologne, Germany), according to the manufacturer's instructions. The siRNA-mediated downregulation of the target gene was assessed by real-time RT-PCR 6 h after transfection, according to the protocol provided by Lonza. Experiments on the release of IL-1β were performed 48 h after transfection.

Whole-cell patch-clamp recordings

For electrophysiological experiments, U937 cells were placed in coated (poly-L-lysine) cell culture dishes (Nunc, Roskilde, Denmark) containing bath solution and incubated for 5 h with LPS (1 μg/ml) at 37°C. Bath solution contained 5.4 mM KCl, 120 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid), and 25 mM glucose (pH 7.4). After incubation with LPS, dishes were mounted on an inverted microscope (Axiovert; Zeiss, Göttingen, Germany), and whole-cell recordings were performed at room temperature. Borosilicate glass capillaries (outer diameter 1.6 mm; Hilgenberg, Malsfeld, Germany) were pulled to patch pipettes with a resistance of 2–4 MΩ by an automated puller (Zeitz, Augsburg, Germany). Pipettes were filled with 120 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 11 mM EGTA, and 20 mM glucose (pH 7.3).

Membrane potential was clamped to −60 mV, and the resulting transmembrane currents were amplified with an EPC 9 amplifier and acquired via an ITC-16 interface with Pulse software (both from HEKA, Lambrecht, Germany). BzATP (100 μM), nicotine (100 μM), and mecamylamine (100 μM) were dissolved in bath solution and applied via a pressure-driven microperfusion system.

Purification and characterization of PC-LPS

Purification and characterization of LPS from the various strains followed a standard protocol (18). Briefly, LPS was isolated from bacterial cells using a phenol/chloroform/light-petroleum method and purified by subsequent ultracentrifugation. Detailed structural knowledge of LPS was obtained using high-field nuclear magnetic resonance and electrospray ionization–mass spectrometry techniques, along with composition and linkage analyses on *O*-deacylated LPS and oligosaccharide samples.

PC-modified BSA

Preparation of PC-modified BSA (PC-BSA) was described previously (19). Briefly, 25 mg (95 μmol) p-aminophenylphosphocholine (Biozol, Eching, Germany) was dissolved in 1 ml ice-cold 1 N hydrochloric acid (Roth, Karlsruhe, Germany). After the addition of 6.5 mg (95 μmol) sodium nitrite (Roth), the mixture was incubated for 10 min under gentle agitation at room temperature. In a second step, 0.5 ml (42.5 μmol) the freshly prepared diazonium phenylphosphorylcholine was added to a solution of 100 mg (1.5 μmol) BSA (Roth) in 5 ml boric acid (100 mM boric acid [pH 9], 150 mM NaCl; Merck, Darmstadt, Germany and Roth, respectively) and incubated under gentle agitation on ice for 12 h.

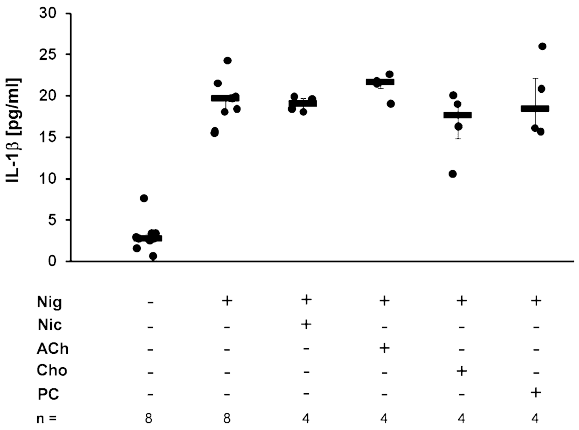

FIGURE 3. Nigericin-mediated release of IL-1β from U937 cells is not inhibited by nicotinic stimulation. U937 cells were primed with LPS for 5 h, and nigericin was added for another 30 min to trigger IL-1β release, which was measured by ELISA. Nigericin-induced (Nig) IL-1β release was unimpaired by nicotine (Nic), ACh, choline (Cho), and PC. Statistical analyses were performed by the Kruskal–Wallis test, followed by the Mann–Whitney rank-sum test.

Finally, PC-BSA was desalted using a Nap5-column (GE Healthcare Bio-Sciences, Uppsala, Sweden), according to the manufacturer's instructions, and stored at −20°C. The efficiency of coupling was quantified after the release of PC upon HF treatment. Choline was measured by HPLC, according to published methods (20). A total of 9 mol PC was incorporated per mol BSA.

PC residues were removed from PC-BSA by treatment with HF. Ten milligrams (150 nmol) of lyophilized PC-BSA was dissolved in 500 μl hydrofluoric acid (48%; Merck) and incubated on ice overnight. The sample was dried under a stream of nitrogen, dissolved in 500 μl water, and lyophilized.

Western blots

Human PBMCs were lysed, and protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein (15 μg) and prestained molecular mass standards (Precision Plus Protein Standards, dual color; Bio-Rad, Hercules, CA) were resolved in 15% reducing SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% skimmed milk powder and 0.01% Tween-20 in PBS. Mouse mAbs to IL-1β (kindly supplied by the National Cancer Institute, Frederick, MD) were diluted 1:20,000 in PBS and 5% skimmed milk powder, whereas polyclonal rabbit Abs to caspase-1 (#2225; Cell Signaling Technology, Danvers, MA) were diluted 1:1,000 in PBS and 2.5% skimmed milk powder. Mouse mAbs to β-actin (A2228; Sigma-Aldrich) were diluted 1:50,000 in PBS and Roti-Block (Roth). After washing in PBS, 0.01% Tween-20, HRP-conjugated rabbit anti-mouse Ig and goat anti-rabbit Ig Abs (both from Dako Cytomation, Glostrup, Denmark), diluted 1:5,000 in PBS, 2.5% skimmed milk powder, and 0.01% Tween-20, were used to detect primary Abs, followed by the chemiluminescent SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL) to detect IL-1β or by the Lumi-Light substrate (Roche, Mannheim, Germany) to detect β-actin. Blots were documented using a digital gel-documentation system (Biozym, Hessisch Oldendorf, Germany).

For the detection of secreted caspase-1 and IL-1β, human PBMCs were stimulated, as described, but in the absence of FCS. Cell culture supernatants were harvested and concentrated by a factor of 10 using Amicon Ultra centrifugal filters (Ultracel 10K; Merck Millipore, Darmstadt, Germany).

Statistics

Data were analyzed with SPSS software (Munich, Germany) by the nonparametric Kruskal–Wallis test, followed by the Mann–Whitney rank-sum test; $p \leq 0.05$ was considered statistically significant. Data obtained from primary leukocytes were analyzed by the Wilcoxon signed-rank test.

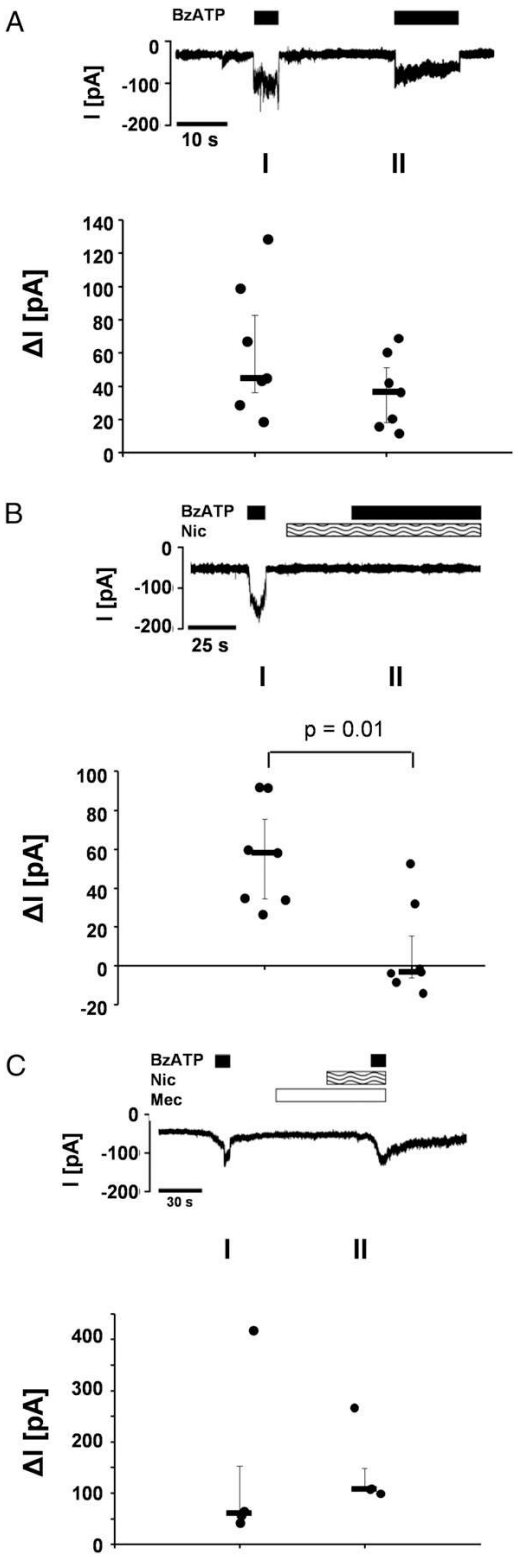

FIGURE 4. BzATP-mediated ion currents are inhibited by nicotine. U937 cells were primed with LPS from *E. coli* for 5 h, and ion fluxes were induced by BzATP. Patch-clamp recordings of U937 cells are depicted (*upper panels*). Two consecutive BzATP applications (I and II) resulted in repetitive current changes (**A**), which are abolished by nicotine (**B**). (**C**) This effect was antagonized by mecamylamine. Experimental groups were compared with the Mann–Whitney rank-sum test.

Results

Endogenous ACh inhibits IL-1β release

We isolated mononuclear leukocytes from the blood vessels of rat renal allografts. These cells expressed the mRNA of pro–IL-1β, as well as CHRNA7, CHRNA9, and CHRNA10, but not the nonfunctional receptor CHRFAMA7A (Fig. 1A). When stimulated with the P2X7-specific agonist BzATP, these leukocytes release only slightly increased amounts of IL-1β (without BzATP: median, 32 pg/ml, range: 14–56 pg/ml; with BzATP: median, 41 pg/ml, range: 31–71 pg/ml; $n = 5$ each). However, when BzATP was applied with cholinesterase, an enzyme that efficiently degrades ACh, significantly more IL-1β was detected in the cell culture supernatant (median, 71 pg/ml, range: 44–116 pg/ml, $n = 5$, $p = 0.05$ compared with BzATP alone) (Fig. 1B). The results from this first set of experiments supported our hypothesis that endogenous ACh inhibits BzATP-mediated release of IL-1β.

Nicotinic ACh receptor activation interferes with BzATP-induced release of IL-1β

In the next set of experiments, we used the human monocytic cell line U937. We primed these cells with LPS from *E. coli*, which resulted in increased pro–IL-1β mRNA levels (Supplemental Fig. 1A). As expected, LPS-primed U937 cells released IL-1β into the culture medium in response to treatment with BzATP for 30 min. However, application of ACh together with BzATP inhibited IL-1β secretion in a dose-dependent manner, whereas IL-6 and TNF-α levels in the supernatant were not reduced (Fig. 2A, data not shown). Of note, the viability of the cells was not impaired (Supplemental Table II).

Similar to ACh, choline and nicotine dose dependently inhibited the release of IL-1β in response to BzATP ($IC_{50} \sim 1$ μM for ACh, $IC_{50} \sim 10$ μM for nicotine and choline) (Fig. 2A, Table I). These results suggested the involvement of CHRNA7 homomers or receptors containing CHRNA9 (21, 22). In agreement with the results published by Chernyavsky et al. (23), mRNAs for CHRNA7, CHRNA9, and CHRNA10 were detected in unprimed and LPS-primed U937 cells. In addition, the inactive duplicate CHRFAM7A was readily detectable (Supplemental Fig. 1B).

Mecamylamine, α-bungarotoxin, and strychnine antagonized the inhibitory effect of nicotine (Fig. 2B), ACh, and choline (data not shown), indicating an involvement of CHRNA7, CHRNA9, and/or CHRNA10. To further corroborate the role of these receptors, we reduced their expression in U937 cells by siRNA (Supplemental Fig. 2A, 2B), which reduced the effect of nicotine by ~50% in all cases, whereas cells treated with control siRNA behaved like untreated cells (Fig. 2C, Supplemental Fig. 2C). Specific gene silencing was confirmed by real-time RT-PCR, which revealed the expected reduced expression of CHRNA9 and CHRNA10 (Supplemental Fig. 2A, 2B). However, the basal mRNA expression of CHRNA7 was too low for quantification. None of the siRNA treatments resulted in an unspecific downregulation of other receptors (Supplemental Fig. 2A, 2B). In view of the concerns raised with respect to the specificity of nicotinic receptor Abs (24), we refrained from attempts to monitor ACh receptor protein expression.

Nicotinic agonists inhibit BzATP-induced ion fluxes

We used the pore-forming toxin to investigate whether the BzATP-induced signaling cascade is inhibited upstream of the formation of

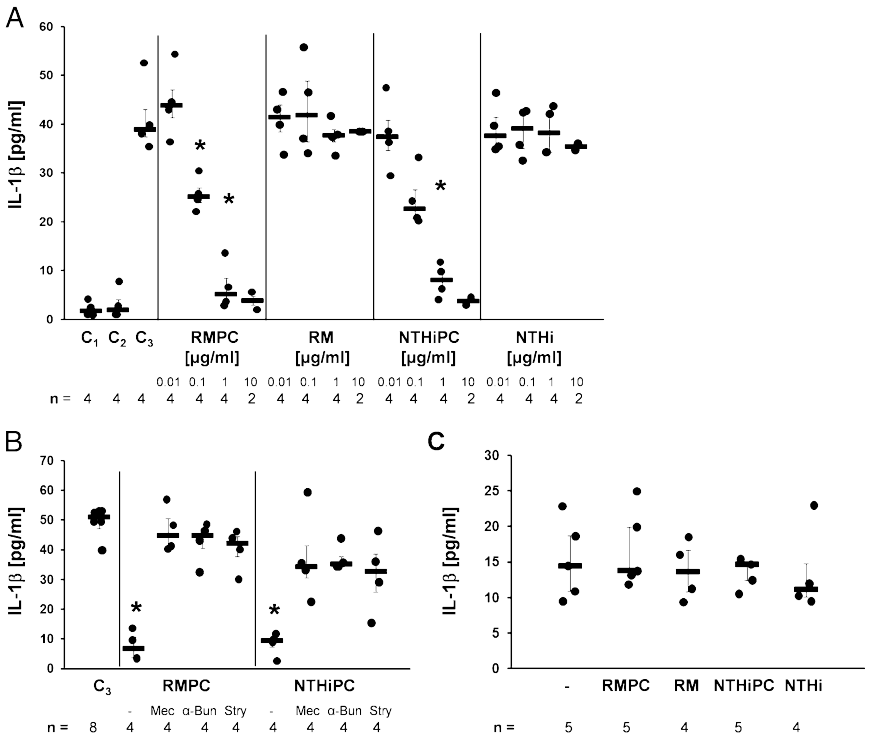

FIGURE 5. BzATP-mediated release of IL-1β from LPS-primed U937 cells is inhibited by PC-modified LPS. U937 cells were primed with LPS from *E. coli* for 5 h, and BzATP was added for another 30 min to trigger IL-1β release. Controls: supernatants from untreated cells (C1), from cells primed with LPS (C2), and from LPS-primed cells stimulated with BzATP (C3). (**A**) PC-LPS from *H. influenzae* strains RM118 (RMPC) and NTHi 1233 (NTHiPC) applied together with BzATP dose dependently inhibited the release of IL-1β. LPS from the corresponding *lic1* mutant strains RM7004-Lic1 (RM) and NTHi1233lic1 (NTHi), which lacks PC modification, was ineffective. (**B**) The inhibitory effect of both PC-LPS variants (1 μg/ml) was antagonized by mecamylamine (Mec), α-bungarotoxin (α-Bun), and strychnine (Stry). (**C**) Nigericin-induced IL-1β release was not influenced by PC-LPS. $^*p \leq 0.05$ versus C3, Kruskal–Wallis test, followed by the Mann–Whitney rank-sum test.

a functional inflammasome complex. Nigericin activates the inflammasome independent of BzATP nigericin and resulted in the release of IL-1β from LPS-primed U937 cells. Nigericin-induced release of IL-1β was unaffected by nicotine, Ach, and choline (Fig. 3).

To investigate the effect of nicotine on ATP-induced ion currents, patch-clamp experiments were performed in LPS-primed U937 cells. As expected, application of BzATP reproducibly induced ion currents (Fig. 4A), which were completely inhibited by nicotine (Fig. 4B). Mecamylamine, in turn, antagonized the effect of nicotine (Fig. 4C); however, nicotine alone did not induce currents.

PC and PC-modified macromolecules inhibit BzATP-induced IL-1β release

We tested whether PC-modified macromolecules also inhibit the release of IL-1β in response to BzATP. PC-LPS from two *H. influenzae* strains, RM118 and NTHi123323, dose dependently and most efficiently inhibited the BzATP-induced release of IL-1β (IC_{50} ~ 0.1 μg/ml, corresponding to ~25 nM PC-LPS) (Fig. 5A, Table I), whereas levels of IL-6 and TNF-α in the cell culture supernatant were not reduced (data not shown). LPS isolated from mutants lacking the PC modification, RM7004-lic1 and NTHi1233lic1 (25), was ineffective (Fig. 5A). The inhibitory effects of LPS from RM118 and NTHi123323 were sensitive to mecamylamine, α-bungarotoxin, and strychnine (Fig. 5B), which again suggested the involvement of CHRNA7, CHRNA9, and/or CHRNA10. Finally, PC-LPS did not change the release of IL-1β in response to nigericin (Fig. 5C). When LPS from RM118 and NTHi123323, as well as from the corresponding *lic1* mutants, was used for priming, mRNA expression of pro–IL-1β, IL-6, and TNF-α was close to baseline (data not shown). In contrast, stimulation with LPS from *E. coli* led to robust induction of cytokine mRNA expression.

Parasitic filarial nematodes secrete PC-modified proteins with anti-inflammatory functions. These proteins can be mimicked by artificial PC-modified proteins (26). We noted an efficient, dose-dependent inhibitory effect on the BzATP-induced release of IL-1β for PC-BSA with an IC_{50} ~10 μg/ml, corresponding to ~140 nM PC-BSA (Fig. 6A, Table I). The average PC:BSA stoichiometry was 9:1. Unmodified BSA and PC-BSA pretreated with hydrofluoric acid to remove the PC modification were inactive (Fig. 6B). In addition, free PC (IC_{50} ~ 10 μM) inhibited the BzATP-dependent release of IL-1β (Fig. 6A, Table I). The effects of PC, PC-LPS, and PC-BSA were antagonized by mecamylamine, α-bungarotoxin, and strychnine (Figs. 5B, 6A), suggesting an involvement of CHRNA7, CHRNA9, and/or CHRNA10. Again, nigericin-induced release of IL-1β was not impaired by PC (Fig. 3).

Nicotinic agonists decrease IL-1β release from primary human blood cells

To confirm that BzATP-mediated release of IL-1β can be inhibited by agonists of CHRNA9 in primary leukocytes, we analyzed PBMCs isolated from healthy human donors. As expected, these cells expressed very low levels of pro–IL-1β mRNA (data not shown). After cell isolation and culture for 3 h, PBMCs expressed the mRNA of pro–IL-1β, CHRNA7, CHRNA9, and CHRNA10 (Supplemental Fig. 1C) and released considerable amounts of IL-1β in response to BzATP (median, 1559 pg/ml, range: 736–7325 pg/ml, n = 5). Addition of ACh or nicotine significantly reduced the release of IL-1β (Fig. 7A). As expected, Western blotting revealed that BzATP reduced the cellular content of pro–IL-1β, and this reduction was attenuated by ACh, nicotine, and PC (Fig. 7B). Mature IL-1β was absent from cell lysates but was detected in cell culture supernatants upon stimulation with BzATP. Release of IL-1β was attenuated in the presence of cholinergic agonists (Fig. 7B, Fig. 8). In the same line, procaspase-1 was detected in cell lysates and active caspase-1 was detected in cell culture

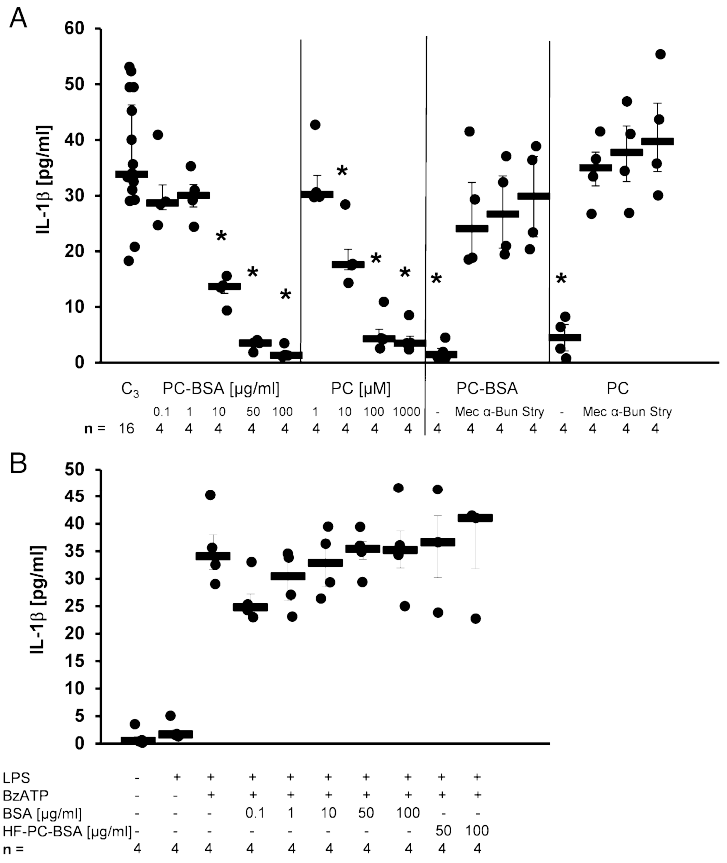

FIGURE 6. BzATP-mediated release of IL-1β from LPS-primed U937 cells is inhibited by PC-BSA and free PC. U937 cells were primed with LPS from *E. coli* for 5 h, and BzATP was added for another 30 min to trigger IL-1β release. (**A**) PC-BSA and free PC dose dependently inhibited BzATP-triggered IL-1β release. The inhibitory effects of PC-BSA (50 μg/ml) and free PC (100 μm) were antagonized by mecamylamine (Mec), α-bungarotoxin (α-Bun), and strychnine (Stry). (**B**) BSA from the same batch and hydrofluoric acid–treated PC-modified BSA (HF-PC-BSA) did not inhibit BzATP-mediated release of IL-1β. U937 cells were primed with LPS. Addition of BzATP (100 μM) for 30 min resulted in a release of IL-1β, which was measured in the cell culture supernatant. Different concentrations of unmodified BSA and HF-PC-BSA were used. *$p \leq 0.05$ versus C3, Kruskal–Wallis test, followed by the Mann–Whitney rank-sum test. C3, supernatants from LPS-primed cells stimulated with BzATP.

supernatants in response to BzATP. Similar to IL-1β, activation and release of caspase-1 were sensitive to cholinergic agonists (Fig. 7B).

When monocytes were isolated by positive selection via magnetic beads, they spontaneously released a certain amount of IL-1β (median, 105 pg/ml, range: 22–181 pg/ml, $n = 6$) within 3.5 h of culture. Stimulation with BzATP for the last 30 min of culture resulted in an additional release of IL-1β (median, 5,088 pg/ml, range: 1,058–14,907 pg/ml, $n = 6$), which was efficiently reduced by the simultaneous addition of nicotine, ACh, choline, and PC (Fig. 7C). Priming with low-dose LPS (5 ng/ml) further increased the amount of IL-1β released in response to BzATP (median, 9,333 pg/ml, range: 2,864–20,268 pg/ml, $n = 6$, $p \leq 0.05$). Also in LPS-primed monocytes, ACh, PC, and choline reduced the ATP-induced release of IL-1β ($p \leq 0.05$), whereas nicotine was only effective in four of six experiments (Fig. 7C). IL-6 and TNF-α were released in similar amounts in the presence or absence of BzATP and nicotinic agonists (Fig. 7C).

Discussion

In this study, we provide evidence for a novel cholinergic mechanism that potently inhibits the ATP-mediated secretion of mature IL-1β from monocytes via CHRNA7, CHRNA9, and/or CHRNA10 (Fig. 8). This mechanism is triggered by canonical endogenous ligands of these receptors, as well as by PC and PC-modified macromolecules produced by bacteria.

Our experiments on activated mononuclear leukocytes isolated from rat renal allografts at the onset of acute rejection already indicated that ACh inhibits BzATP-induced release of IL-1β. We showed previously that mononuclear leukocytes accumulating in the blood vessels of renal allografts produce ACh (11, 12). Therefore, we assumed that endogenous ACh blocks the ATP-induced increased secretion of IL-1β. In fact, addition of cholinesterase, which efficiently destroys endogenous ACh, enabled IL-1β release in response to ATP. This result supported our hypothesis that leukocytic ACh interferes with ATP-mediated secretion of IL-β in vivo.

Because activated leukocytes from experimental rat renal allografts are difficult to obtain and to manipulate, they are inappropriate to elucidate a novel mechanism of action. Therefore, the following experiments were performed on the human monocytic cell line U937 or on primary blood leukocytes. In line with our hypothesis, IL-1β secretion from LPS-primed U937 cells was efficiently inhibited by ACh and nicotine. Because choline also was effective, we hypothesized that CHRNA7 homomers, CHRNA9 homomers, or CHRNA9/CHRNA10 heteromers mediate inhibition. Indeed, mecamylamine, a general nicotinic blocker, as well as α-bungarotoxin and strychnine, antagonists of CHRNA7 and CHRNA9 (18, 27), antagonized cholinergic inhibition of IL-1β release. The pivotal role of CHRNA7, CHRNA9, and CHRNA10 was further corroborated by gene silencing, which significantly attenuated the cholinergic inhibitory effect in all cases. In conclusion, stimulation of the evolutionarily ancient CHRNA7, CHRNA9, and/or CHRNA10 efficiently inhibit ATP-mediated secretion of IL-1β from LPS-primed U937 cells.

In the following experiments, we investigated which step in the ATP-induced signaling cascade is regulated by nicotinic receptor stimulation. A first and sufficient step in ATP-mediated

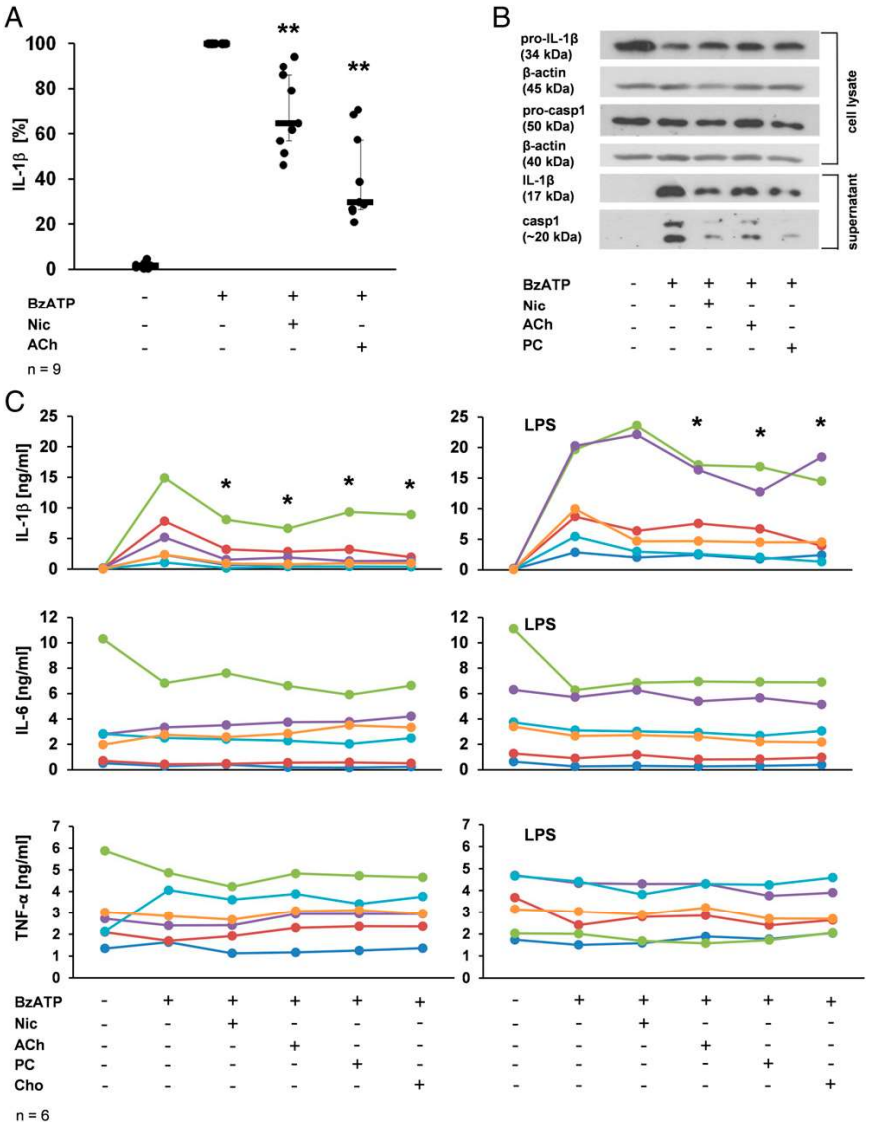

FIGURE 7. Cholinergic inhibition of IL-1β release from primary PBMCs and monocytes. (**A**) BzATP induced the release of IL-1β from PBMCs, which was inhibited by nicotine (Nic) and ACh. Release of IL-1β in response to BzATP was set to 100%. (**B**) Intracellular pro–IL-1β and procaspase-1 (pro-casp1) were detected by Western blotting in lysates of PBMCs treated with BzATP in the presence or absence of Nic, ACh, or PC; β-actin served as loading control. Cleaved IL-1β and caspase-1 (casp1) were assessed in respective cell culture supernatants. Typical results of one experiment out of four are depicted. (**C**) Primary human monocytes were purified by positive selection. Addition of LPS increased the amount of IL-1β released in response to BzATP ($p \leq 0.05$, $n = 6$). ACh, choline (Cho), and PC inhibited the BzATP-induced release of IL-1β from freshly isolated monocytes ($p \leq 0.05$) compared with application of BzATP alone but did not influence the release of IL-6 and TNF-α. Data are presented as individual data points, and the results from individual donors are depicted in the same color. $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ versus BzATP alone, Wilcoxon signed-rank test.

maturation of pro–IL-1β is the induction of a K^+ efflux resulting in a decreased intracellular K^+ concentration and inflammasome activation (3, 4, 28). Because nigericin, a toxin produced by *Streptomyces hygroscopicus*, forms pores for K^+ ions in the cell membrane, it induces NLRP3 inflammasome assembly in the absence of extracellular ATP (29). In fact, ATP-independent, nigericin-induced release of IL-1β was not impaired by stimulation of nicotinic receptors. In neurons, nicotinic ACh receptors physically interact with P2X ATP receptors, resulting in cross-inhibition of ion fluxes (30, 31). Accordingly, patch-clamp experiments using LPS-primed U937 cells directly showed a mecamylamine-sensitive nicotinic inhibition of BzATP-induced ion currents. These findings led to the conclusion that the ATP-mediated signaling is inhibited by nicotinic receptors and that inflammasome assembly triggered by ATP-independent stimuli is unimpaired. However, nicotine alone did not induce ion currents, indicating that nicotinic ACh receptors expressed by U937 cells do not form functional ion channels as was described previously for monocytes and other leukocytes (11, 32, 33). At such unconventional metabotropic nicotinic ACh receptors, antagonists of canonical ionotropic receptors can gain agonist activity (33), which is in line with the present observation that nicotine shares activity with ACh and choline in inhibiting BzATP-induced IL-1β release while acting as an antagonist of ionotropic CHRNA9/CHRNA10 (22). More research is needed to answer the question of how these metabotropic nicotinic receptors are composed, and we do not know whether they form multimers or whether CHRNA7, CHRNA9, and CHRNA10 can be combined together in functional heteromers.

To ensure that cholinergic inhibition of BzATP-mediated IL-1β release also operates in primary human cells, we isolated PBMCs and enriched monocytes from the blood of healthy human volunteers. In line with our data on U937 cells, nicotine, ACh, choline, and PC reduced BzATP-induced release of IL-1β and caspase-1 from primary PBMCs and purified monocytes. ATP-dependent release of caspase-1 from human monocytes was described previously (34). However, after priming of primary monocytes with low-dose LPS, nicotine was inactive in two of six experiments, but the endogenous nicotinic agonists ACh,

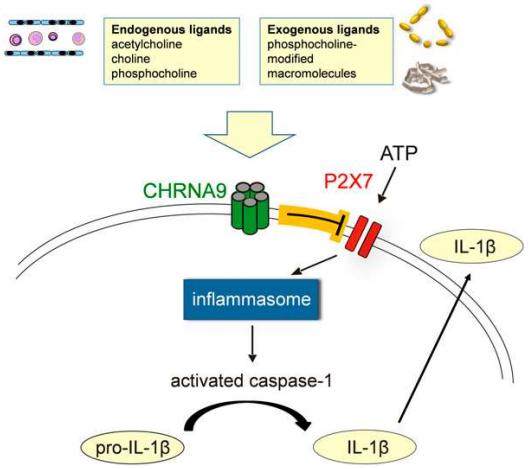

FIGURE 8. Schematic summary of the (phospho-) cholinergic inhibition of ATP-induced inflammasome activation in monocytes. Endogenous ligands of CHRNA7, CHRNA9, and/or CHRNA10, such as ACh, choline, or PC, inhibit ATP-dependent release of IL-1β. PC-modified macromolecules of parasitic and bacterial origin are efficient agonists of CHRNA9, inhibiting the ATP-dependent release of IL-1β. It remains to be clarified whether CHRNA9 actually forms conventional pentamers, as shown in the schematic drawing.

choline, and PC were active. These results support the idea that the described mechanism is active in vivo and suggest that it might be an interesting therapeutic target to prevent IL-1β release into the blood. Indeed, a choline-rich diet, which increases blood choline levels, was shown to improve survival in experimental endotoxin shock (35).

The observation that PBMCs and monocytes from healthy human donors express pro–IL-1β after 3 h of culture, but not immediately after isolation, deserves discussion. Most investigators report that release of IL-1β from human monocytes depends on priming (36–38). Our data suggest that cells are primed during isolation, which induces transient pro–IL-1β expression during subsequent culture. Priming might have happened during gradient centrifugation (39) or adherence to tissue culture dishes (40). Hence, monocytes in the blood of healthy humans are probably devoid of pro–IL-1β. However, during systemic inflammation leading to monocyte activation in vivo, increased blood ATP levels, which are typical of major trauma (41), may cause a substantial release of monocytic IL-1β into the blood. In this situation, specific agonists of CHRNA7, CHRNA9, and CHRNA10 might be useful to prevent shock.

PC-modified macromolecules are produced by numerous pro- and eukaryotic pathogens. They enable colonization of the host, most probably by binding to the receptor for platelet activation factor (15). In addition, PC-modified molecules exert pronounced anti-inflammatory functions, the mechanisms of which are still elusive (14). Because inflammasome activation plays a pivotal role in the host defense against numerous pathogens (1), we speculated that the described inhibitory pathway was hijacked by pathogens for immune evasion. Indeed, PC-LPS isolated from two virulent wild-type *H. influenzae* strains was the most efficient agonist, inhibiting BzATP-mediated secretion of IL-1β with a very low IC_{50} (~25 nM). The inhibitory effect was probably mediated by the PC modification, because LPS from two corresponding *lic1* mutants devoid of PC was completely ineffective. The use of specific antagonists and gene silencing again supported the involvement of CHRNA7, CHRNA9, and/or CHRNA10. In line with published data, PC-modified LPS from *H. influenzae* was almost ineffective in inducing pro–IL-1β, IL-6, and TNF-α mRNA expression (42). However, this was independent of the presence of a PC modification.

Along the same line, PC-BSA, a synthetic model compound for PC-modified secretory products from helminths, also was a potent agonist with low IC_{50} (~140 nM), and this activity was lost upon treatment with hydrofluoric acid, which removes the PC modification. Free PC had a similar efficiency to choline in inhibiting BzATP signaling, with an $IC_{50} \sim 10$ μM. Chemical binding of PC to macromolecules seemed to potentiate its effectiveness. It is conceivable that bound PC is more stable than its free form and that cellular uptake is prevented. Hence, we describe novel agonists of receptors containing CHRNA7, CHRNA9, and/or CHRNA10. Furthermore, we identified a mechanism that explains, at least in part, the anti-inflammatory effects of PC-modified products of bacterial and eukaryotic parasites. In view of the importance of IL-1β for host defense and its pleiotropic proinflammatory functions (1, 2), we consider this a biologically relevant mechanism by which PC-modified macromolecules modulate inflammation. Because antibiotic resistance is a major clinical problem and bacterial and parasitic infections are still among the leading causes of morbidity and mortality worldwide, the development of specific nicotinic antagonists as antibacterial and antiparasitic medicaments might be a promising approach.

In conclusion, PC, PC-modified macromolecules, and canonical cholinergic agonists act upon CHRNA7, CHRNA9, and/or CHRNA10 to inhibit ATP-mediated IL-1β release from human and rat mononuclear blood leukocytes. This novel inhibitory pathway is induced by endogenous cholinergic agonists and seems to be efficiently hijacked by bacteria and parasites.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Vladimer, G. I., R. Marty-Roix, S. Ghosh, D. Weng, and E. Lien. 2013. Inflammasomes and host defenses against bacterial infections. *Curr. Opin. Microbiol.* 16: 23–31.
2. Dinarello, C. A., A. Simon, and J. W. van der Meer. 2012. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat. Rev. Drug Discov.* 11: 633–652.
3. Gross, O., C. J. Thomas, G. Guarda, and J. Tschopp. 2011. The inflammasome: an integrated view. *Immunol. Rev.* 243: 136–151.
4. Rathinam, V. A., S. K. Vanaja, and K. A. Fitzgerald. 2012. Regulation of inflammasome signaling. *Nat. Immunol.* 13: 333–342.
5. Grahames, C. B., A. D. Michel, I. P. Chessell, and P. P. Humphrey. 1999. Pharmacological characterization of ATP- and LPS-induced IL-1beta release in human monocytes. *Br. J. Pharmacol.* 127: 1915–1921.
6. Kawashima, K., T. Fujii, Y. Moriwaki, H. Misawa, and K. Horiguchi. 2012. Reconciling neuronally and nonneuronally derived acetylcholine in the regulation of immune function. *Ann. N. Y. Acad. Sci.* 1261: 7–17.
7. Olofsson, P. S., M. Rosas-Ballina, Y. A. Levine, and K. J. Tracey. 2012. Rethinking inflammation: neural circuits in the regulation of immunity. *Immunol. Rev.* 248: 188–204.
8. Borovikova, L. V., S. Ivanova, M. Zhang, H. Yang, G. I. Botchkina, L. R. Watkins, H. Wang, N. Abumrad, J. W. Eaton, and K. J. Tracey. 2000. Vagus

nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 405: 458–462.
9. Rosas-Ballina, M., P. S. Olofsson, M. Ochani, S. I. Valdés-Ferrer, Y. A. Levine, C. Reardon, M. W. Tusche, V. A. Pavlov, U. Andersson, S. Chavan, et al. 2011. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science* 334: 98–101.
10. de Jonge, W. J., E. P. van der Zanden, F. O. The, M. F. Bijlsma, D. J. van Westerloo, R. J. Bennink, H. R. Berthoud, S. Uematsu, S. Akira, R. M. van den Wijngaard, and G. E. Boeckxstaens. 2005. Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat. Immunol.* 6: 844–851.
11. Hecker, A., Z. Mikulski, K. S. Lips, U. Pfeil, A. Zakrzewicz, S. Wilker, P. Hartmann, W. Padberg, I. Wessler, W. Kummer, and V. Grau. 2009. Pivotal Advance: Up-regulation of acetylcholine synthesis and paracrine cholinergic signaling in intravascular transplant leukocytes during rejection of rat renal allografts. *J. Leukoc. Biol.* 86: 13–22.
12. Wilczynska, J., U. Pfeil, A. Zakrzewicz, H. Dietrich, C. Körner, A. Hecker, I. Wessler, W. Padberg, W. Kummer, and V. Grau. 2011. Acetylcholine and chronic vasculopathy in rat renal allografts. *Transplantation* 91: 263–270.
13. Mikulski, Z., P. Hartmann, G. Jositsch, Z. Zasłona, K. S. Lips, U. Pfeil, H. Kurzen, J. Lohmeyer, W. G. Clauss, V. Grau, et al. 2010. Nicotinic receptors on rat alveolar macrophages dampen ATP-induced increase in cytosolic calcium concentration. *Respir. Res.* 11: 133.
14. Grabitzki, J., and G. Lochnit. 2009. Immunomodulation by phosphocholine–biosynthesis, structures and immunological implications of parasitic PC-epitopes. *Mol. Immunol.* 47: 149–163.
15. Clark, S. E., and J. N. Weiser. 2013. Microbial modulation of host immunity with the small molecule phosphorylcholine. *Infect. Immun.* 81: 392–401.
16. Schweda, E. K., J. C. Richards, D. W. Hood, and E. R. Moxon. 2007. Expression and structural diversity of the lipopolysaccharide of *Haemophilus influenzae*: implication in virulence. *Int. J. Med. Microbiol.* 297: 297–306.
17. Grau, V., O. Stehling, H. Garn, and B. Steiniger. 2001. Accumulating monocytes in the vasculature of rat renal allografts: phenotype, cytokine, inducible no synthase, and tissue factor mRNA expression. *Transplantation* 71: 37–46.
18. Schweda, E. K., and J. C. Richards. 2003. Structural profiling of short-chain lipopolysaccharides from *Haemophilus influenzae. Methods Mol. Med.* 71: 161–183.
19. Baltar, P., F. Romarís, J. Estévez, J. Leiro, and F. M. Ubeira. 1998. Carrier-dependent suppression of the anti-phosphorylcholine plaque-forming cell response in *Trichinella*-infected mice is mediated by anti-hapten IgG1 antibodies. *Exp. Parasitol.* 90: 95–102.
20. Mohr, F., M. Zimmermann, and J. Klein. 2013. Mice heterozygous for AChE are more sensitive to AChE inhibitors but do not respond to BuChE inhibition. *Neuropharmacology* 67: 37–45.
21. Papke, R. L., M. Bencherif, and P. Lippiello. 1996. An evaluation of neuronal nicotinic acetylcholine receptor activation by quaternary nitrogen compounds indicates that choline is selective for the alpha 7 subtype. *Neurosci. Lett.* 213: 201–204.
22. Verbitsky, M., C. V. Rothlin, E. Katz, and A. B. Elgoyhen. 2000. Mixed nicotinic-muscarinic properties of the alpha9 nicotinic cholinergic receptor. *Neuropharmacology* 39: 2515–2524.
23. Chernyavsky, A. I., J. Arredondo, M. Skok, and S. A. Grando. 2010. Auto/paracrine control of inflammatory cytokines by acetylcholine in macrophage-like U937 cells through nicotinic receptors. *Int. Immunopharmacol.* 10: 308–315.
24. Moser, N., N. Mechawar, I. Jones, A. Gochberg-Sarver, A. Orr-Urtreger, M. Plomann, R. Salas, B. Molles, L. Marubio, U. Roth, et al. 2007. Evaluating the suitability of nicotinic acetylcholine receptor antibodies for standard immunodetection procedures. *J. Neurochem.* 102: 479–492.
25. Schweda, E. K., J. R. Brisson, G. Alvelius, A. Martin, J. N. Weiser, D. W. Hood, E. R. Moxon, and J. C. Richards. 2000. Characterization of the phosphocholine-substituted oligosaccharide in lipopolysaccharides of type b *Haemophilus influenzae. Eur. J. Biochem.* 267: 3902–3913.
26. Harnett, W., and M. M. Harnett. 2006. Filarial nematode secreted product ES-62 is an anti-inflammatory agent: therapeutic potential of small molecule derivatives and ES-62 peptide mimetics. *Clin. Exp. Pharmacol. Physiol.* 33: 511–518.
27. Baker, E. R., R. Zwart, E. Sher, and N. S. Millar. 2004. Pharmacological properties of alpha 9 alpha 10 nicotinic acetylcholine receptors revealed by heterologous expression of subunit chimeras. *Mol. Pharmacol.* 65: 453–460.
28. Muñoz-Planillo, R., P. Kuffa, G. Martínez-Colón, B. L. Smith, T. M. Rajendiran, and G. Núñez. 2013. K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 38: 1142–1153.
29. Graven, S. N., S. Estrada-O, and H. A. Lardy. 1966. Alkali metal cation release and respiratory inhibition induced by nigericin in rat liver mitochondria. *Proc. Natl. Acad. Sci. USA* 56: 654–658.
30. Searl, T. J., R. S. Redman, and E. M. Silinsky. 1998. Mutual occlusion of P2X ATP receptors and nicotinic receptors on sympathetic neurons of the guinea-pig. *J. Physiol.* 510: 783–791.
31. Khakh, B. S., J. A. Fisher, R. Nashmi, D. N. Bowser, and H. A. Lester. 2005. An angstrom scale interaction between plasma membrane ATP-gated P2X2 and alpha4beta2 nicotinic channels measured with fluorescence resonance energy transfer and total internal reflection fluorescence microscopy. *J. Neurosci.* 25: 6911–6920.
32. Peng, H., R. L. Ferris, T. Matthews, H. Hiel, A. Lopez-Albaitero, and L. R. Lustig. 2004. Characterization of the human nicotinic acetylcholine receptor subunit alpha (alpha) 9 (CHRNA9) and alpha (alpha) 10 (CHRNA10) in lymphocytes. *Life Sci.* 76: 263–280.
33. Razani-Boroujerdi, S., R. T. Boyd, M. I. Dávila-García, J. S. Nandi, N. C. Mishra, S. P. Singh, J. C. Pena-Philippides, R. Langley, and M. L. Sopori. 2007. T cells express alpha7-nicotinic acetylcholine receptor subunits that require a functional TCR and leukocyte-specific protein tyrosine kinase for nicotine-induced Ca2+ response. *J. Immunol.* 179: 2889–2898.
34. Laliberte, R. E., J. Eggler, and C. A. Gabel. 1999. ATP treatment of human monocytes promotes caspase-1 maturation and externalization. *J. Biol. Chem.* 274: 36944–36951.
35. Rivera, C. A., M. D. Wheeler, N. Enomoto, and R. G. Thurman. 1998. A choline-rich diet improves survival in a rat model of endotoxin shock. *Am. J. Physiol.* 275: G862–G867.
36. Agostini, L., F. Martinon, K. Burns, M. F. McDermott, P. N. Hawkins, and J. Tschopp. 2004. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* 20: 319–325.
37. Ward, J. R., P. W. West, M. P. Ariaans, L. C. Parker, S. E. Francis, D. C. Crossman, I. Sabroe, and H. L. Wilson. 2010. Temporal interleukin-1beta secretion from primary human peripheral blood monocytes by P2X7-independent and P2X7-dependent mechanisms. *J. Biol. Chem.* 285: 23147–23158.
38. Netea, M. G., C. A. Nold-Petry, M. F. Nold, L. A. Joosten, B. Opitz, J. H. van der Meer, F. L. van de Veerdonk, G. Ferwerda, B. Heinhuis, I. Devesa, et al. 2009. Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* 113: 2324–2335.
39. Jahr, H., G. Pfeiffer, B. J. Hering, K. Federlin, and R. G. Bretzel. 1999. Endotoxin-mediated activation of cytokine production in human PBMCs by collagenase and Ficoll. *J. Mol. Med.* 77: 118–120.
40. Kelley, J. L., M. M. Rozek, C. A. Suenram, and C. J. Schwartz. 1987. Activation of human blood monocytes by adherence to tissue culture plastic surfaces. *Exp. Mol. Pathol.* 46: 266–278.
41. Gordon, J. L. 1986. Extracellular ATP: effects, sources and fate. *Biochem. J.* 233: 309–319.
42. Choi, J., A. D. Cox, J. Li, W. McCready, and M. Ulanova. 2014. Activation of innate immune responses by *Haemophilus influenzae* lipooligosaccharide. *Clin. Vaccine Immunol.* 21: 769–776.

Supplemental Table S1: Primers used for real-time RT-PCR

Gene	Gene bank accession number	Forward primer (5'-3')	Reverse primer (5'-3')	Product (bp)
rat pro-IL-1β	NM_031512.2	116-135	216-196	101
rat CHRNA7	NM_012832.3	343-363	515-496	173
rat CHRNA9	NM_022930.1	416-436	534-514	119
rat CHRNA10	NM_022639.1	201-224	324-303	124
rat PBGD	NM_013168.2	583-602	697-678	115
human pro-IL-1β	NM_000576.2	527-546	659-640	133
human IL-6	NM_000600.3	522-541	622-604	100
human CHRFAM7A	NM_148911.1	539-560	640-619	102
human CHRNA7	NM_000746.4	416-436	557-536	142
human CHRNA9	NM_017581.3	428-449	543-524	116
human CHRNA10	NM_020402.2	905-923	1025-1005	121
human TNF-α	NM_000594.3	314-336	447-428	133
human PBGD	NM_000190.3	159-178	228-209	70

bp, base pairs; CHRNA, nicotinic acetylcholine receptor alpha; PBGD, porphobilinogen deaminase

Supplemental Table S2: Release of lactate dehydrogenase (LDH)

Reagents	Mean ± SD	n
-	3.4 ± 2.2%	23
LPS	5.6 ± 3.7%	23
LPS, BzATP	9.1 ± 2.2%	4
LPS, BzATP, ACh 0.01 µM	8.4 ± 2.2%	4
LPS, BzATP, ACh 0.1 µM	8.3 ± 2.3%	4
LPS, BzATP, ACh 1 µM	9.7 ± 2.4%	4
LPS, BzATP, ACh 10 µM	9.6 ± 2.6%	4
LPS, BzATP, choline 1 µM	4.0 ± 1.5%	4
LPS, BzATP, choline 10 µM	3.6 ± 0.9%	4
LPS, BzATP, choline 100 µM	3.3 ± 0.8%	4
LPS, BzATP, choline 1000 µM	4.0 ± 1.2%	4
LPS, BzATP, nicotine 0.1 µM	1.5 ± 1.0%	3
LPS, BzATP, nicotine 1 µM	1.5 ± 0.8%	3
LPS, BzATP, nicotine 10 µM	2.2 ± 1.2%	3
LPS, BzATP, nicotine 100 µM	2.0 ±0.6%	3
LPS, BzATP, PC 1 µM	3.8 ± 0.9%	4
LPS, BzATP, PC 10 µM	3.2 ± 1.4%	4
LPS, BzATP, PC 100 µM	3.3 ± 1.2%	4
LPS, BzATP, PC 1000 µM	3.0 ± 0.9%	4
LPS, BzATP, RMPC 0.01 µg/ml	2.5 ± 1.2%	4
LPS, BzATP, RMPC 0.1µg/ml	2.4 ± 1.0%	4
LPS, BzATP, RMPC 1µg/ml	2.4 ± 0.9%	4
LPS, BzATP, RMPC 10 µg/ml	2.7 ±1.7%	4
LPS, BzATP, RM 0.01 µg/ml	2.1 ± 0.6%	4
LPS, BzATP, RM 0.1 µg/ml	2.2 ± 0.7%	4
LPS, BzATP, RM 1µg/ml	2.3 ± 1.0%	4
LPS, BzATP, RM 10 µg/ml	2.5 ± 1.5%	4
LPS, BzATP, NTHiPC 0.01µg/ml	2.0 ± 0.7%	4
LPS, BzATP, NTHiPC 0.1µg/ml	2.1 ± 0.6%	4
LPS, BzATP, NTHiPC 1 µg/ml	1.8 ± 0.9 %	4
LPS, BzATP, NTHiPC 10 µg/ml	2.4 ± 0.9%	4
LPS, BzATP, NTHi 0.01µg/ml	2.0 ± 1.0%	4
LPS, BzATP, NTHi 0.1µg/ml	2.0 ± 1.0%	4
LPS, BzATP, NTHi 1 µg/ml	2.0 ± 1.2%	4
LPS, BzATP, NTHi 10 µg/ml	2.4 ± 1.1%	4

U937 cells were primed with lipopolysaccharide (LPS) from *Escherichia coli* for 5 h. BzATP was added for another 30 min in the presence or absence of nicotinic agonists or variants of LPS from different *Haemophilus influenzae* strains. LDH release into the cell culture supernatant is given as % of the total release. ACh, acetylcholine; NTHi, LPS from the *lic1*-mutant strain NTHi1233lic1; NTHiPC, PC-modified LPS from *Haemophilus influenzae* strain NTHi 1233; PC, phosphocholine; RM, LPS from the *lic1*-mutant strain RM7004-Lic1; RMPC, PC-modified LPS from *Haemophilus influenzae* strain RM118.

Figure S1

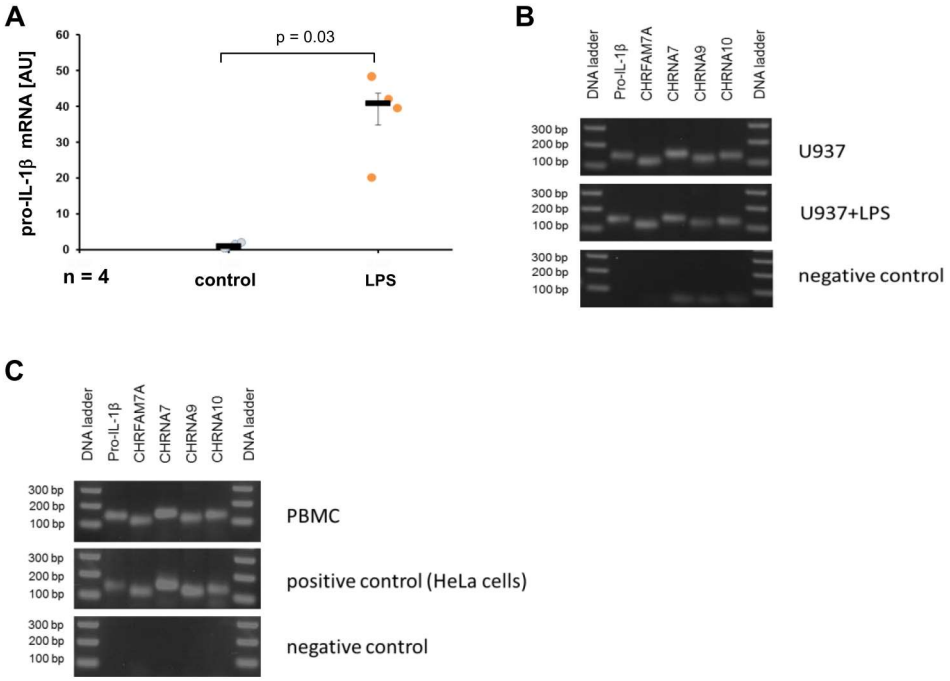

Fig. S1: Expression of pro-IL-1β and acetylcholine receptor mRNA. (**A**) U937 cells were incubated for 5 h with 1 µg/ml lipopolysaccharide (LPS) and pro-IL-1β mRNA expression was measured by real-time RT-PCR and compared to untreated control cells. Data are presented as individual data points, bars indicate median, whiskers percentiles 25 and 75; Mann-Whitney rank sum test. (**B**) Nicotinic acetylcholine receptors CHRNA7, CHRNA9 and CHRNA10 as well as the inactive duplicate CHRFAM7A were detected by real-time RT-PCR. Products obtained by real-time RT-PCR from untreated and LPS-primed U937 were separated in agarose gels. (**C**) Real-time RT-PCR products from freshly isolated human peripheral blood mononuclear cells (PBMC). In negative controls, cDNA was replaced by water.

Figure S2

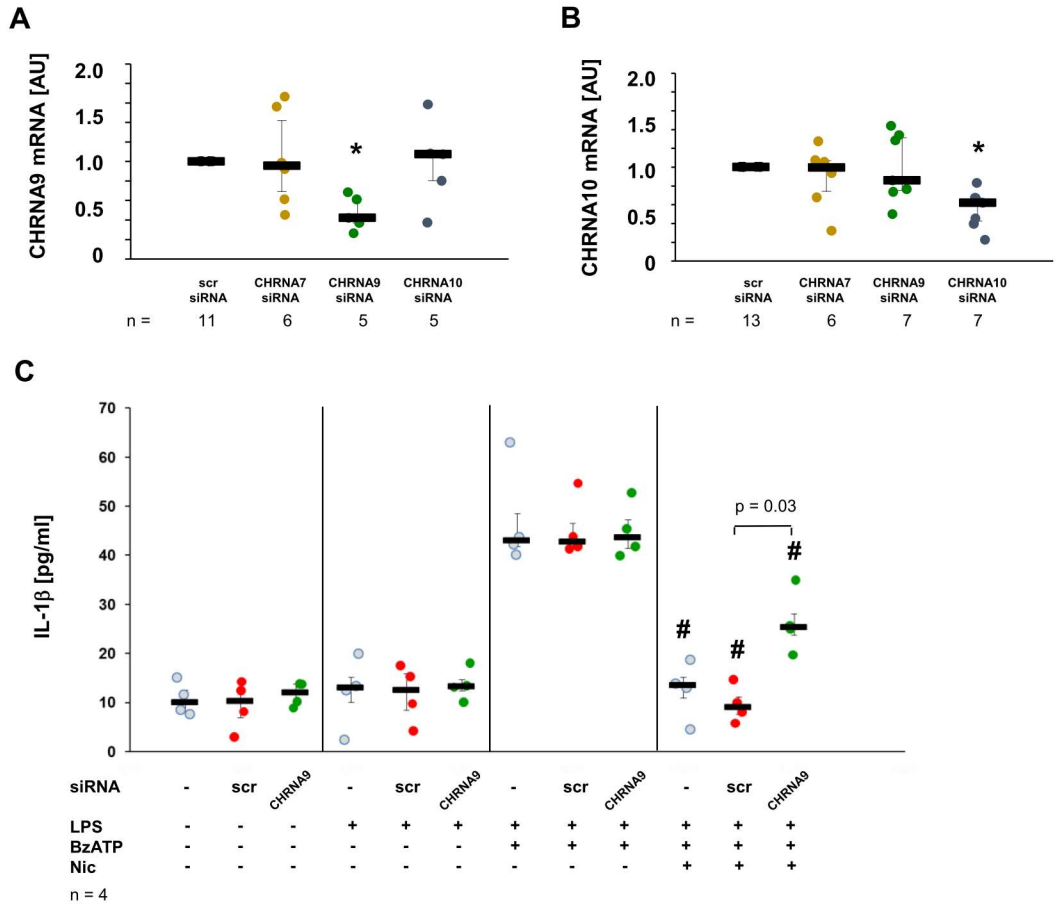

Fig. S2: Reduction of CHRNA7, CHRNA9 and CHRNA10 mRNA expression by siRNA transfection. U937 cells were treated with control siRNA (scr) or siRNA targeting nicotinic receptors and the mRNA expression of (**A**) CHRNA9 and (**B**) CHRNA10 was measured by real-time RT-PCR 6 h later. The mRNA expression of CHRNA7 was too low for quantification. (**C**) Control cells without siRNA, transfection with control siRNA (scr) or with siRNA targeting CHRNA9 did not affect the release of IL-1β from unprimed cells, from cells primed with LPS, and from LPS-primed cells stimulated with BzATP. The inhibitory effect of nicotine, however, was blunted in cells treated with siRNA against CHRNA9 but not after transfection of scrambled siRNA. A part of these data is also shown in Figure 2 C. Data are presented as individual data points, bars indicate median, whiskers percentiles 25 and 75; * $p \leq 0.05$ (**A-B**) versus scr siRNA; # $p \leq 0.05$ significantly different from respective cells treated with LPS and BzATP but not with nicotine, Mann-Whitney rank sum test (**A-B**) or Kruskal-Wallis test followed by Mann-Whitney rank sum test (**C**).

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Canonical and Novel Non-Canonical Cholinergic Agonists Inhibit ATP-Induced Release of Monocytic Interleukin-1β via Different Combinations of Nicotinic Acetylcholine Receptor Subunits α7, α9 and α10

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Recently, we discovered a cholinergic mechanism that inhibits the adenosine triphosphate (ATP)-dependent release of interleukin-1β (IL-1β) by human monocytes via nicotinic acetylcholine receptors (nAChRs) composed of α7, α9 and/or α10 subunits. Furthermore, we identified phosphocholine (PC) and dipalmitoylphosphatidylcholine (DPPC) as novel nicotinic agonists that elicit metabotropic activity at monocytic nAChR. Interestingly, PC does not provoke ion channel responses at conventional nAChRs composed of subunits α9 and α10. The purpose of this study is to determine the composition of nAChRs necessary for nicotinic signaling in monocytic cells and to test the hypothesis that common metabolites of phosphatidylcholines, lysophosphatidylcholine (LPC) and glycerophosphocholine (G-PC), function as nAChR agonists. In peripheral blood mononuclear cells from nAChR gene-deficient mice, we demonstrated that inhibition of ATP-dependent release of IL-1β by acetylcholine (ACh), nicotine and PC depends on subunits α7, α9 and α10. Using a panel of nAChR antagonists and siRNA technology, we confirmed the involvement of these subunits in the control of IL-1β release in the human monocytic cell line U937. Furthermore, we showed that LPC (C16:0) and G-PC efficiently inhibit ATP-dependent release of IL-1β. Of note, the inhibitory effects mediated by LPC and G-PC depend on nAChR subunits α9 and α10, but only to a small degree on α7. In *Xenopus laevis* oocytes heterologously expressing different combinations of human α7, α9 or α10 subunits, ACh induced canonical ion channel activity, whereas LPC, G-PC and PC did not. In conclusion,

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we demonstrate that canonical nicotinic agonists and PC elicit metabotropic nAChR activity in monocytes via interaction of nAChR subunits α7, α9 and α10. For the metabotropic signaling of LPC and G-PC, nAChR subunits α9 and α10 are needed, whereas α7 is virtually dispensable. Furthermore, molecules bearing a PC group in general seem to regulate immune functions without perturbing canonical ion channel functions of nAChR.

Keywords: acetylcholine, *CHRNA*, glycerophosphocholine, inflammasome, interleukin-1beta, lysophosphatidylcholine, nicotine and phosphocholine

INTRODUCTION

Interleukin-1β (IL-1β) is a potent pro-inflammatory cytokine of innate immunity that importantly contributes to host defense against pathogens (Vladimer et al., 2013). Production and secretion of IL-1β are strictly controlled and typically require two independent danger signals (Rathinam et al., 2012). The presence of potential pathogens is commonly sensed by Toll-like receptors that lead to cytoplasmic expression of pro-IL-1β. A prototypical second signal is extracellular adenosine triphosphate (ATP) originating from the cytoplasm of damaged host cells (Trautmann, 2009). ATP binds to the ionotropic P2X7 receptor, which enables efflux of potassium ions. This induces the assembly of the NLRP3 (NLR family, pyrin domain containing protein 3) inflammasome and activation of the protease caspase-1, which eventually cleaves pro-IL-1β and enables the release of mature bioactive IL-1β (Ferrari et al., 2006; Gross et al., 2011; Ozaki et al., 2015). Excessive systemic release of IL-1β can be dangerous for the host (Dinarello et al., 2012). Elevated systemic levels of IL-1β can cause fever, shock, systemic inflammatory response syndrome (SIRS) and can lead to life-threatening multi-organ damage (Cauwels et al., 2014). Mechanisms controlling IL-1β maturation are of substantial clinical interest but so far have remained largely unexplored.

Recently, we demonstrated that activation of nicotinic acetylcholine (ACh) receptors (nAChRs) by classical ligands like ACh, nicotine (Nic) and choline (Cho) inhibits ATP signaling (Hecker et al., 2009, 2015; Mikulski et al., 2010; Richter et al., 2016), activation of caspase-1 and release of IL-1β by human monocytes (Hecker et al., 2015; Richter et al., 2016). Interestingly, activated monocytes, T cells and vascular endothelial cells can endogenously produce ACh, which presumably acts in an autocrine or paracrine fashion (Hecker et al., 2009; Wilczynska et al., 2011; Kawashima et al., 2012). In addition to conventional nAChR agonists, we demonstrated that free phosphocholine (PC), a common metabolite of phosphatidylcholine and PC-modified macromolecules can also effectively inhibit the ATP-dependent release of IL-1β by human and murine monocytes (Hecker et al., 2015; Richter et al., 2016). Gene-silencing experiments and pharmacological studies demonstrated that nAChR subunits α7, α9 and/or α10 are involved in the cholinergic inhibition of IL-1β release (Hecker et al., 2015). The nAChR subunits α9 and α10 contribute to the inhibitory effect of PC (Richter et al., 2016). It is however unclear, if the effect of PC also depends on subunit α7 and if receptor subunits α7, α9 and α10 cooperate in monocytic cells or exert redundant functions. Similarly, dipalmitoylphosphatidylcholine (DPPC), the main lipid component of pulmonary surfactant, inhibits ATP-driven inflammasome activation in monocytes. DPPC signals via the nAChR subunit α9 in combination with either subunit α7 or α10 (Backhaus et al., 2017).

Phosphatidylcholines possess a PC head group and two non-polar fatty acid chains. Enzymatic removal of one fatty acid results in lysophosphatidylcholine (LPC), which can be further metabolized to glycerophosphocholine (G-PC). LPC has been shown to modulate responses of the innate and adaptive immune system by various non-cholinergic mechanisms (Kabarowski et al., 2002; Stock et al., 2006; Carneiro et al., 2013). The potential involvement of LPC and G-PC, two molecules bearing a PC head group, in the cholinergic regulation of ATP-mediated IL-1β release by monocytes has not yet been tested.

The purpose of this study was to investigate if nAChR subunits α7, α9 and α10 interact in monocytes and which nAChR subunit composition is necessary for ACh-, Nic- and PC-mediated inhibition of IL-1β release. Furthermore, we hypothesize that LPC and G-PC, both PC-bearing metabolites of phosphatidylcholines, can function as agonists of nAChRs in monocytes and contribute to the regulation of IL-1β release. In addition, we characterize, which nAChR subunits are required for the inhibitory effect mediated by LPC and G-PC. Finally, we investigate if LPC, G-PC and PC induce ionotropic functions at heterologously expressed canonical nAChRs.

MATERIALS AND METHODS

Animals

This study was carried out in accordance with the recommendations of the NIH "Guide for the Care and Use of Laboratory Animals". The protocols for animal experiments were approved by the Regierungspräsidium Giessen, Hesse, Germany (permit number 549_M; R.P. Nr. Gi 20/23-Nr. A12/2011; R.P. Nr. Gi 20/23-Nr. A10/2011). Adult female *Xenopus laevis* (Xenopus-Express, Le Bourg, France) as well as male and female wild-type (WT) and *Chrna7* (C57B1/6J), *Chrna9* (129S-*Chrna9*^{tm1Bedv}/J) and *Chrna10* (129S4-*Chrna10*^{tm1Bedv}/Mmucd) gene-deficient mice

were used. The generation and characterization of every gene-deficient mouse strain was described before (Orr-Urtreger et al., 1997; Vetter et al., 1999, 2007; Whiteaker et al., 2007). *Chrna 7* gene-deficient mice were obtained from the Jackson Laboratories after being re-derived, whereas *Chrna 9* and *Chrna 10* gene-deficient mice were kindly provided by Prof. Douglas E. Vetter. The genotype of every mouse was evaluated by PCR. The corresponding WT background strain for every gene-deficient mouse was used as a control.

Mononuclear Leukocytes from Mice

Mouse peripheral blood mononuclear cells (PBMCs) were freshly isolated from heparinized blood obtained from WT, *Chrna7*, *Chrna9*, or *Chrna10* gene-deficient mice using Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden; 1.082 g/ml) density gradient centrifugation. Mononuclear cells were cultured for 2 h in 24-well-plates at 37°C, 5% CO_2, in RPMI 1640 (Gibco/Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS Superior EU, Biochrom GmbH, Berlin, Germany) and 2 mM L-glutamine (GlutaMAX^{TM}, Gibco/Life Technologies). Thereafter, cells were stimulated with 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate trieethylammonium salt (BzATP, 100 μM, Sigma-Aldrich, Taufkirchen, Germany) for 30 min, in the presence or absence of ACh chloride (10 μM), PC chloride calcium salt tetrahydrate (PC, 100 μM) or nicotine (Nic, 100 μM; all purchased from Sigma-Aldrich, Taufkirchen, Germany). Cell culture supernatants were collected and stored at −20°C until IL-1β and lactate dehydrogenase (LDH) measurement.

U937 Cells

The human histocytic lymphoma cell line U937 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured according to the protocol described for mouse PBMCs. Cells in the log-phase of growth were transferred to 24-well plates (1×10^6 cells/ml and per well) and primed with 1 μg/ml LPS from *Escherichia coli* (L2654; Sigma-Aldrich) for 5 h. Thereafter, BzATP (100 μM) was applied for 30 min, in the presence or absence of ACh (10 μM), Nic (100 μM), PC (100 μM), L-α-G-PC (Sigma-Aldrich; 0.1 μM to 1000 μM) or 1-palmitoyl-*sn*-glycero-3-phosphocholine LPC (C16:0), Sigma-Aldrich; 0.1 μM to 100 μM). To test the involvement of different subunits of nAChR, the following antagonists were applied: mecamylamine hydrochloride (Mec, 100 μM, Sigma-Aldrich), α-bungarotoxin (α-Bun, 1 μM, Tocris Bioscience, Bristol, UK), strychnine hydrochloride (Stry, 10 μM, Sigma-Aldrich) or conotoxins ArIB [V11L, V16D] (500 nM) or RgIA4 (200 nM; Ramarao and Cohen, 1998; Whiteaker et al., 2007; Innocent et al., 2008; Richter et al., 2016; Romero et al., 2017).

Knock-Down of α7, α9 or α10 nAChR Subunit Expression

The expression of human nAChR subunits α7, α9 or α10 was attenuated using siRNA technology. Cells were transfected with ON-TARGETplus human *CHRNA7* (α7), *CHRNA9* (α9) or *CHRNA10* (α10) SMARTpool siRNA (Thermo Fisher Scientific, Schwerte, Germany) or with ON-TARGETplus non-targeting control pool (Thermo Fisher Scientific) as a negative control. Cells were transfected with 30 pmol siRNA/1 $\times$ 10^6 cells using the Amaxa Cell Line Nucleofector Kit C and the Nucleofector II Device (both from Lonza, Cologne, Germany) according to the manufacturer's instructions. The siRNA-mediated down-regulation of target genes was assessed 48 h after transfection by real-time RT-PCR.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from 1×10^6 U937 cells using the RNesay Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One microgram of RNA was reversely transcribed using M-MLV H$^-$ Reverse Transcriptase and 1 μg of random hexamer primers (Promega, Mannheim, Germany).

Real-Time RT-PCR

To confirm efficient and selective knock-down of nAChR subunits α7, α9 or α10 targeted selectively with siRNA, real-time RT-PCR was performed (n = 4 per experimental group, each sample was assessed in duplicates) using the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and Platinum SYBER green qPCR Super Mix-UDG (Invitrogen, Karlsruhe, Germany). The hydroxymethylbilane synthase (HMBS) gene was selected as a reference gene, as it was reported not to be regulated in monocytes under various culture conditions and stimulations (Moosig et al., 2006). Primers specific for the detection of *HMBS*, α7, α9 and α10 nAChR subunits were synthesized by MWG Biotech (Ebersberg, Germany) and their sequences have been published before (Hecker et al., 2015). Changes in the mRNA expression levels of nAChR were calculated by the $2^{\Delta CT}$ method, where ΔCT represents the difference between the CT value of *HMBS* gene and the CT value of gene of interest. The mean of the mRNA expression values from cells transfected with non-targeting (scrambled) siRNA was set to one and the values from cells transfected with siRNA specific for nAChR were calculated accordingly.

ELISA

The IL-1β concentration in the cell culture supernatants was determined by mouse- or human- specific Quantikine Immunoassays (R&D Systems, Mineapolis, MN, USA) according to the manufacturer's instructions.

LDH Measurement

The viability of the cells was estimated by measurement of LDH in cell culture supernatants by the Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. To assess the proportion of dead cells, a maximum LDH release control was generated. For this purpose, an equivalent number of U937 cells were disrupted by freezing them twice at −80°C. The LDH value of the sample of interest was compared with the total content of LDH in the control sample, which was set to 100% and the relative cell death was calculated accordingly.

Isolation and Culture of Oocytes

Xenopus laevis oocytes were purchased from Ecocyte Bioscience (Castrop-Rauxel, Germany) or obtained from adult female frogs (Xenopus-Express, Le Bourg, France) and prepared as previously described (Richter et al., 2016). Defolliculated oocytes were stored in oocyte Ringer's solution (ORi) containing (in mM) 90 NaCl, 1 KCl, 2 $CaCl_2$, 5 HEPES (4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid), 2.5 pyruvate, 20 mg/ml penicillin and 25 mg/ml streptomycin (pH 7.4) at 16°C. All chemicals used for ORi preparation were purchased from Fluka (Deisenhofen, Germany), except for HEPES, penicillin and streptomycin (Sigma-Aldrich).

Heterologous Expression of Human nAChRs in Oocytes and Electrophysiological Whole-Cell Measurements

The cDNA clones of human α9, human α10 and human 43 kDa receptor-associated protein of the synapse (*RAPSN*) in the pTNT vector were obtained from Eurofins Genomics (Ebersberg, Germany). Capped cRNA was synthesized using an *in vitro* transcription kit (T7 RiboMAX^{TM} Large Scale RNA Production System Kit, Promega, Mannheim, Germany). The cRNA encoding human α7 was kindly provided by G. Schmalzing (Department of Molecular Pharmacology, RWTH Aachen University, Aachen, Germany). In brief, a plasmid containing the entire coding region of human α7 (GenBank NM_ 000746.5, Addgene plasmid #62276; Wang et al., 2014) was purchased from Addgene (Teddington, UK[1]) and subcloned via gateway PCR from the pcDNA3.1 vector into a gateway-modified version of the oocyte expression vector pNKS2 (Gloor et al., 1995). The plasmid was linearized downstream of the vector-provided polyA tail with XhoI and transcribed with SP6 RNA polymerase into capped and polyadenylated cRNA, as previously described (Stolz et al., 2015).

Oocytes were injected with cRNA encoding nAChR subunit α7 (20 ng/oocyte) or with cRNA combinations for subunits α7 and α9 (20 ng/oocyte, each), or subunits α9 and α10 (20 ng/ oocyte, each), or α7, α9 and α10 (16, 19, 19 ng/oocyte, respectively). *RAPSN* (5 ng/oocyte) was co-injected in all experiments to increase the expression levels and to obtain stable nAChR expression (Froehner et al., 1990). The cRNA was dissolved in nuclease-free water and injected in a volume of 50.6 nl per oocyte. In all two-electrode voltage-clamp (TEVC) experiments representative controls were performed with oocytes that were injected with the same amount of nuclease-free water.

TEVC measurements were performed on cRNA- or water-injected oocytes (incubation time 3–5 days). The membrane voltage was clamped to −60 mV using a TEVC amplifier (Warner Instruments, Hamden, CT, USA), and transmembrane currents (I_M) were low-pass filtered at 1000 Hz (Frequency Devices 902, Haverhill, MA, USA) and recorded with a strip chart recorder (Kipp and Zonen, Delft, Netherlands). Oocytes were placed in a perfusion chamber and perfused (gravity driven) with ORi without pyruvate and antibiotics (pH 7.4). In all experimental groups, measurements were performed on oocytes isolated from at least two different *Xenopus laevis* individuals.

For experiments examining the inhibition and recovery from inhibition of ACh-gated currents by LPC and G-PC, oocytes were injected with a 1:1 ratio of cRNA encoding human α9 and α10 or α7 alone and incubated at 17°C for 2–3 days prior to use. Oocytes were placed in a 30 µl chamber and continuously perfused by gravity with a solution containing (in mM) 96 NaCl, 2.5 KCl, 1.8 $CaCl_2$ and 1 $MgCl_2$ (pH 7.4). Stock solutions of LPC or G-PC were prepared in water and diluted with perfusion solution to a final concentration of 1 µM or 100 µM, respectively. The membranes were clamped at a holding potential of −70 mV and stimulated with 1 s pulses of 100 µM ACh once every 60 s until a steady-state baseline response was observed. Then, the perfusion solution was switched to one containing LPC or G-PC and the ACh-evoked responses were monitored for changes in amplitude and the data for inhibition of the ACh-evoked responses were normalized to three averaged control pulses, in the absence of LPC or G-PC, and analyzed with an exponential decay equation. The data for inhibition and recovery of inhibition by LPC or G-PC were best fit with single exponential equations.

Statistical Analysis and Data Processing

Data were analyzed using SPSS software (Munich, Germany) by Wilcoxon signed-rank test or by the non-parametric Kruskal-Wallis test, followed by the Mann-Whitney rank-sum test. A p value below 0.05 was considered as statistically significant and marked with *. Data were visualized using program Inkscape version 0.92 (Free and Open Source Software licensed under the GPL).

RESULTS

Single Gene Deletions of *Chrna7*, *Chrna9* or *Chrna10* Fully Abolish ACh-, Nic- or PC-Mediated Inhibition of BzATP-Induced IL-1β Release by Mouse PBMCs

ACh and Nic, known canonical agonists of nAChRs, as well as PC have been reported to inhibit ATP-mediated IL-1β release by human monocytes by a mechanism involving nAChRs (Hecker et al., 2015; Richter et al., 2016). To further determine the involvement of nAChR subunits α7, α9 and/or α10 in this mechanism, PBMCs from WT mice and mice deficient in single nAChR subunit genes were investigated. PBMCs isolated from WT mice or from *Chrna7*, *Chrna9* or *Chrna10* gene-deficient mice spontaneously released low amounts of IL-1β into the cell culture medium (**Figures 1A–C**). In response to stimulation with BzATP, an agonist of ATP receptor P2X7, the concentration of IL-1β was increased (**Figures 1A–C**). ACh, Nic and PC significantly inhibited the BzATP-induced release of IL-1β from WT PBMCs (**Figures 1A–C**, $p \leq 0.05$, $n \geq 4$). In sharp contrast to cells isolated from WT animals, ACh, Nic and PC did not change BzATP-induced release of IL-1β from PBMCs obtained from *Chrna7* gene-deficient mice (**Figure 1A**, $n \geq 5$). Similarly,

[1]https://www.addgene.org/62276/

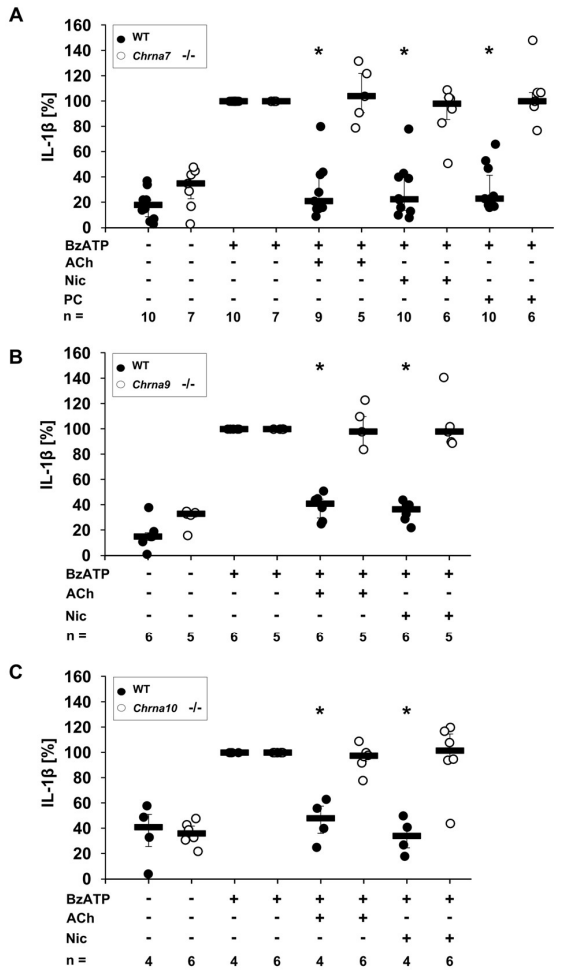


FIGURE 1 | Release of interleukin-1β (IL-1β) by mouse peripheral blood mononuclear cells (PBMCs) from wild-type (WT) mice and mice deficient in *Chrna7*, *Chrna9* or *Chrna10*. Freshly isolated PBMCs were left untreated or stimulated with 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium salt (BzATP, 100 μM) for 30 min, in the presence or absence of acetylcholine (ACh, 10 μM) **(A)**, nicotine (Nic, 100 μM) **(B)** or phosphocholine (PC, 100 μM) **(C)**. BzATP induced the release of IL-1β in PBMCs obtained from all mouse strains. ACh, Nic and PC significantly inhibited the IL-1β release by PBMCs from WT mice **(A–C)**. In contrast, the IL-1β release by PBMCs from mice deficient in *Chrna7* was not affected by ACh, Nic and PC **(A)**. Similarly, a deficiency in *Chrna9* **(B)** or *Chrna10* **(C)** abolished the inhibitory effect of ACh and Nic. The IL-1β concentration was determined by ELISA. In each experiment, the IL-1β concentrations obtained after stimulation with BzATP alone were set to 100% and all other values were calculated accordingly. Data are presented as individual data points, bars represent median, whiskers percentiles 25 and 75. *$p \leq 0.05$ significantly different from samples where BzATP was given alone, Wilcoxon signed-rank test.

the ACh- and Nic-mediated inhibition of IL-1β release was fully abolished in PBMCs from *Chrna9* (**Figure 1B**, $n \geq 5$) or *Chrna10* (**Figure 1C**, $n \geq 4$) gene-deficient mice. Previously, we demonstrated that the inhibitory effect of PC is also abolished in PBMCs deficient in *Chrna9* and *Chrna10* (Richter et al., 2016). Cell death, estimated by measurement of LDH release, remained below 9% in all experiments (data not shown).

The nAChR Subunits α7, α9 and α10 Are Necessary for ACh-, Nic- or PC-Mediated Inhibition of BzATP-Induced IL-1β Release by U937 Cells

To corroborate the results obtained in mouse PBMCs and to confirm that the inhibitory effects of ACh, Nic and PC depend on the activation of nAChRs composed of α7, α9 and/or α10 subunits in human monocytic cells as well, a panel of nAChR antagonists was used. As expected, ACh, Nic or PC completely inhibited the IL-1β release from human monocytic U937 cells primed with LPS and stimulated with BzATP (**Figures 2A–C**, $p \leq 0.05$, $n = 4$). In the presence of the general nAChR antagonist Mec (Philip et al., 2012), the inhibitory effect of ACh and PC was lost (**Figures 2A,C**). Similarly, the nAChR antagonists α-Bun and Stry, that preferentially inhibit nAChRs containing α7 or α9 subunits (McIntosh et al., 2009; Kudryavtsev et al., 2015), effectively blocked the inhibitory effects of ACh and PC. As published before, Nic was also ineffective in the presence of Mec, α-Bun or Stry (Hecker et al., 2015). In line with these observations, ArIB [V11L, V16D], a selective antagonist of α7 nAChRs (Whiteaker et al., 2007; Innocent et al., 2008), abolished the inhibitory effects of ACh and Nic (**Figures 2A,B**), whereas the effect of PC was strongly diminished but not fully inhibited (**Figure 2C**). Suppression of the activity of the nAChR subunit α9 by the treatment with the selective α-conotoxin RgIA4 (Vincler et al., 2006; Romero et al., 2017) abolished the inhibitory effect mediated by ACh or Nic (**Figures 2A,B**). It has been shown before, that the inhibitory effect of PC was prevented in the presence of RgIA4 (Richter et al., 2016). Of note, the application of nAChR antagonists alone had no effect on the IL-1β release (Hecker et al., 2015; Richter et al., 2016).

To further confirm the importance of nAChR subunits α7, α9 and/or α10 in the ACh-, and PC-mediated inhibition of BzATP-induced IL-1β secretion, the expression of these subunits was down-regulated in U937 cells by siRNA. As expected, ACh and PC fully inhibited IL-1β release from U937 cells transfected with scrambled siRNA (**Figures 3A,B**, $p \leq 0.05$, $n = 4$), whereas silencing the expression of the α7 subunit blunted the inhibitory potential of ACh and PC in U937 cells primed with LPS and stimulated with BzATP (**Figures 3A,B**). Similarly, a reduced expression of α9 or α10 subunits blocked the inhibitory mechanism mediated by ACh (**Figure 3A**). Of importance, gene-silencing did not impair BzATP-mediated IL-1β release (**Figures 3A,B**).

To control for the efficiency and specificity of subunit knock-down, the mRNA expression of nAChR subunits α9 and α10 was quantified by real-time RT-PCR (**Figures 4A–D**). As already reported (Hecker et al., 2015), transfection of siRNAs targeting the α9 or the α10 nAChR subunit resulted in a significant down-regulation of mRNA of subunit α9 (**Figure 4A**, $p \leq 0.05$, $n = 4$) and α10 (**Figure 4C**, $p \leq 0.05$, $n = 4$) respectively, when compared with cells transfected with scrambled siRNA.

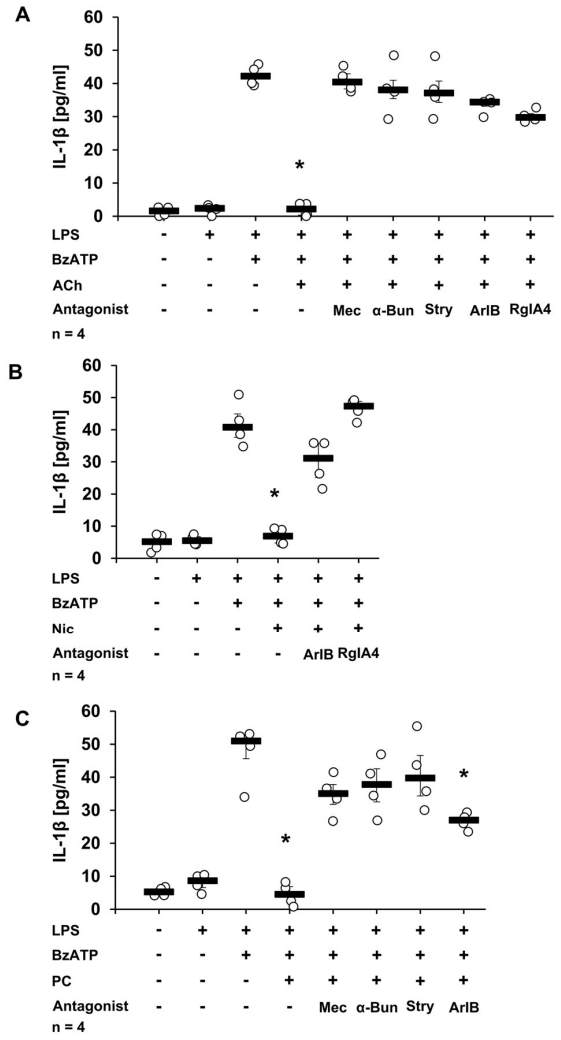


FIGURE 2 | Secretion of IL-1β by U937 cells in the presence of nicotinic acetylcholine receptor (nAChR) antagonists and ACh, Nic or PC. U937 cells were primed with lipopolysaccharide (LPS; 1 μg/ml) for 5 h and BzATP (100 μM) was given for additional 30 min, in the presence or absence of ACh (10 μM) **(A)**, Nic (100 μM) **(B)** or PC (100 μM) **(C)**. BzATP induced the release of IL-1β, which was inhibited in the presence of ACh, Nic or PC **(A–C)**. The inhibitory potential of ACh **(A)** was abolished by mecamylamine (Mec, 100 μM), α-bungarotoxin (α-Bun, 1 μM), strychnine (Stry, 10 μM), ArIB [V11L, V16D] (500 nM) or RgIA4 (200 nM). Addition of ArIB or RgIA4 antagonized the effects of Nic **(B)**. Similarly the effect of PC was blunted by Mec, α-Bun, Stry and ArIB **(C)**. The IL-1β concentration was quantified by ELISA. Data were analyzed by Kruskal-Wallis test followed by Mann-Whitney rank sum test and presented as individual data points, bars represent median, whiskers percentiles 25 and 75. $*p \leq 0.05$ significantly different from samples where BzATP was given alone.

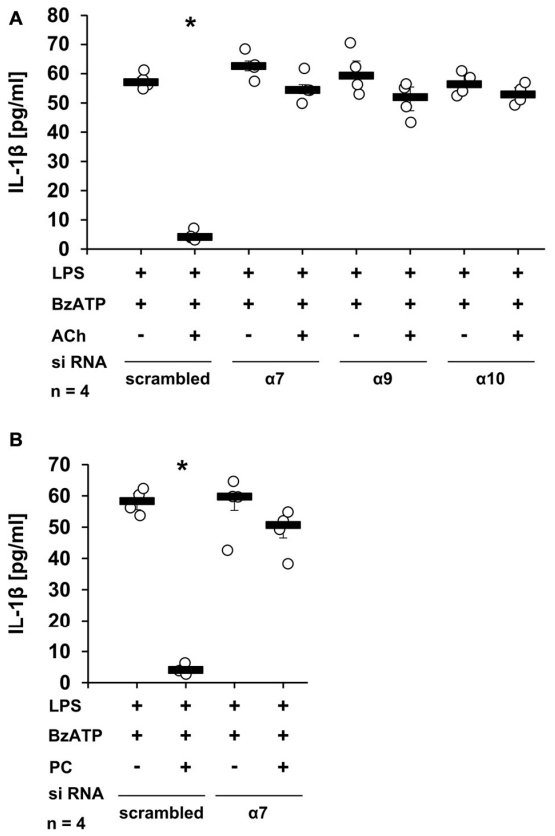


FIGURE 3 | Down-regulation of nAChR subunits α7, α9 or α10 by siRNA abolished the inhibitory potential of ACh or PC. U937 cells were transfected with siRNA targeting *CHRNA7* (α7), *CHRNA9* (α9), *CHRNA10* (α10) or with scrambled siRNA. Forty-eight hours after transfection, cells were primed with lipopolysaccharide (LPS; 1 μg/ml) for 5 h and BzATP (100 μM) was given for additional 30 min in the presence or absence of ACh (10 μM) **(A)** or PC (100 μM) **(B)**. BzATP induced the release of IL-1β, which was inhibited in the presence of ACh or PC in cells transfected with scrambled siRNA. Down-regulation of the nAChR subunit α7 blunted the effect of ACh **(A)** or PC **(B)**. Similarly, reduced expression of the α9 and α10 nAChR subunits diminished the inhibitory potential of ACh **(A)**. The IL-1β concentration was measured by ELISA. Data were analyzed by Kruskal-Wallis test followed by Mann-Whitney rank sum test and presented as individual data points, bars represent median, whiskers percentiles 25 and 75. $*p \leq 0.05$ significantly different from samples where BzATP was given alone.

In contrast, transfection with siRNA targeting nAChR subunits α7 or α10 did not change the α9 mRNA expression (**Figure 4B**). Similarly, transfection with siRNA specific for the α7 or α9 nAChR subunits did not influence the expression of subunit α10 (**Figure 4D**). The mRNA expression of nAChR subunit α7 was detected at a very low level, which could not be reliably quantified. Due to the lack of specific antibodies (Moser et al., 2007; Rommel et al., 2015) it is problematic to investigate protein expression levels of nAChR subunits.

LPC and G-PC Dose-Dependently Inhibit BzATP-Induced IL-1β Release by U937 Cells

To test whether other metabolites of phosphatidylcholines, besides PC, could also inhibit BzATP-induced IL-1β release

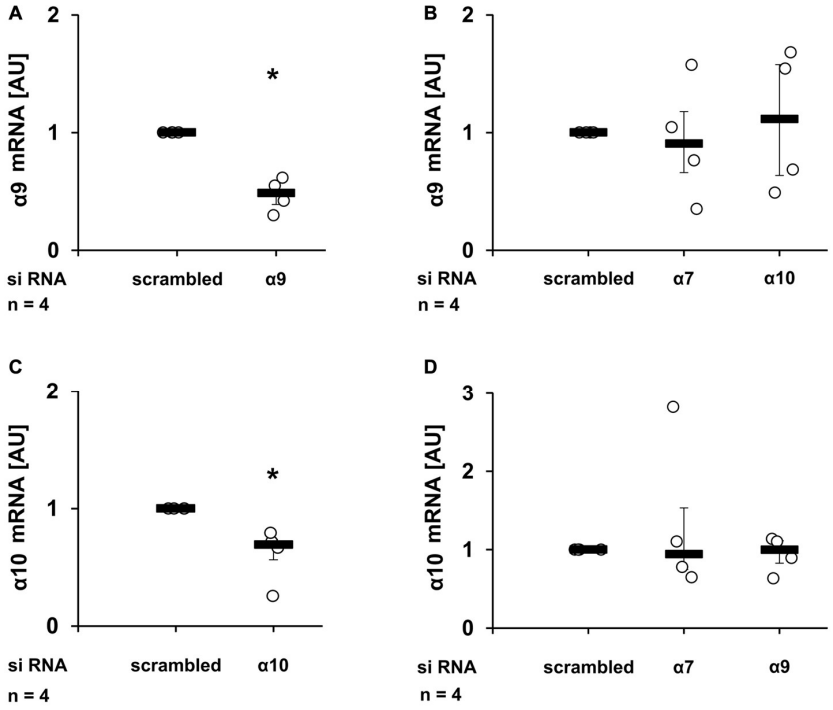


FIGURE 4 | Reduction of mRNA expression of nAChR subunits α9 and α10 upon siRNA transfection. U937 cells were transfected with scrambled siRNA or with siRNA specific for *CHRNA7* (α7), *CHRNA9* (α9) or *CHRNA10* (α10). Forty-eight hours after transfection, the mRNA expression of α9 **(A,B)** or α10 **(C,D)** was analyzed by real-time RT-PCR. Transfection efficiently **(A,C)** and selectively **(B,D)** down-regulated the mRNA expression of the gene targeted with gene-specific siRNA. Data were analyzed by Mann-Whitney rank sum test and presented as individual data points, bars represent median, whiskers percentiles 25 and 75. $^*p \leq 0.05$ significantly different from samples transfected with scrambled siRNA.

in U937 cells, the inhibitory effects of LPC and G-PC were examined. LPC (IC_{50} ~1 μM; **Figure 5A**, $p \leq 0.05$, $n = 5$) as well as G-PC (IC_{50} ~1 μM; **Figure 5B**, $p \leq 0.05$, $n \geq 4$) dose-dependently inhibited BzATP-induced IL-1β release without affecting the viability of the cells, as measured by LDH release (data not shown). Application of LPC (100 μM) or G-PC (100 μM) alone did not induce IL-1β release.

The nAChR Subunits α9 and α10 Are Mandatory for LPC- or G-PC-Mediated Inhibition of BzATP-Induced IL-1β Release by U937 Cells

To analyze the involvement of nAChR subunits α7, α9 and/or α10 in the LPC- and G-PC-mediated inhibition of BzATP-induced IL-1β secretion, a panel of nAChR antagonists was applied. The inhibitory effects of LPC and G-PC were fully prevented in the presence of Mec, α-Bun, Stry or RgIA4. In contrast, ArIB, the nAChR α7 specific antagonist, at best slightly attenuated the inhibitory effects mediated by LPC and G-PC (**Figures 6A,B**, $p \leq 0.05$, $n = 4$). To further confirm these results, the expression of nAChR subunits α7, α9 or α10 was selectively down-regulated using siRNA (**Figures 7A,B**, $p \leq 0.05$, $n = 4$). Silencing of single nAChR subunits, or transfection of scrambled siRNA, did not influence the BzATP-induced IL-1β release. Importantly, silencing the expression of nAChR subunits α9 or α10 effectively abolished the inhibitory potential of LPC and G-PC. In contrast, down-regulation of the α7 nAChR subunit only slightly impaired the inhibitory effects of LPC and G-PC (**Figures 7A,B**, $p \leq 0.05$, $n = 4$).

LPC and G-PC Do Not Induce Ion Channel Functions at Heterologously Expressed nAChRs

Next, we investigated the effect of LPC and G-PC at canonical ionotropic nAChRs. For this purpose, we used *Xenopus laevis* oocytes as a heterologous expression system for different combinations of human α7, α9 and α10 nAChR subunits and performed TEVC measurements to assess ion channel functions. We demonstrated previously, that PC does not evoke ion currents at heterologously expressed homomeric α9 and heteromeric α9α10 nAChR (Richter et al., 2016). Here we tested the effects of PC at different combinations of human α7, α9 and

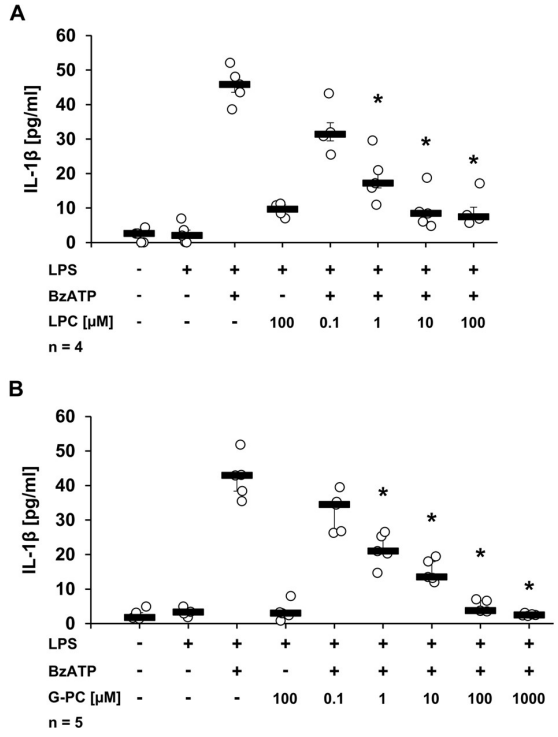


FIGURE 5 | Dose-dependent inhibition of IL-1β release by lysophosphatidylcholine (LPC) or glycerophosphocholine (G-PC). U937 cells were primed with LPS (1 μg/ml) for 5 h and BzATP (100 μM) was applied for additional 30 min, in the presence or absence of different concentrations of LPC (0.1–100 μM) or G-PC (0.1–1000 μM). The BzATP-induced IL-1β release was inhibited in the presence of LPC or G-PC. The IL-1β concentration was determined by ELISA. Data were analyzed by Kruskal-Wallis test followed by Mann-Whitney rank sum test and presented as individual data points, bars represent median, whiskers percentiles 25 and 75. *$p \leq 0.05$ significantly different from samples where BzATP was given alone.

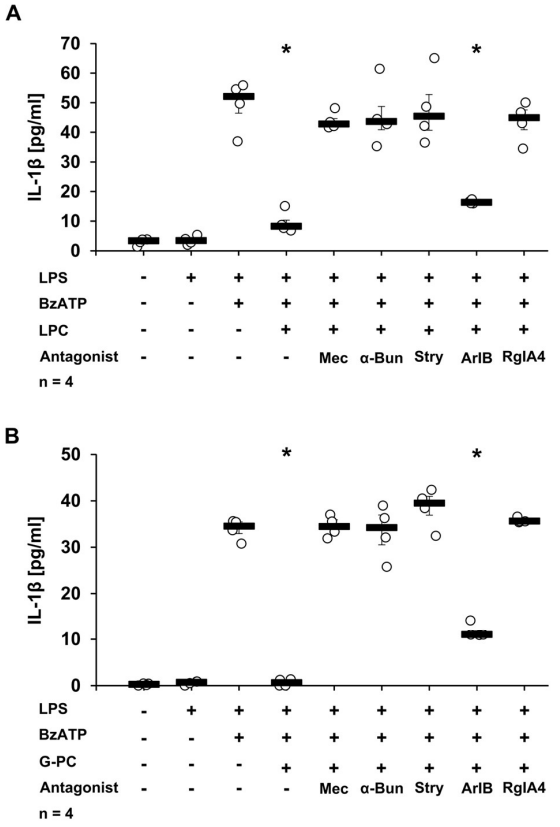


FIGURE 6 | Secretion of IL-1β by U937 cells in the presence of nAChR antagonists and LPC or G-PC. U937 cells were primed with LPS (1 μg/ml) for 5 h and BzATP (100 μM) was given for additional 30 min, in the presence or absence of LPC (10 μM) **(A)** or G-PC (100 μM) **(B)**. LPC or G-PC inhibited efficiently the BzATP-mediated IL-1β release. The inhibitory potential of LPC and G-PC was abolished by mecamylamine (Mec, 100 μM), α-bungarotoxin (α-Bun, 1 μM), strychnine (Stry, 10 μM), or RgIA4 (200 nM). In contrast, addition of ArIB [V11L, V16D] (500 nM), an α7 nAChR subunit-specific antagonist, did not abolish the inhibitory effect mediated by LPC or G-PC. The IL-1β concentration was quantified by ELISA. Data were analyzed by Kruskal-Wallis test followed by Mann-Whitney rank sum test and presented as individual data points, bars represent median, whiskers percentiles 25 and 75. *$p \leq 0.05$ significantly different from samples where BzATP was given alone.

α10 nAChR subunits. To control for the expression of nAChR in all experiments, the nAChR agonist ACh (100 μM; Papke et al., 1996; Azam and McIntosh, 2012) was used as a positive control.

The first set of experiments was performed on oocytes that were injected with cRNA encoding for α7, α9 and α10 nAChR subunits (**Figure 8**). As expected, application of ACh (ACh1) evoked currents (ΔI_M) that were reversible and repeatable by subsequent ACh application (ACh2; **Figure 8A**). The ACh-induced effects (ACh1, ACh2) were not significantly different ($n = 17$, $p = 0.14$; **Figures 8A,E**). Next, LPC (100 μM) was tested on oocytes expressing nAChR subunits α7, α9 and α10 (**Figure 8B**). While LPC (2 min) did not elicit ion currents, subsequent application of ACh resulted in significant current responses ($n = 8$, $p = 0.001$; **Figures 8B,E**). In the same way, G-PC (100 μM; **Figure 8C**) and PC (1 mM; **Figure 8D**) were tested. Again, neither G-PC nor PC induced ion currents, while application of ACh induced a current response (G-PC: $n = 6$, $p = 0.001$, **Figures 8C,E**; PC: $n = 8$, $p = 0.001$; **Figures 8D,E**). In water-injected control oocytes, ACh ($n = 4$), LPC ($n = 6$), G-PC ($n = 4$) and PC ($n = 6$) and had no impact on ΔI_M (**Figures 8F–I**).

In addition, we performed experiments on oocytes that were injected with different combinations of human nAChR subunits (**Figure 9**). In oocytes expressing homomeric α7 nAChRs, application of LPC, G-PC or PC had no impact on ΔI_M, while ACh induced significant current responses (**Figures 9A,B**; LPC: $n = 6$, $p = 0.001$; G-PC: $n = 6$, $p = 0.001$; PC: $n = 6$, $p = 0.001$). The same results were obtained in experiments on oocytes co-expressing nAChR subunits α7 and α9 (LPC: $n = 6$, $p = 0.001$; G-PC: $n = 7$, $p = 0.001$; PC: $n = 6$, $p = 0.001$; **Figures 9C,D**). It was previously

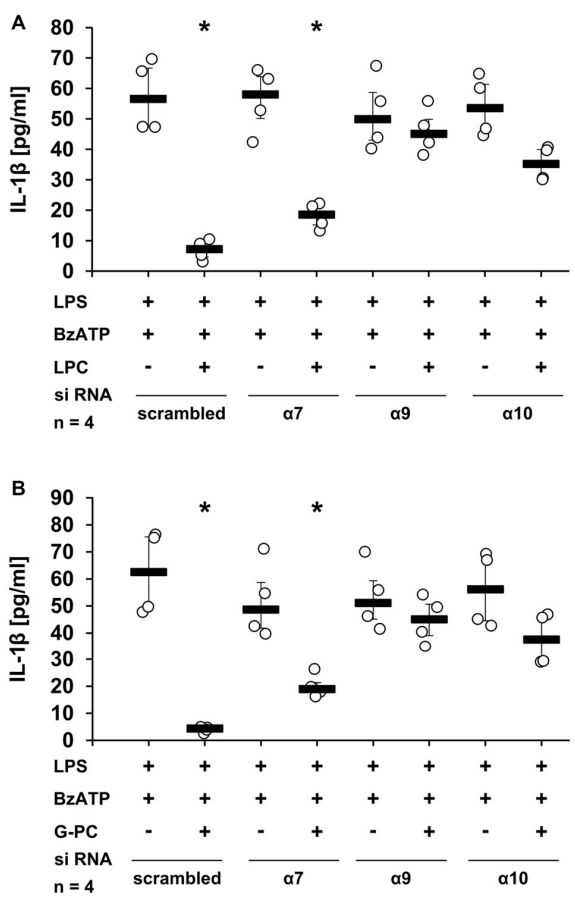


FIGURE 7 | Down-regulation of nAChR subunits α9 or α10 by siRNA abolished the inhibitory potential of LPC and G-PC. U937 cells were transfected with siRNA targeting *CHRNA7* (α7), *CHRNA9 (*α9), *CHRNA10* (α10) or with scrambled siRNA. Forty-eight hours after transfection cells were primed with LPS (1 μg/ml) for 5 h and BzATP (100 μM) was given for additional 30 min in the presence or absence of LPC (10 μM) **(A)** and G-PC (100 μM) **(B)**. BzATP induced the release of IL-1β, which was inhibited in the presence of LPC or G-PC in cells transfected with scrambled siRNA. Reduced expression of α9 or α10 subunits abrogated the inhibitory potential of LPC and G-PC. In contrast, down-regulation of the α7 subunit expression only slightly inhibited the LPC- or G-PC-mediated mechanism. The IL-1β concentration was measured by ELISA. Data were analyzed by Kruskal-Wallis test followed by Mann-Whitney rank sum test and presented as individual data points, bars represent median, whiskers percentiles 25 and 75. *$p \leq 0.05$ significantly different from samples where BzATP was given alone.

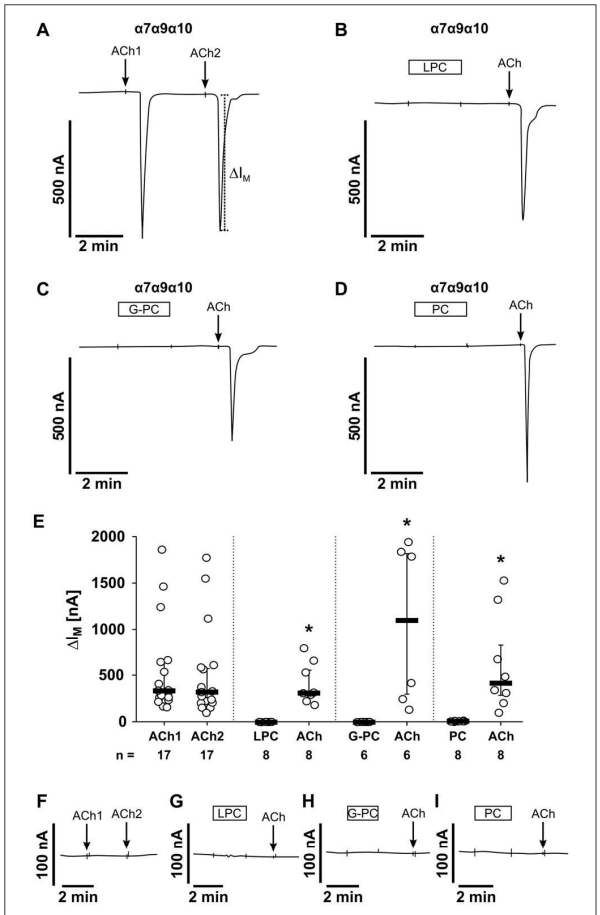


FIGURE 8 | LPC, G-PC and PC do not induce ion channel functions at oocytes co-expressing nAChR subunits α7, α9 and α10. Two-electrode voltage-clamp (TEVC) measurements were performed on *Xenopus laevis* oocytes that heterologously co-expressed human α7, α9 and α10 nAChR subunits. **(A)** Representative current curve. The application of ACh (100 μM; 1 s) induced repetitive responses of transmembrane ion currents (ΔI_M; ACh1 and ACh2). **(B–D)** Initial application of LPC (100 μM; 2 min) **(B)**, G-PC (100 μM; 2 min) **(C)** as well as PC (100 μM; 2 min) **(D)** had no impact on ΔI_M, whereas ACh induced a current response. **(E)** Graphical representation of the results of experiments as shown in **(A–D)**. **(F–I)** In water-injected control oocytes neither repeated application of ACh (n = 16), nor application of LPC (n = 6), G-PC (n = 4) or PC (n = 6) had any effect on ΔI_M. All changes of ΔI_M induced by cholinergic stimulation are shown as individual data points, bars represent median, whiskers percentiles 25 and 75. Statistical analyses were performed using the Wilcoxon signed-rank test. *$p \leq 0.05$ significantly different from the ΔI_M values before.

shown, that PC does not induce ion channel functions on heterologously expressed heteromeric α9α10 nAChR (Richter et al., 2016). Here, we investigated the impact of LPC and G-PC on heteromeric α9α10 nAChR (**Figures 9E,F**). Again, application of LPC and G-PC had no effect on ΔI_M, while ACh induced a significant current response (**Figures 9E,F**; LPC: $n = 6$, $p = 0.001$; G-PC: $n = 6$, $p = 0.001$). In all experiments the ACh-induced effect was repeatable (ACh1, ACh2) and not significantly different compared to the first response (α7: $n = 9$, $p = 0.432$; α7α9: $n = 7$, $p = 0.645$; α9α10: $n = 6$, $p = 0.65$; **Figures 9B,D,F**). LPC, G-PC, PC and ACh did not induce changes in ion currents of water-injected control oocytes (data not shown).

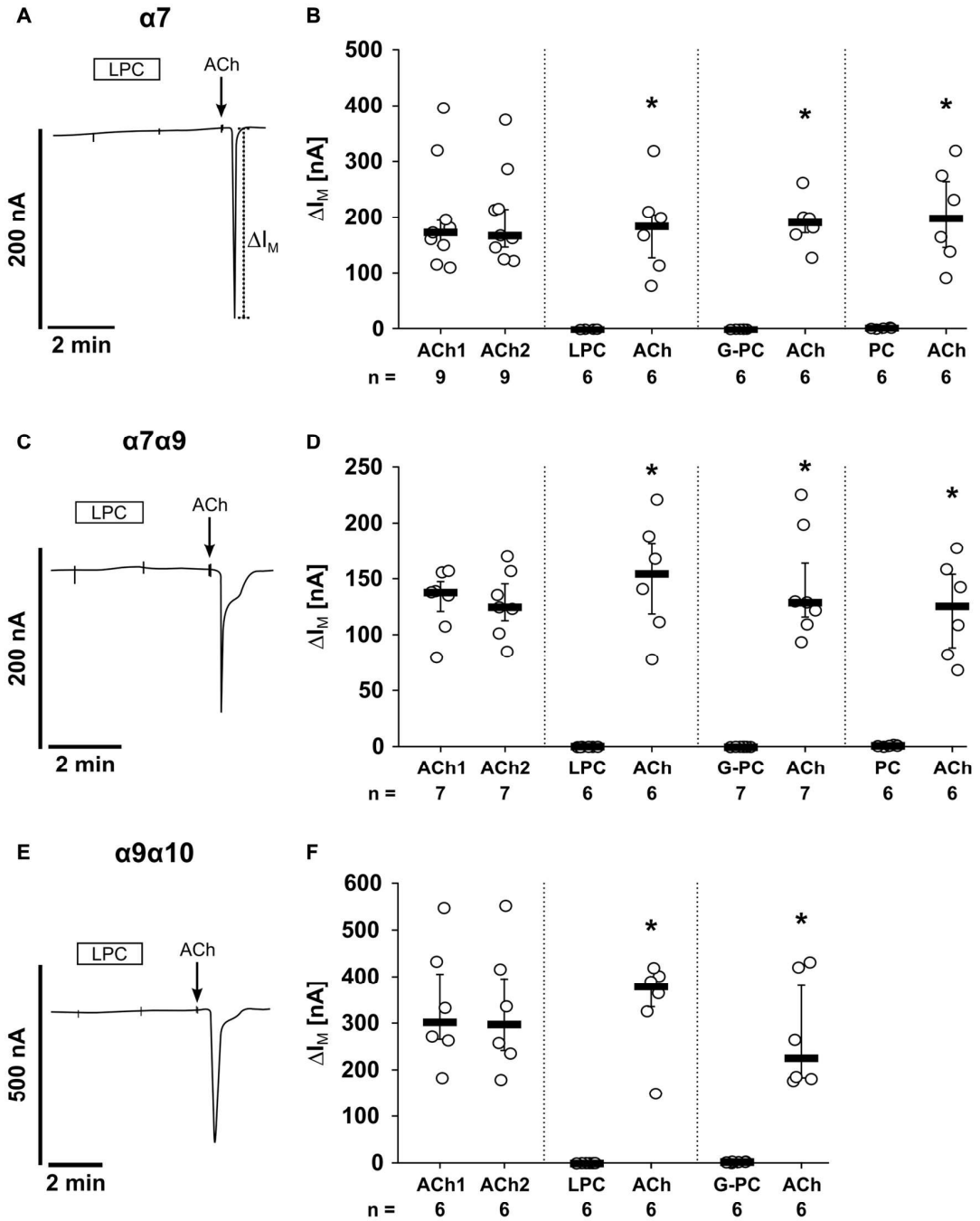


FIGURE 9 | LPC, G-PC and PC do not induce ion channel activity at heterologously expressed nAChR subunits. TEVC measurements were performed on *Xenopus laevis* oocytes that expressed the human α7 nAChR subunit alone **(A,B)**, as well as co-expressed human α7 and α9 **(C,D)** or human α9 and α10 **(E,F)** nAChR subunits. In all experiments, application of ACh (100 μM; 1 s) induced repetitive stimulations of the transmembrane ion current (ΔI_M; ACh1 and ACh2). In contrast, initial application of LPC (100 μM; 2 min), G-PC (100 μM; 2 min) as well as PC (100 μM; 2 min) had no impact on I_M, whereas ACh induced a current response. All changes of ΔI_M induced by cholinergic stimulation are shown as individual data points, bars represent median, whiskers percentiles 25 and 75. Statistical analyses were performed using the Wilcoxon signed-rank test. *$p \leq 0.05$ significantly different from the ΔI_M values before.

Inhibition of ACh-Induced Currents by LPC and G-PC

Previously, we demonstrated that PC blunts classical ion channel responses at α9α10 nAChR heterologously expressed by *Xenopus laevis* oocytes (Richter et al., 2016). To test if LPC and G-PC have an impact on ACh-induced ion currents of nAChR, we performed TEVC measurements and monitored current responses induced by ACh (100 μM, 1 s pulses) in presence or absence of LPC or G-PC (**Figure 10**). In experiments on oocytes expressing heteromeric α9α10 nAChR, LPC (1 μM) decreased the ACh-evoked responses to 69 ± 2% (n = 12) of control values after a 12 min exposure to the compound (**Figures 10A,B**). Thereafter, LPC was washed out and the current responses recovered to 101 ± 2% (n = 12) after 12 min (**Figures 10A,B**). Similar experiments were performed on oocytes expressing homomeric α7 nAChR (**Figure 10C**). In presence of LPC, the ACh-evoked currents were reduced to 89 ± 7% (n = 6) of control values after a 12 min perfusion (**Figure 10C**). The responses recovered to 101 ± 6% (n = 6) of control values after a 12 min wash-out period (**Figure 10C**). In oocytes that expressed heteromeric α9α10 nAChR, G-PC (100 μM) decreased the ACh-induced currents to 60 ± 7% (n = 6) of control values (**Figure 10D**). After a 12 min wash-out period of G-PC, the ACh-induced responses recovered to 90 ± 2% (n = 6) of control values (**Figure 10D**).

DISCUSSION

Anti-inflammatory effects of nAChR agonists in macrophages were repeatedly described and the term "anti-inflammatory cholinergic reflex" was coined more than a decade ago. It was suggested that ACh of vagal origin activates nAChR composed of subunit α7 and controls the synthesis of pro-IL-1β and other pro-inflammatory cytokines on the transcriptional and post-transcriptional level (Borovikova et al., 2000; de Jonge et al., 2005; Rosas-Ballina et al., 2011; Olofsson et al., 2012). We recently discovered that Nic, Cho and ACh efficiently reduce the ATP-induced inflammasome activation and IL-1β maturation in human monocytes. Ligand-binding to monocytic nAChRs containing subunits α7, α9 and/or α10 triggers a metabotropic response that efficiently inhibits ATP-signaling at P2X7 receptor (Hecker et al., 2015). Similar cholinergic effects on monocytes are also provoked by PC, PC-modified macromolecules and DPPC (Hecker et al., 2015; Richter et al., 2016; Backhaus et al., 2017). In the current study: (i) we demonstrate that LPC and G-PC, common metabolites of phosphatidylcholines, also activate monocytic nAChR; (ii) we determine the nAChR subunit requirements of different conventional and non-conventional nicotinic agonists; and (iii) we provide evidence that LPC, G-PC and PC do not evoke ion currents at heterologously expressed nAChR, but rather act as silent agonists.

We provide evidence that LPC and G-PC function as nAChR agonists that dose-dependently and efficiently inhibit the BzATP-mediated IL-1β release by LPS-primed human monocytic U937 cells. Interestingly, the IC_{50} of both compounds is one order of magnitude lower than that of PC (Hecker et al., 2015) or DPPC (Backhaus et al., 2017). Importantly, effective concentrations of LPC, G-PC and PC can be detected in human plasma (Ilcol et al., 2002, 2005; Drobnik et al., 2003; Quehenberger et al., 2010). As LPC and G-PC are metabolites of phosphatidylcholines, different cell signaling pathways involving phospholipases as well as exposure to insect or snake venoms rich in phospholipases (Harris and Scott-Davey, 2013) may trigger this anti-inflammatory pathway. There are some conflicting data on immune-modulatory functions of LPC (Liu-Wu et al., 1998; Kabarowski et al., 2002; Stock et al., 2006; Carneiro et al., 2013), but none of these studies suggested a cholinergic mechanism. Anti-inflammatory effects of LPC, including a reduction of peritoneal IL-1β levels, were reported for experimental sepsis in the mouse and these effects seemed to depend on the presence of 18 carbon molecules in the acyl chain (Yan et al., 2004), whereas we used LPC with a chain length of 16 in our experiments. Other studies suggested an induction of pro-IL-1β in human monocytes during long-term incubation with LPC with a carboxyl chain length of 16 and 18 (Liu-Wu et al., 1998) and a LPC- and Ca^{2+}-dependent induction of IL-1β in LPS-primed microglial cells (Stock et al., 2006). These conflicting results might be explained by the different experimental protocols, the different cell types investigated and by the potential presence of pyrogenic contaminations that induce the expression of IL-1β.

In order to define which nAChR subunits are required for the inhibitory function of ACh, Nic and PC on the BzATP-driven release of IL-1β, we used gene-deficient mice, a panel of nAChR antagonists and performed gene-silencing experiments. We knew from previous pharmacological and gene-silencing studies that the effect of Nic is mediated via nAChR containing subunits α7, α9 and/or 10 (Hecker et al., 2015). It remained, however, unclear, if all three receptor subunits are mandatory or if they exert redundant functions. Regarding the signaling of PC, experiments on gene-deficient mice as well as pharmacological and gene-silencing studies revealed that nAChR subunits α9 and α10 are mandatory (Richter et al., 2016) but in this case the role of subunit α7 was still unclear and nothing was known about the nAChR subunit requirements for the ACh mediated effects. First, we investigated PBMCs from WT mice and mice deficient in the genes for nAChR subunits α7, α9 or α10. The results of these experiments suggested that all three receptor subunits are mandatory for the inhibition of IL-1β release by ACh, Nic and PC. In the same line, Mec, a general nicotinic antagonist (Philip et al., 2012) as well as α-Bun and Stry, antagonists of nAChR containing subunits α7 and α9 (McIntosh et al., 2009; Kudryavtsev et al., 2015), efficiently reverted the inhibitory effects of ACh, Nic (Hecker et al., 2015) and PC in LPS-primed human U937 cells. Similarly, RgIA4, a selective antagonist of nAChR subunit α9 (Vincler et al., 2006; Romero et al., 2017) and ArIB, a selective antagonist of nAChR subunit α7 (Innocent et al., 2008), fully abolished the inhibitory effect mediated by ACh, Nic and PC (Richter et al., 2016). These data were further corroborated by siRNA experiments performed here and in previous studies (Hecker et al., 2015; Richter et al., 2016). We conclude from these data that all three nAChR subunits α7,

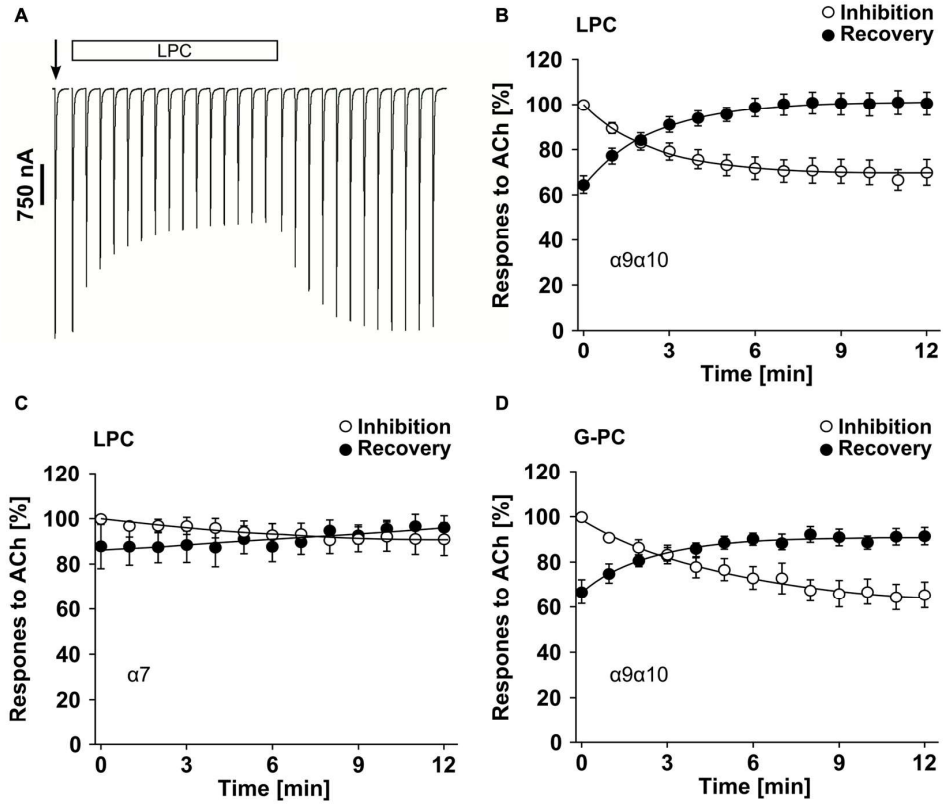


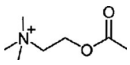
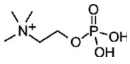
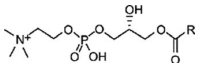
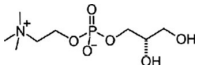
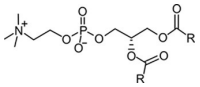
FIGURE 10 | LPC and G-PC desensitize ACh-gated currents mediated by nAChRs heterologously expressed in *Xenopus laevis* oocytes. TEVC experiments were performed on oocytes that expressed human homomeric α7 or heteromeric α9α10 nAChRs. **(A)** Representative traces of ACh-gated currents mediated by α9α10 nAChRs illustrating the inhibitory effect of LPC (1 μM). The current traces represent 30 s recordings each and are shown concatenated omitting the 30 s gap between each individual trace. The oocytes were continuously perfused with control solution and stimulated with 1 s pulses of ACh (100 μM) once per min until steady-state baseline responses were observed (indicated by the arrow). Subsequently, the control solution was switched to one containing LPC and the ACh-gated currents were monitored for changes in amplitude for 12 min. Thereafter, the perfusion solution was switched back to control and the ACh-gated currents were monitored for recovery. **(B)** Graphical representation of the experimental results shown in **(A)** (n = 12). **(C)** Similar experiments as those in **(A,B)** were performed on α7 nAChR expressing oocytes to assess the effects of LPC on ACh-gated currents mediated by this nAChR subtype (n = 6). **(D)** Analysis of inhibition and recovery from inhibition of ACh-gated currents by G-PC (100 μM) on α9α10 nAChR expressing oocytes (n = 6). The error bars in all graphs denote the standard error of mean (SEM) from the indicated numbers of oocytes. The data in **(B–D)** were analyzed with single exponential equations and the X-axes represent the temporal duration of each experimental condition (absence or presence of LPC or G-PC).

α9 and α10 are needed for signaling of ACh, Nic and PC in monocytic cells (**Table 1**).

Next, we investigated the receptor requirements for the more complex compounds LPC and G-PC by using both nAChR antagonists and gene-silencing of nAChR subunits by siRNA in human U937 cells. In contrast to ACh, Nic and PC, we provide evidence that only nAChR subunits α9 and α10 are mandatory for the inhibitory mechanism mediated by LPC and G-PC. There might be a biologically insignificant contribution of subunit α7 because both gene silencing und ArIB seemed to provoke a subtle impairment of the inhibitory effects of LPC and G-PC. This discrepancy might be explained by differences in the structure and the physicochemical properties among these compounds (**Table 1**). Interestingly, the recently reported inhibitory effects of DPPC on ATP-driven inflammasome activation also strictly depended on the α9 subunit, but in contrast to LPC and G-PC, the α10 subunit is not mandatory but it can be functionally replaced by subunit α7 (Backhaus et al., 2017).

With all due caution, we propose that the α9 nAChR subunit is the critical subunit involved in the metabotropic regulation of inflammasome activation, irrespective of the structure of the nicotinic agonist. The α9 nAChR subunit interacts with α7 and/or α10 subunits depending on the complexity of the nAChR agonist. Small agonists such as ACh or PC need all three subunits α7, α9 and α10 for signaling, the agonists LPC and G-PC depend on α9 and α10, without an essential contribution

TABLE 1 | Concentration of agonists causing 50% inhibition (IC_{50}) of the adenosine triphosphate (ATP)-mediated release of interleukin-1β (IL-1β) in monocytes and the nAChR composition needed for the signaling.

Endogenous agonist	Approximate IC_{50}	Required nAChR subunits
ACh	10 μM (Hecker et al., 2015)	α7, α9, α10
		
PC	10 μM (Hecker et al., 2015)	α7, α9, α10
		
LPC	1 μM	α9, α10
		
G-PC	1 μM	α9, α10
		
DPPC	10 μM (Backhaus et al., 2017)	α9 and α7 or α10
		

ACh, acetylcholine; DPPC, dipalmitoylphosphatidylcholine; G-PC, glycerophosphocholine; LPC, lysophosphatidylcholine; nAChR, nicotinic ACh receptor; R, fatty acid residue C16:0.

of subunit α7 (**Table 1**). The more bulky and hydrophobic phosphatidylcholines like DPPC, signal alternatively via subunit combination α7α9 or via α9α10 (Backhaus et al., 2017; **Table 1**). We have to admit that although this generalization is tempting, we only tested LPC (16:0) and DPPC up to now, but different variants of LPC and phosphatidylcholines have to be investigated before establishing a principle, and these are many and varied.

The following experiments were designed to clarify if LPC, G-PC and PC interact with canonical nAChRs that function as ligand-gated ion channels. We have previously shown that PC does not evoke ion currents in *Xenopus laevis* oocytes expressing human α9 or α9α10 nAChRs. Here, we investigated the short-term effect of LPC, G-PC and PC on ionotropic nAChR functions using *Xenopus laevis* oocytes as a heterologous expression system for human α7 nAChR as well as the expression of combinations of α7, α9 and α10 subunits. Due to the lack of specific antibodies to the nAChR investigated (Moser et al., 2007; Rommel et al., 2015), we were not able to test if all nAChR subunits were co-expressed in our experimental setup, but we were able to demonstrate that ACh induced characteristic ion currents in oocytes injected with cDNA for nAChR but not in control oocytes. In addition, we do not know which homomeric or heteromeric nAChR species form in co-injected oocytes. Of note, LPC, G-PC or PC did not induce ion currents in any of the experimental settings, wheras the classical nAChR agonist ACh did.

Although PC does not induce ion currents at classical nAChR, it seems to function as a silent agonist that desensitizes α9α10 nAChR (Richter et al., 2016). Hence, we investigated the long-term effects of LPC and G-PC on heterologously expressed nAChR. While the brief application of 100 μM LPC did not evoke responses in oocytes expressing nAChRs, application of LPC (100 μM) induced a non-specific increase of the baseline holding current during continuous perfusion of the compound. Such effects have been described before and might be due to a detergent-like action of LPC (Ikeuchi et al., 1997). Therefore, we lowered the LPC concentration in the long-term experiments to 1 μM, a concentration that still inhibited the ATP-induced IL-1β release in U937 cells.

In the inhibition and recovery from inhibition measurements, we found that in human α9α10 nAChR-expressing oocytes, ACh-mediated current responses were blunted in the presence of LPC and G-PC. The slow kinetics of inhibition and recovery from inhibition by LPC and G-PC suggest that both compounds might also function as silent agonists of the heteromeric α9α10 nAChR. However, we cannot formally exclude that LPC and G-PC act as partial antagonists at these nAChR. Interestingly, while LPC decreased the ACh-evoked current responses to 69 ± 2% in heteromeric α9α10 nAChR expressing oocytes, the ACh-evoked currents in homomeric α7 nAChR expressing oocytes were decreased only to 89 ± 7%. These findings are in accordance with our observation on the monocytic U937 cells, in which the immuno-modulatory function of LPC requires the expression of nAChR subunits α9 and α10 but not α7. In summary, LPC, G-PC and PC are potent agonists of non-classical nicotinic receptors of monocytes that provoke metabotropic functions. LPC, G-PC and PC do not induce ion currents, but they seem to act as silent agonists.

Receptor subunits α7, α9 and α10 belong to a unique class of evolutionary conserved nAChR, which in contrast to all other nAChR can form pentameric homomers (Franchini and Elgoyhen, 2006; Lipovsek et al., 2012, 2014). Homomeric α7 and α9 nAChR were also described (Elgoyhen et al., 1994), whereas the human α10 subunit alone does not form functional receptors (Elgoyhen et al., 2001). The α9 and α10 nAChR subunits can co-assemble to heteromers with pharmacological properties different from α9 homomers (Boffi et al., 2017). Heteromers of α7 and α10 have been evidenced in rat sympathetic neurons (Lips et al., 2006). Furthermore a complex interaction of nAChR subunits α7, α9 or α10 in regulating vestibular afferent gain was shown (Morley et al., 2017). In a mast cell line, interaction of nAChR subunits α7, α9 and α10 was described in the context of nicotinic inhibition of FcεRI-induced leukotriene and cytokine production (Mishra et al., 2010). However, exceedingly low nanomolar concentrations of Nic were effective in this mast cell line, which is not the case for the inhibition of ATP-induced release of IL-1β by human U937 cells (Hecker et al., 2015). In all leukocyte subtypes tested so far, nAChR subunits do not form conventional ligand-gated ion channels,

but rather exert metabotropic function (Peng et al., 2004; Razani-Boroujerdi et al., 2007; Hecker et al., 2009, 2015; Mishra et al., 2010; Richter et al., 2016; Valbuena and Lerma, 2016). We published before, that Nic, Cho, PC and DPPC exert their immuno-modulatory functions via metabotropic inhibition of P2X7 receptor function (Hecker et al., 2015; Richter et al., 2016; Backhaus et al., 2017). However, the structure of non-canonical nAChR receptors exerting metabotropic functions is largely unknown; it is even unclear, if leukocytic nAChR form multimers.

There is increasing evidence, that signaling via nAChR subunit α7 induces Ca^{2+} signals and that signaling-related proteins including G proteins can interact with a cytoplasmic loop of this subunit (Kabbani et al., 2013; King and Kabbani, 2016; Valbuena and Lerma, 2016). Similar protein-protein interactions might also occur for subunit α9. In addition, direct interactions of nAChR containing subunit α6 with purinergic receptors P2X2 and P2X3 have been reported (Limapichat et al., 2014; Wieskopf et al., 2015). This interesting and emerging field of metabotropic nicotinic signaling certainly deserves more scientific attention.

Excessive release of IL-1β into the circulation in response to trauma-associated extracellular ATP is useless and dangerous, because the cytokine is swept away from the site of injury and can cause severe life-threatening SIRS and multi-organ damage (Stoecklein et al., 2012; Lord et al., 2014). As monocytes are the major source of IL-1β within the circulation (Arango Duque and Descoteaux, 2014), the inhibitory mechanisms described in this study might be of high clinical relevance. ATP-driven inflammasome activation is typical for the undesired sterile inflammation that causes a high morbidity and mortality worldwide, whereas several ATP-independent pathways lead to the desired inflammasome activation needed for defense against infection (Cauwels et al., 2014). Of note, nAChR subunit α9 plays a central role in the control of ATP-driven inflammasome activation in monocytes, whereas subunit α7 controls the expression of pro-inflammatory cytokines more generally (Borovikova et al., 2000). The specific α9-dependend pathway seems to be an interesting target that might enable the prevention of SIRS caused by multiple traumata and major surgery without inhibiting host defense against infections. As we demonstrated that the cholinergic inhibition of ATP-driven IL-1β release is also active in mice, this concept can and should be evaluated experimentally *in vivo*.

This study has several limitations and more research is needed to further corroborate our concept. One major technical issue is the lack of specific antibodies to the nAChR subunits under investigation that prevents an unequivocal proof of the specificity and efficiency of gene-silencing as well as the proof of the heterologous co-expression of nAChR subunits. At present, we cannot conclude that all LPC species act in the same way like LPC (16:0) and the same holds true for phosphatidylcholines, where only DPPC was investigated previously (Backhaus et al., 2017). It also remains to be tested, if LPC and G-PC stimulate ion currents in monocytic cells, although this is improbable as PC and DPPC do not (Richter et al., 2016; Backhaus et al., 2017). Additionally, more research is needed to identify the signaling pathways involved in the inhibitory mechanism mediated by LPC, G-PC and PC. Finally, we can only speculate on the relevance of our findings *in vivo*.

In conclusion, we demonstrated that canonical nAChR agonists as well as PC inhibit ATP-induced release of IL-1β by human and mouse mononuclear cells via nAChR subunits α7, α9 and α10. In contrast, LPC (16:0) and G-PC, more complex metabolites of phosphatidylcholines, elicit the inhibitory mechanism in human monocytes via interaction of the two nAChR subunits α9 and α10. Independent of the complexity of the compound, neither LPC, G-PC nor PC induce ionotropic functions at heterologously expressed nAChR, suggesting that PC-bearing molecules in general regulate immuno-modulatory functions without inducing canonical ion channel function at nAChR. This selective activity opens the opportunity for the development of novel anti-inflammatory therapies without inducing ionotropic functions of nAChRs.

AUTHOR CONTRIBUTIONS

AZ and KR participated in research design, performance of experiments, interpretation of the data and writing of the manuscript. AA, SW, KS, BF and AJH: performance of experiments, interpretation of the data and editing of the manuscript. GK-C, MA and WP participated in research design, interpretation of the data and editing of the manuscript. JMM and VG participated in research design, interpretation of the data and writing of the manuscript.

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REFERENCES

Arango Duque, G., and Descoteaux, A. (2014). Macrophage cytokines: involvement in immunity and infectious diseases. *Front. Immunol.* 5:491. doi: 10.3389/fimmu.2014.00491

Azam, L., and McIntosh, J. M. (2012). Molecular basis for the differential sensitivity of rat and human α9α10 nAChRs to α-conotoxin RgIA. *J. Neurochem.* 122, 1137–1144. doi: 10.1111/j.1471-4159.2012.07867.x

Backhaus, S., Zakrzewicz, A., Richter, K., Damm, J., Wilker, S., Fuchs-Moll, G., et al. (2017). Surfactant inhibits ATP-induced release of interleukin-1β; via nicotinic acetylcholine receptors. *J. Lipid Res.* 58, 1055–1066. doi: 10.1194/jlr.M071506

Boffi, J. C., Marcovich, I., Gill-Thind, J. K., Corradi, J., Collins, T., Lipovsek, M. M., et al. (2017). Differential contribution of subunit interfaces to α9α10 nicotinic acetylcholine receptor function. *Mol. Pharmacol.* 91, 250–262. doi: 10.1124/mol.116.107482

Borovikova, L. V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G. I., Watkins, L. R., et al. (2000). Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 405, 458–462. doi: 10.1038/35013070

Carneiro, A. B., Iaciura, B. M., Nohara, L. L., Lopes, C. D., Veas, E. M., Mariano, V. S., et al. (2013). Lysophosphatidylcholine triggers TLR2- and TLR4-mediated signaling pathways but counteracts LPS-induced NO synthesis in peritoneal macrophages by inhibiting NF-κB translocation and MAPK/ERK phosphorylation. *PLoS One* 8:e76233. doi: 10.1371/journal.pone.0076233

Cauwels, A., Rogge, E., Vandendriessche, B., Shiva, S., and Brouckaert, P. (2014). Extracellular ATP drives systemic inflammation, tissue damage and mortality. *Cell Death Dis.* 5:e1102. doi: 10.1038/cddis.2014.70

de Jonge, W. J., van der Zanden, E. P., The, F. O., Bijlsma, M. F., van Westerloo, D. J., Bennink, R. J., et al. (2005). Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat. Immunol.* 6, 844–851. doi: 10.1038/ni1229

Dinarello, C. A., Simon, A., and van der Meer, J. W. (2012). Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat. Rev. Drug Discov.* 11, 633–652. doi: 10.1038/nrd3800

Drobnik, W., Liebisch, G., Audebert, F. X., Frohlich, D., Gluck, T., Vogel, P., et al. (2003). Plasma ceramide and lysophosphatidylcholine inversely correlate with mortality in sepsis patients. *J. Lipid Res.* 44, 754–761. doi: 10.1194/jlr.m200401-jlr200

Elgoyhen, A. B., Johnson, D. S., Boulter, J., Vetter, D. E., and Heinemann, S. (1994). α9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell* 79, 705–715. doi: 10.1016/0092-8674(94)90555-x

Elgoyhen, A. B., Vetter, D. E., Katz, E., Rothlin, C. V., Heinemann, S. F., and Boulter, J. (2001). α10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proc. Natl. Acad. Sci. U S A* 98, 3501–3506. doi: 10.1073/pnas.051622798

Ferrari, D., Pizzirani, C., Adinolfi, E., Lemoli, R. M., Curti, A., Idzko, M., et al. (2006). The P2X7 receptor: a key player in IL-1 processing and release. *J. Immunol.* 176, 3877–3883. doi: 10.4049/jimmunol.176.7.3877

Franchini, L. F., and Elgoyhen, A. B. (2006). Adaptive evolution in mammalian proteins involved in cochlear outer hair cell electromotility. *Mol. Phylogenet. Evol.* 41, 622–635. doi: 10.1016/j.ympev.2006.05.042

Froehner, S. C., Luetje, C. W., Scotland, P. B., and Patrick, J. (1990). The postsynaptic 43K protein clusters muscle nicotinic acetylcholine receptors in Xenopus oocytes. *Neuron* 5, 403–410. doi: 10.1016/0896-6273(90)90079-u

Gloor, S., Pongs, O., and Schmalzing, G. (1995). A vector for the synthesis of cRNAs encoding Myc epitope-tagged proteins in *Xenopus laevis* oocytes. *Gene* 160, 213–217. doi: 10.1016/0378-1119(95)00226-v

Gross, O., Thomas, C. J., Guarda, G., and Tschopp, J. (2011). The inflammasome: an integrated view. *Immunol. Rev.* 243, 136–151. doi: 10.1111/j.1600-065x.2011.01046.x

Harris, J. B., and Scott-Davey, T. (2013). Secreted phospholipases A2 of snake venoms: effects on the peripheral neuromuscular system with comments on the role of phospholipases A2 in disorders of the CNS and their uses in industry. *Toxins (Basel)* 5, 2533–2571. doi: 10.3390/toxins5122533

Hecker, A., Küllmar, M., Wilker, S., Richter, K., Zakrzewicz, A., Atanasova, S., et al. (2015). Phosphocholine-modified macromolecules and canonical nicotinic agonists inhibit ATP-induced IL-1β release. *J. Immunol.* 195, 2325–2334. doi: 10.4049/jimmunol.1400974

Hecker, A., Mikulski, Z., Lips, K. S., Pfeil, U., Zakrzewicz, A., Wilker, S., et al. (2009). Pivotal advance: up-regulation of acetylcholine synthesis and paracrine cholinergic signaling in intravascular transplant leukocytes during rejection of rat renal allografts. *J. Leukoc. Biol.* 86, 13–22. doi: 10.1189/jlb.1107722

Ikeuchi, Y., Nishizaki, T., Matsuoka, T., and Sumikawa, K. (1997). Long-lasting enhancement of ACh receptor currents by lysophospholipids. *Brain Res. Mol. Brain Res.* 45, 317–320. doi: 10.1016/s0169-328x(96)00292-6

Ilcol, Y. O., Dilek, K., Yurtkuran, M., and Ulus, I. H. (2002). Changes of plasma free choline and choline-containing compounds' concentrations and choline loss during hemodialysis in ESRD patients. *Clin. Biochem.* 35, 233–239. doi: 10.1016/s0009-9120(02)00298-9

Ilcol, Y. O., Ozbek, R., Hamurtekin, E., and Ulus, I. H. (2005). Choline status in newborns, infants, children, breast-feeding women, breast-fed infants and human breast milk. *J. Nutr. Biochem.* 16, 489–499. doi: 10.1016/j.jnutbio.2005.01.011

Innocent, N., Livingstone, P. D., Hone, A., Kimura, A., Young, T., Whiteaker, P., et al. (2008). α-conotoxin *Arenatus* IB[V11L,V16D] [corrected] is a potent and selective antagonist at rat and human native α7 nicotinic acetylcholine receptors. *J. Pharmacol. Exp. Ther.* 327, 529–537. doi: 10.1124/jpet.108.142943

Kabarowski, J. H., Xu, Y., and Witte, O. N. (2002). Lysophosphatidylcholine as a ligand for immunoregulation. *Biochem. Pharmacol.* 64, 161–167. doi: 10.1016/s0006-2952(02)01179-6

Kabbani, N., Nordman, J. C., Corgiat, B. A., Veltri, D. P., Shehu, A., Seymour, V. A., et al. (2013). Are nicotinic acetylcholine receptors coupled to G proteins? *Bioessays* 35, 1025–1034. doi: 10.1002/bies.201300082

Kawashima, K., Fujii, T., Moriwaki, Y., Misawa, H., and Horiguchi, K. (2012). Reconciling neuronally and nonneuronally derived acetylcholine in the regulation of immune function. *Ann. N Y Acad. Sci.* 1261, 7–17. doi: 10.1111/j.1749-6632.2012.06516.x

King, J. R., and Kabbani, N. (2016). Alpha7 nicotinic receptor coupling to heterotrimeric G proteins modulates RhoA activation, cytoskeletal motility, and structural growth. *J. Neurochem.* 138, 532–545. doi: 10.1111/jnc.13660

Kudryavtsev, D., Shelukhina, I., Vulfius, C., Makarieva, T., Stonik, V., Zhmak, M., et al. (2015). Natural compounds interacting with nicotinic acetylcholine receptors: from low-molecular weight ones to peptides and proteins. *Toxins* 7, 1683–1701. doi: 10.3390/toxins7051683

Limapichat, W., Dougherty, D. A., and Lester, H. A. (2014). Subtype-specific mechanisms for functional interaction between α6β4* nicotinic acetylcholine receptors and P2X receptors. *Mol. Pharmacol.* 86, 263–274. doi: 10.1124/mol.114.093179

Lipovsek, M., Fierro, A., Pérez, E. G., Boffi, J. C., Millar, N. S., Fuchs, P. A., et al. (2014). Tracking the molecular evolution of calcium permeability in a nicotinic acetylcholine receptor. *Mol. Biol. Evol.* 31, 3250–3265. doi: 10.1093/molbev/msu258

Lipovsek, M., Im, G. J., Franchini, L. F., Pisciottano, F., Katz, E., Fuchs, P. A., et al. (2012). Phylogenetic differences in calcium permeability of the auditory hair cell cholinergic nicotinic receptor. *Proc. Natl. Acad. Sci. U S A* 109, 4308–4313. doi: 10.1073/pnas.1115488109

Lips, K. S., König, P., Schätzle, K., Pfeil, U., Krasteva, G., Spies, M., et al. (2006). Coexpression and spatial association of nicotinic acetylcholine receptor subunits α7 and α10 in rat sympathetic neurons. *J. Mol. Neurosci.* 30, 15–16. doi: 10.1385/jmn:30:1:15

Liu-Wu, Y., Hurt-Camejo, E., and Wiklund, O. (1998). Lysophosphatidylcholine induces the production of IL-1β by human monocytes. *Atherosclerosis* 137, 351–357. doi: 10.1016/s0021-9150(97)00295-5

Lord, J. M., Midwinter, M. J., Chen, Y. F., Belli, A., Brohi, K., Kovacs, E. J., et al. (2014). The systemic immune response to trauma: an overview of pathophysiology and treatment. *Lancet* 384, 1455–1465. doi: 10.1016/s0140-6736(14)60687-5

McIntosh, J. M., Absalom, N., Chebib, M., Elgoyhen, A. B., and Vincler, M. (2009). α9 nicotinic acetylcholine receptors and the treatment of pain. *Biochem. Pharmacol.* 78, 693–702. doi: 10.1016/j.bcp.2009.05.020

Mikulski, Z., Hartmann, P., Jositsch, G., Zaslona, Z., Lips, K. S., Pfeil, U., et al. (2010). Nicotinic receptors on rat alveolar macrophages dampen ATP-induced increase in cytosolic calcium concentration. *Respir. Res.* 11:133. doi: 10.1186/1465-9921-11-133

Mishra, N. C., Rir-sima-ah, J., Boyd, R. T., Singh, S. P., Gundavarapu, S., Langley, R. J., et al. (2010). Nicotine inhibits Fc epsilon RI-induced cysteinyl leukotrienes and cytokine production without affecting mast cell degranulation through α7/α9/α10-nicotinic receptors. *J. Immunol.* 185, 588–596. doi: 10.4049/jimmunol.0902227

Moosig, F., Damm, F., Knorr-Spahr, A., Ritgen, M., Zeuner, R. A., Kneba, M., et al. (2006). Reduced expression of C1q-mRNA in monocytes from patients with systemic lupus erythematosus. *Clin. Exp. Immunol.* 146, 409–416. doi: 10.1111/j.1365-2249.2006.03225.x

Morley, B. J., Lysakowski, A., Vijayakumar, S., Menapace, D., and Jones, T. A. (2017). Nicotinic acetylcholine receptors regulate vestibular afferent gain and activation timing. *J. Comp. Neurol.* 525, 1216–1233. doi: 10.1002/cne.24131

Moser, N., Mechawar, N., Jones, I., Gochberg-Sarver, A., Orr-Urtreger, A., Plomann, M., et al. (2007). Evaluating the suitability of nicotinic acetylcholine receptor antibodies for standard immunodetection procedures. *J. Neurochem.* 102, 479–492. doi: 10.1111/j.1471-4159.2007.04498.x

Olofsson, P. S., Rosas-Ballina, M., Levine, Y. A., and Tracey, K. J. (2012). Rethinking inflammation: neural circuits in the regulation of immunity. *Immunol. Rev.* 248, 188–204. doi: 10.1111/j.1600-065x.2012.01138.x

Orr-Urtreger, A., Göldner, F. M., Saeki, M., Lorenzo, I., Goldberg, L., De Biasi, M., et al. (1997). Mice deficient in the α7 neuronal nicotinic acetylcholine receptor lack α-bungarotoxin binding sites and hippocampal fast nicotinic currents. *J. Neurosci.* 17, 9165–9171.

Ozaki, E., Campbell, M., and Doyle, S. L. (2015). Targeting the NLRP3 inflammasome in chronic inflammatory diseases: current perspectives. *J. Inflamm. Res.* 8, 15–27. doi: 10.2147/jir.s51250

Papke, R. L., Bencherif, M., and Lippiello, P. (1996). An evaluation of neuronal nicotinic acetylcholine receptor activation by quaternary nitrogen compounds indicates that choline is selective for the α7 subtype. *Neurosci. Lett.* 213, 201–204. doi: 10.1016/s0304-3940(96)12889-5

Peng, H., Ferris, R. L., Matthews, T., Hiel, H., Lopez-Albaitero, A., and Lustig, L. R. (2004). Characterization of the human nicotinic acetylcholine receptor subunit α (α) 9 (CHRNA9) and α (α) 10 (CHRNA10) in lymphocytes. *Life Sci.* 76, 263–280. doi: 10.1016/j.lfs.2004.05.031

Philip, N. S., Carpenter, L. L., Tyrka, A. R., and Price, L. H. (2012). The nicotinic acetylcholine receptor as a target for antidepressant drug development. *ScientificWorldJournal* 2012:104105. doi: 10.1100/2012/104105

Quehenberger, O., Armando, A. M., Brown, A. H., Milne, S. B., Myers, D. S., Merrill, A. H., et al. (2010). Lipidomics reveals a remarkable diversity of lipids in human plasma. *J. Lipid Res.* 51, 3299–3305. doi: 10.1194/jlr.M009449

Ramarao, M. K., and Cohen, J. B. (1998). Mechanism of nicotinic acetylcholine receptor cluster formation by rapsyn. *Proc. Natl. Acad. Sci. U S A* 95, 4007–4012. doi: 10.1073/pnas.95.7.4007

Rathinam, V. A., Vanaja, S. K., and Fitzgerald, K. A. (2012). Regulation of inflammasome signaling. *Nat. Immunol.* 13, 333–342. doi: 10.1038/ni.2237

Razani-Boroujerdi, S., Boyd, R. T., Dávila-Garcia, M. I., Nandi, J. S., Mishra, N. C., Singh, S. P., et al. (2007). T cells express α7-nicotinic acetylcholine receptor subunits that require a functional TCR and leukocyte-specific protein tyrosine kinase for nicotine-induced Ca^{2+} response. *J. Immunol.* 179, 2889–2898. doi: 10.4049/jimmunol.179.5.2889

Richter, K., Mathes, V., Fronius, M., Althaus, M., Hecker, A., Krasteva-Christ, G., et al. (2016). Phosphocholine-an agonist of metabotropic but not of ionotropic functions of α9-containing nicotinic acetylcholine receptors. *Sci. Rep.* 6:28660. doi: 10.1038/srep28660

Romero, H. K., Christensen, S. B., Di Cesare Mannelli, L., Gajewiak, J., Ramachandra, R., Elmslie, K. S., et al. (2017). Inhibition of α9α10 nicotinic acetylcholine receptors prevents chemotherapy-induced neuropathic pain. *Proc. Natl. Acad. Sci. U S A* 114, E1825–E1832. doi: 10.1073/pnas.1621433114

Rommel, F. R., Raghavan, B., Paddenberg, R., Kummer, W., Tumala, S., Lochnit, G., et al. (2015). Suitability of nicotinic acetylcholine receptor α7 and muscarinic acetylcholine receptor 3 antibodies for immune detection: evaluation in murine skin. *J. Histochem. Cytochem.* 63, 329–339. doi: 10.1369/0022155415575028

Rosas-Ballina, M., Olofsson, P. S., Ochani, M., Valdés-Ferrer, S. I., Levine, Y. A., Reardon, C., et al. (2011). Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science* 334, 98–101. doi: 10.1126/science.1209985

Stock, C., Schilling, T., Schwab, A., and Eder, C. (2006). Lysophosphatidylcholine stimulates IL-1β release from microglia via a $P2X_7$ receptor-independent mechanism. *J. Immunol.* 177, 8560–8568. doi: 10.4049/jimmunol.177.12.8560

Stoecklein, V. M., Osuka, A., and Lederer, J. A. (2012). Trauma equals danger—damage control by the immune system. *J. Leukoc. Biol.* 92, 539–551. doi: 10.1189/jlb.0212072

Stolz, M., Klapperstück, M., Kendzierski, T., Detro-Dassen, S., Panning, A., Schmalzing, G., et al. (2015). Homodimeric anoctamin-1, but not homodimeric anoctamin-6, is activated by calcium increases mediated by the P2Y1 and P2X7 receptors. *Pflugers Arch.* 467, 2121–2140. doi: 10.1007/s00424-015-1687-3

Trautmann, A. (2009). Extracellular ATP in the immune system: more than just a "danger signal". *Sci. Signal.* 2:pe6. doi: 10.1126/scisignal.256pe6

Valbuena, S., and Lerma, J. (2016). Non-canonical signaling, the hidden life of ligand-gated ion channels. *Neuron* 92, 316–329. doi: 10.1016/j.neuron.2016.10.016

Vetter, D. E., Katz, E., Maison, S. F., Taranda, J., Turcan, S., Ballestero, J., et al. (2007). The α10 nicotinic acetylcholine receptor subunit is required for normal synaptic function and integrity of the olivocochlear system. *Proc. Natl. Acad. Sci. U S A* 104, 20594–20599. doi: 10.1073/pnas.0708545105

Vetter, D. E., Liberman, M. C., Mann, J., Barhanin, J., Boulter, J., Brown, M. C., et al. (1999). Role of α9 nicotinic ACh receptor subunits in the development and function of cochlear efferent innervation. *Neuron* 23, 93–103. doi: 10.1016/s0896-6273(00)80756-4

Vincler, M., Wittenauer, S., Parker, R., Ellison, M., Olivera, B. M., and McIntosh, J. M. (2006). Molecular mechanism for analgesia involving specific antagonism of α9α10 nicotinic acetylcholine receptors. *Proc. Natl. Acad. Sci. U S A* 103, 17880–17884. doi: 10.1073/pnas.0608715103

Vladimer, G. I., Marty-Roix, R., Ghosh, S., Weng, D., and Lien, E. (2013). Inflammasomes and host defenses against bacterial infections. *Curr. Opin. Microbiol.* 16, 23–31. doi: 10.1016/j.mib.2012.11.008

Wang, Y., Xiao, C., Indersmitten, T., Freedman, R., Leonard, S., and Lester, H. A. (2014). The duplicated α7 subunits assemble and form functional nicotinic receptors with the full-length α7. *J. Biol. Chem.* 289, 26451–26463. doi: 10.1074/jbc.M114.582858

Whiteaker, P., Christensen, S., Yoshikami, D., Dowell, C., Watkins, M., Gulyas, J., et al. (2007). Discovery, synthesis, and structure activity of a highly selective α7 nicotinic acetylcholine receptor antagonist. *Biochemistry* 46, 6628–6638. doi: 10.1021/bi7004202

Wieskopf, J. S., Mathur, J., Limapichat, W., Post, M. R., Al-Qazzaz, M., Sorge, R. E., et al. (2015). The nicotinic α6 subunit gene determines variability in chronic pain sensitivity via cross-inhibition of P2X2/3 receptors. *Sci. Transl. Med.* 7:287ra272. doi: 10.1126/scitranslmed.3009986

Wilczynska, J., Pfeil, U., Zakrzewicz, A., Dietrich, H., Körner, C., Hecker, A., et al. (2011). Acetylcholine and chronic vasculopathy in rat renal allografts. *Transplantation* 91, 263–270. doi: 10.1097/TP.0b013e3182038654

Yan, J. J., Jung, J. S., Lee, J. E., Lee, J., Huh, S. O., Kim, H. S., et al. (2004). Therapeutic effects of lysophosphatidylcholine in experimental sepsis. *Nat. Med.* 10, 161–167. doi: 10.1038/nm989

Conflict of Interest Statement: Certain conotoxins, including RgIA4 have been patented by the University of Utah; JMM is an inventor on these patents.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Phosphocholine – an agonist of metabotropic but not of ionotropic functions of α9-containing nicotinic acetylcholine receptors

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We demonstrated previously that phosphocholine and phosphocholine-modified macromolecules efficiently inhibit ATP-dependent release of interleukin-1β from human and murine monocytes by a mechanism involving nicotinic acetylcholine receptors (nAChR). Interleukin-1β is a potent pro-inflammatory cytokine of innate immunity that plays pivotal roles in host defence. Control of interleukin-1β release is vital as excessively high systemic levels cause life threatening inflammatory diseases. In spite of its structural similarity to acetylcholine, there are no other reports on interactions of phosphocholine with nAChR. In this study, we demonstrate that phosphocholine inhibits ion-channel function of ATP receptor P2X7 in monocytic cells via nAChR containing α9 and α10 subunits. In stark contrast to choline, phosphocholine does not evoke ion current responses in *Xenopus laevis* oocytes, which heterologously express functional homomeric nAChR composed of α9 subunits or heteromeric receptors containing α9 and α10 subunits. Preincubation of these oocytes with phosphocholine, however, attenuated choline-induced ion current changes, suggesting that phosphocholine may act as a silent agonist. We conclude that phophocholine activates immuno-modulatory nAChR expressed by monocytes but does not stimulate canonical ionotropic receptor functions.

Phosphocholine (PC) is a precursor as well as a degradation product of phosphatidylcholine (lecithin), a major phospholipid of eukaryotic bio-membranes[1,2]. Beside the important role of PC for eukaryotic bio-membranes, PC moieties can be covalently attached to proteins and glycolipids of plants[3], fungi[4], eukaryotic parasites[5], and some pathogenic bacteria[6,7]. PC and PC-modified macromolecules interact with C-reactive protein (CRP), a classical acute phase reactant[8,9], or with natural antibodies[7,10]. These interactions may induce complement fixation and other effector mechanisms involved in host defence. In contrast, PC-modified macromolecules are also known to provoke strong anti-inflammatory effects and contribute to immune evasion of parasites[5,11]. For example, PC-modified lipopolysaccharide (LPS) from *Haemophilus influenzae* contributes to bacterial virulence and enables persistent host colonization[6,12,13]. The mechanisms, regarding how PC-modified macromolecules initiate immune evasion, are not fully understood. PC-modifications on the surface of *H. influenzae* might be a kind of molecular mimicry preventing activation of *Toll-like* receptors (TLR)[14,15]. Interestingly, *Myd88*, an adaptor molecule involved in signalling of TLR and interleukin-1 (IL-1) seems to be of importance, as wild-type and PC-deficient *H. influenzae* strains are cleared at the same pace in *Myd88*-deficient mice[15].

Two isoforms of the pro-inflammatory cytokine IL-1 exist, IL-1α and IL-1β, which signal via the same receptors and play a central role in host defence against infections[16]. IL-1α mainly acts locally within infected tissues, whereas IL-1β exerts more systemic effects[16]. High concentrations of circulating IL-1β, however, can cause

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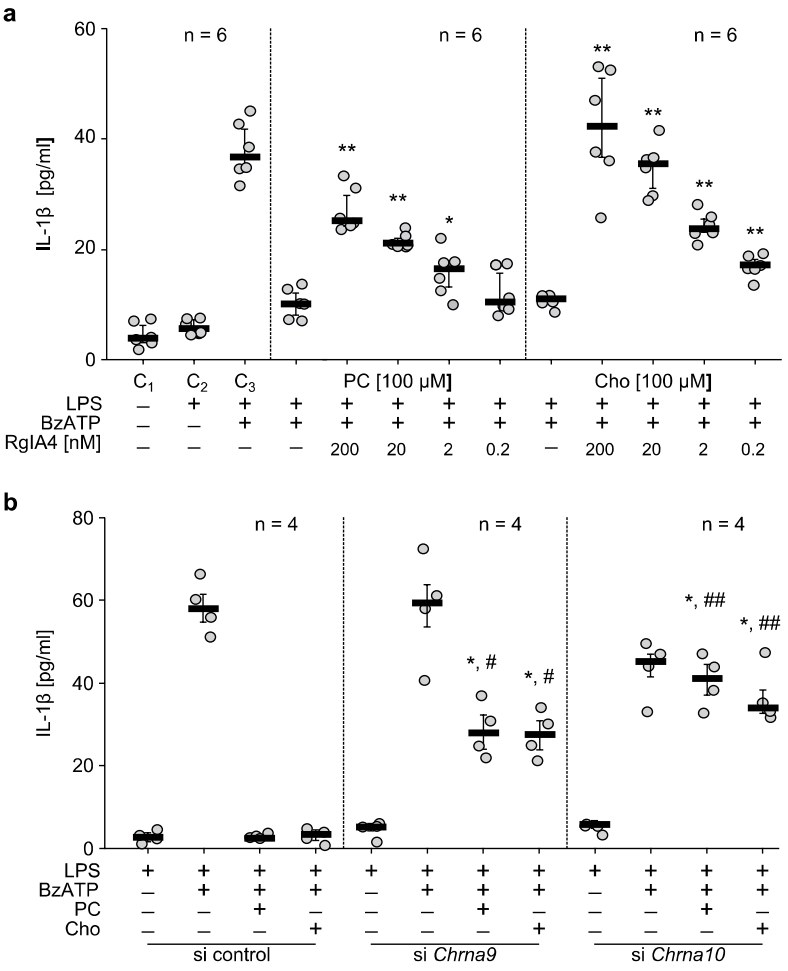

Figure 1. Choline and phosphocholine inhibit BzATP-induced IL-1β release from U937 cells via nicotinic acetylcholine receptor containing α9 and α10 subunits. (**a**) Human monocytic U937 cells were primed with lipopolysaccharide (LPS, 1 µg/ml, 5 h) followed by stimulation with BzATP (100 µM, 30 min) in the presence or absence of phosphocholine (PC, 100 µM), choline (Cho, 100 µM) and/or different concentrations of the α-conotoxin RgIA4, a specific antagonist of α9α10 nicotinic acetylcholine receptors (nAChR). Interleukin-1β (IL-1β) released into the culture medium was measured by enzyme linked immunosorbent assay (ELISA). In control experiments, cells were left untreated (C1), primed with LPS (C2) or with LPS followed by BzATP (C3). In the presence of PC as well as Cho the IL-1β release was inhibited. The inhibitory effect of PC and Cho was dose-dependently antagonized by RgIA4 (*$P \leq 0.05$, **$P \leq 0.01$, significantly different from cells treated with PC or Cho alone, Mann-Whitney rank-sum test). (**b**) In LPS-primed U937 cells that were transfected with control siRNA (si) the BzATP-stimulated IL-1β release was inhibited by PC and Cho. In cells transfected with siRNA to Chrna9 or Chrna10, the effects of PC and Cho were blunted (*$P \leq 0.05$, different from cells treated with LPS and BzATP; $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, different from respective experiments on cells treated with control siRNA; Kruskal-Wallis followed by Mann-Whitney rank-sum test). Data are presented as individual data points, bar represents median, whiskers percentiles 25 and 75.

life-threatening systemic inflammatory response syndrome (SIRS)[17]. Hence, IL-1β release is tightly controlled and the underlying mechanisms are of significant clinical interest. Production of mature IL-1β by human monocytes and macrophages typically depends on two consecutive danger signals such as LPS and extracellular ATP[18,19]. LPS activates TLR-4 and induces the biosynthesis of pro-IL-1β that remains within the cytoplasm, unless the cell becomes activated by another danger signal. Extracellular ATP originating from damaged neighbouring cells typically binds to ATP-receptor P2X7, induces efflux of K^+ ions, assembly of the NLRP3 inflammasome, activation of the proteolytic activity of caspase-1, cleavage of pro-IL-1β, and release of mature bioactive IL-1β[18,19].

Recently, we demonstrated that free PC and PC-modified macromolecules dose-dependently inhibit ATP-induced release of IL-1β by human monocytic cells[20]. In the same study we showed that a similar effect is provoked by nicotine, acetylcholine (ACh) and choline (Cho)[20]. The inhibitory effect of PC moieties is

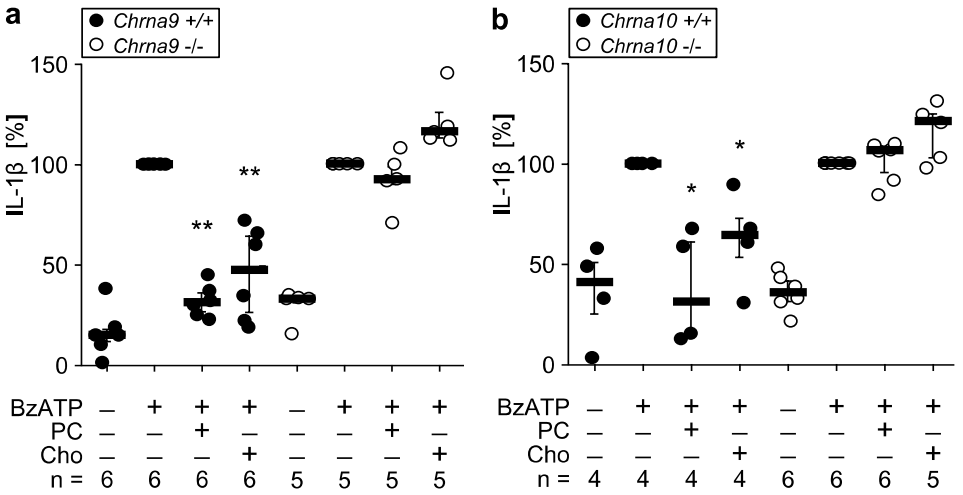

Figure 2. Choline and phosphocholine do not inhibit BzATP-induced IL-1β release from mononuclear leukocytes of Chrna9 and Chrna10 gene-deficient mice. (**a**,**b**) Mononuclear leukocytes were isolated from Chrna9 and Chrna10 gene-deficient mice (white circle; Chrna9 −/−; Chrna10 −/−) and corresponding wild-type mice (black circle; Chrna9 +/+; Chrna10 +/+). BzATP (100 μM) induced release of interleukin-1β (IL-1β) was investigated in the presence of phosphocholine (PC; 100 μM) or choline (Cho; 100 μM). PC and Cho suppressed BzATP-induced release of IL-1β in all WT strains investigated. In sharp contrast, no inhibition of IL-1β release was seen in Chrna9 −/− and Chrna10 −/− mice deficient in α9 or α10 subunit containing nicotinic acetylcholine receptors, suggesting that both subunits are needed (*$P \leq 0.05$, **$P \leq 0.01$, significantly different from cells treated with PC or Cho alone, Mann-Whitney rank-sum test). Data are presented as individual data points, bar represents median, whiskers percentiles 25 and 75.

antagonized by mecamylamine (Mec), α-bungarotoxin and strychnine, suggesting that nicotinic acetylcholine receptors (nAChR) containing α9 and/or α10 subunits are involved in signalling[20]. Interestingly, nicotine abolishes ATP-induced ion channel functions of P2X7 receptors in U937 cells, a human monocytic cell line, but does not provoke ion currents itself[20].

The purpose of this study was to test the hypothesis that binding of PC and Cho to nAChR inhibit P2X7 receptor function similar to nicotine. In addition, we hypothesised that PC is a novel agonist of nAChR containing α9 and/or α10 subunits and directly compare the effects of PC to the well-known α9* (* indicates the possible presence of additional nAChR subunits) agonist Cho[21]. We provide evidence that PC and Cho induce metabotropic effects via α9α10*-containing nAChR in monocytic cells that result in an inhibition of P2X7 receptor function. Canonical ionotropic functions of α9* nAChR, however, are triggered by Cho but strikingly not by PC.

Results

PC and Cho inhibit BzATP-induced IL-1β release via α9 and α10 nAChR subunits. To test the hypothesis, that the inhibitory effects of PC and Cho are mediated via nAChR containing α9 and/or α10 subunits, we used an analogue of α-conotoxin RgIA (RgIA4, synonym CSP-4), a potent and selective antagonist of human α9* nAChR[22,23]. Human monocytic U937 cells were primed with LPS for 5 h followed by stimulation with the selective P2X7 agonist BzATP (2′(3′)-O-(4-benzoyl-benzoyl)ATP trieethylammonium salt)[24] for another 30 min in the presence or absence of PC, Cho and/or RgIA4. Thereafter, IL-1β released into the culture medium was measured by an enzyme linked immunosorbent assay (ELISA; Fig. 1). As expected, PC or Cho (100 μM each) completely inhibited BzATP-induced IL-1β release (Fig. 1a). RgIA4 fully antagonized the inhibitory effect of PC and Cho in a dose-dependent manner (IC_{50} about 10 nM; $n = 6$; $P \leq 0.05$; Fig. 1a).

To corroborate these results, we transfected U937 cells with small interfering RNA (siRNA) to silence the expression of α9 and α10 nAChR subunits or with scrambled control siRNA. The efficiency of this treatment in U937 cells was shown before[20]. Transfection of control siRNA neither impaired BzATP-induced release of IL-1β nor altered the inhibitory effects of PC and Cho ($n = 4$; $P \leq 0.05$; Fig. 1b). In contrast, when the expression of α9 or α10 subunits was silenced by siRNA the inhibitory effect of PC as well as Cho was blunted ($n = 4$; $P \leq 0.05$; Fig. 1b).

Furthermore, we investigated BzATP-induced IL-1β release by peripheral blood mononuclear leukocytes (PBMC) isolated from *Chrna9* and from *Chrna10* gene-deficient mice (*Chrna9* −/−; *Chrna10* −/−) as well as from two corresponding wild-type (WT) strains (*Chrna9* +/+; *Chrna10* +/+). BzATP consistently induced release of IL-1β from WT and gene-deficient PBMC ($n \geq 4$; Fig. 2). PC or Cho (100 μM each) significantly reduced BzATP-induced IL-1β release from PBMC isolated from *Chrna9* +/+ ($n = 6$; $P \leq 0.01$) and *Chrna10* +/+ ($n = 4$; $P \leq 0.05$) mouse strains (Fig. 2). In contrast, PC and Cho were ineffective in PBMC from *Chrna9* −/− or *Chrna10* −/− mice ($n \geq 5$; $P \geq 0.05$; Fig. 2).

Cell treatment	Cell death [%] Mean ± SEM	n
–	2.14 ± 0.87	6
LPS	2.23 ± 0.91	6
LPS, BzATP	1.65 ± 0.67	6
LPS, BzATP, PC	1.76 ± 0.72	6
LPS, BzATP, PC, RgIA4 200 nM	2.38 ± 0.97	6
LPS, BzATP, PC, RgIA4 20 nM	1.48 ± 0.60	6
LPS, BzATP, PC, RgIA4 2 nM	2.30 ± 0.94	6
LPS, BzATP, PC, RgIA4 0.2 nM	1.76 ± 0.72	6
LPS, BzATP, Cho	1.30 ± 0.53	6
LPS, BzATP, Cho, RgIA4 200 nM	1.50 ± 0.61	6
LPS, BzATP, Cho, RgIA4 20 nM	1.54 ± 0.63	6
LPS, BzATP, Cho, RgIA4 2 nM	1.66 ± 0.68	6
LPS, BzATP, Cho, RgIA4 0.2 M	2.09 ± 0.85	6
si control: LPS	4.96 ± 2.48	4
si control: LPS, BzATP	5.04 ± 2.52	4
si control: LPS, BzATP, PC	5.41 ± 2.71	4
si control: LPS, BzATP, Cho	5.13 ± 2.57	4
si *Chrna9*: LPS	4.55 ± 2.27	4
si *Chrna9*: LPS, BzATP	5.20 ± 2.60	4
si *Chrna9*: LPS, BzATP, PC	5.13 ± 2.56	4
si *Chrna9*: LPS, BzATP, Cho	5.00 ± 2.50	4
si *Chrna10*: LPS	2.48 ± 1.01	6
si *Chrna10*: LPS, BzATP	3.25 ± 1.33	6
si *Chrna10*: LPS, BzATP, PC	3.00 ± 1.22	6
si *Chrna10*: LPS, BzATP, Cho	2.96 ± 1.21	6

Table 1. Lactate dehydrogenase (LDH) release of U937 cells. Human monocytic U937 cells were primed for 5 h with lipopolysaccharide (LPS, from *Escherichia coli*; 1 μg/ml). Subsequent, BzATP (100 μM) was applied for another 30 min in presence or absence of the nAChR agonist phosphocholine (PC; 100 μM) or choline (Cho; 100 μM) or α-conotoxin RgIA4. In some experiments the expression of nAChR containing the α9 or α10 subunit was reduced by using the small interfering RNA (si) transfection. At the end of the experiments, LDH was measured in the cell culture supernatants and is given as % of total release (mean ± standard error of mean, SEM).

At the end of each experiment, lactate dehydrogenase (LDH) levels were determined to test for cell viability. As shown in Table 1 and Table 2, LDH values remained below 10% of the total release, irrespective of the experiment performed.

PC and Cho inhibit BzATP-induced ion current stimulation in U937 cells. To investigate if BzATP-induced ion current stimulation due to P2X7 receptor activation is inhibited by PC and Cho, we performed whole-cell patch-clamp measurements on LPS-primed U937 cells. As shown previously[20], application of BzATP (100 μM) consistently induced ion currents (Fig. 3a,d). BzATP-induced ion current stimulation was reversible by washout and repeatable (Fig. 3a,d). No significant changes were detected when comparing the amplitude (ΔI_{BzATP}) of the first BzATP-induced response with the second (n = 10, P = 0.241; Fig. 3d), indicating that the receptors do not desensitise under these conditions. In the next set of experiments BzATP was first applied alone, which provoked ion currents (Fig. 3b). After washout, the cells were preincubated with Cho (100 μM) for 30 s, followed by an additional application of BzATP (Fig. 3b). Cho alone did not cause any changes in current (n = 7, Fig. 3b). Moreover, Cho abolished BzATP-induced current stimulation (n = 7, P = 0.018; Fig. 3b,d). In the next experiments, cells were preincubated with the nAChR antagonist Mec (100 μM), followed by application of Cho (Fig. 3c). Under these conditions (Mec + Cho) a BzATP-induced current stimulation was detectable and the first and second BzATP-induced effects did not differ (n = 5, P = 0.5; Fig. 3c,d).

We performed the same kind of experiments using PC (1 mM) instead of Cho (Fig. 4). PC alone did not induce ion current changes. The BzATP-induced effect was abolished in presence of PC (n = 6, P = 0.028; Fig. 4a,d). Furthermore, the inhibitory effect of PC was antagonized by preincubation of the cells with Mec and the first and second BzATP-induced effect did not differ (n = 6, P = 0.209; Fig. 4b,d). Taken together, we were able to show that PC and Cho inhibit BzATP-induced ion fluxes in U937 cells.

Previous experiments examining the inhibition of BzATP-induced IL-1β release by PC provided evidence that nAChR containing subunits α9 and/or α10 are involved (Fig. 1). To confirm these data, we performed whole-cell patch-clamp measurements in the presence of RgIA4 (200 nM) (Fig. 4c). For this purpose, the BzATP-induced current was determined (n = 6; Fig. 4c). After washout of BzATP, cells were preincubated with RgIA4 and followed by PC application (Fig. 4c). Subsequent application of BzATP activated a current that was similar to the

Genotype	Cell treatment	Cell death [%] Mean ± SEM	n
Chrna9 +/+	–	6.90 ± 1.50	6
Chrna9 +/+	BzATP	6.97 ± 1.37	6
Chrna9 +/+	BzATP, PC	6.62 ± 1.06	6
Chrna9 +/+	BzATP, Cho	6.71 ± 1.23	6
Chrna9 −/−	–	8.64 ± 0.93	5
Chrna9 −/−	BzATP	7.63 ± 1.09	5
Chrna9 −/−	BzATP, PC	7.96 ± 0.91	5
Chrna9 −/−	BzATP, Cho	8.62 ± 1.06	5
Chrna10 +/+	–	4.38 ± 0.95	4
Chrna10 +/+	BzATP	5.04 ± 2.04	4
Chrna10 +/+	BzATP, PC	4.81 ± 1.96	4
Chrna10 +/+	BzATP, Cho	5.96 ± 1.89	4
Chrna10 −/−	–	6.35 ± 1.18	6
Chrna10 −/−	BzATP	6.42 ± 1.13	6
Chrna10 −/−	BzATP, PC	6.40 ± 1.30	6
Chrna10 −/−	BzATP, Cho	7.68 ± 1.38	5

Table 2. Lactate dehydrogenase (LDH) measurement of peripheral blood mononuclear leukocytes (PBMC). Peripheral blood mononuclear leukocytes (PBMC) were isolated from *Chrna9* gene-deficient (*Chrna9* −/−) and *Chrna10* (*Chrna10* −/−) gene-deficient mice as well as from two corresponding wild-type (WT) strains. LDH was measured in the cell culture supernatants at the end of the experiments, and is given as % of total release (mean ± standard error of mean, SEM). Untreated cells are marked by "**–**". BzATP (100 μM) was applied in the presence and absence of phosphocholine (PC; 100 μM) or choline (Cho; 100 μM).

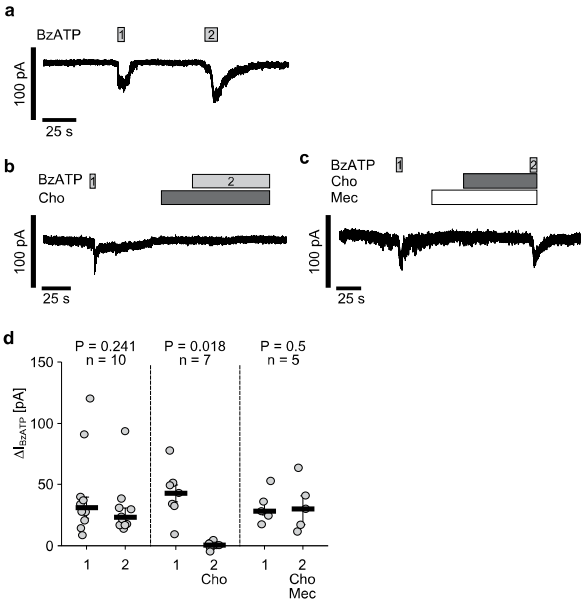

Figure 3. Choline inhibits BzATP-induced ion current stimulation in U937 cells. Whole-cell patch-clamp measurements were performed on human monocytic U937 cells primed with lipopolysaccharide (1 μg/ml, 5 h). Depicted are representative current curves (**a,c**). (**a,d**) In control experiments, consecutive application of the P2X7 receptor agonist BzATP (100 μM, grey bar) induced repetitive ion current stimulations (BzATP1 and 2). (**b,d**). After washout of the first BzATP stimulus, choline (Cho, 100 μM, dark grey bars) was applied. In presence of Cho, BzATP did not change the ion current. (**c,d**) Mecamylamine (Mec, 100 μM, white bar) antagonized the inhibitory effect of Cho. All BzATP-induced current changes (ΔI_{BzATP}) are shown as individual data points, bars represent median, whiskers percentiles 25 and 75. Statistical analyses were performed using the Wilcoxon signed-rank test.

preceding BzATP current (n = 6, P = 0.173; Fig. 4c,d), suggesting that RgIA4 fully antagonized the inhibitory

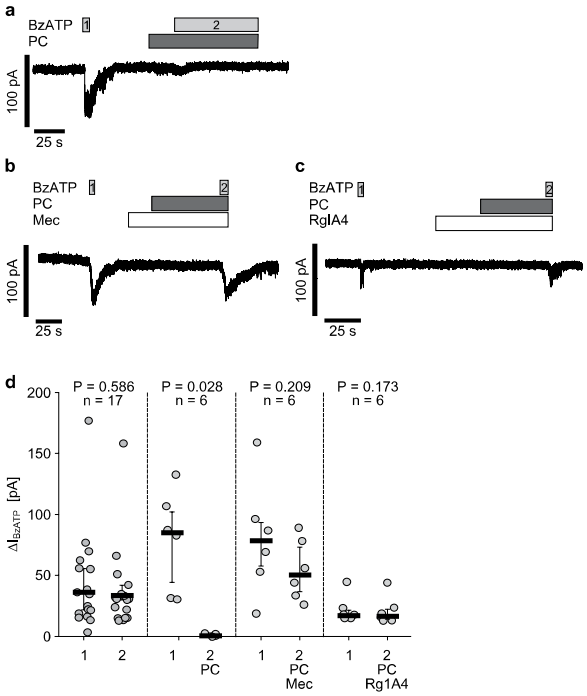

Figure 4. The inhibitory effect of phosphocholine on BzATP-mediated ion current stimulation is antagonized by α-conotoxin RgIA4. Whole-cell patch-clamp measurements were performed on human monocytic U937 cells primed with lipopolysaccharide (1 μg/ml, 5 h). (**a**) Application of the P2X7 receptor agonist BzATP (100 μM, grey bar) induced an ion current stimulation (BzATP1). After washout of BzATP1, phosphocholine (PC, 1 mM, dark grey bars) was applied. In presence of PC, BzATP did not change the ion current (BzATP2). (**b,c,d**) Mecamylamine (Mec, 100 μM, white bar, (**b**) as well as RgIA4 (200 nM, white bar; (**c**) antagonized the inhibitory effect of PC. (**d**) In parallel performed control experiments application of BzATP induced repetitive ion current stimulations that did not differ (current curve not shown). All ΔI_{BzATP} values are shown as individual data points, bars represent median, whiskers percentiles 25 and 75. Statistical analyses were performed using the Wilcoxon signed-rank test.

effect of PC.

PC does not induce ion current stimulation in *Xenopus laevis* oocytes expressing α9 or α9α10 nAChR. Cho is an agonist of nAChR containing α9 subunits[21]. To test if PC also evokes ion currents at canonical α9 containing receptors, human α9 subunit as well as a combination of human α9 and α10 subunits were heterologously expressed in *Xenopus laevis* oocytes. Two-electrode voltage-clamp (TEVC) measurements were performed to assess ion channel functions of nAChR.

In oocytes expressing homomeric α9 nAChR, application of Cho (Cho1) resulted in a rapid stimulation of the transmembrane current (I_M; Fig. 5a,c). The effect of Cho was reversible upon washout (Fig. 5a). Subsequent application of Cho (Cho2) activated a current that was significantly smaller than the preceding Cho-induced current (n = 14, P = 0.005; Fig. 5a,c). In contrast, when PC was applied for 2 min, no ion currents were provoked (Fig. 5b). As an internal positive control, Cho was applied at the end of each experiment resulting in a significant current stimulation (n = 12, P = 0.002; Fig. 5c).

The same kinds of experiments were performed on oocytes co-expressing nAChR subunits α9 and α10. Application of Cho induced a current stimulation (Fig. 5d,f). In comparison to the Cho-induced effect in oocytes expressing α9 (Fig. 5a), the current stimulation was faster and shorter (Fig. 5d). The Cho effect was repeatable and significantly blunted compared to the first response (n = 12, P = 0.003; Fig. 5f). Again, application of PC had no impact on the current while Cho induced a current stimulation (n = 10, P = 0.005; Fig. 5e,f). Neither application of Cho (n = 17) nor PC (n = 11) induced changes in currents of water injected control oocytes, which did neither express human α9 nor α10 nAChR subunits (Fig. 5g,h).

PC interacts with heterologously expressed α9α10 nAChR. Since PC did not evoke ion currents in oocytes expressing homomeric α9 and heteromeric α9α10 nAChR, we questioned whether PC might function as a silent desensitiser or as an antagonist of α9* nAChR containing subtypes. Therefore, we performed an additional set of experiments designed to monitor the effects of PC on the Cho-evoked responses over time (Fig. 6a,b). Oocytes expressing heteromeric α9α10 nAChR were stimulated with Cho once per min until a

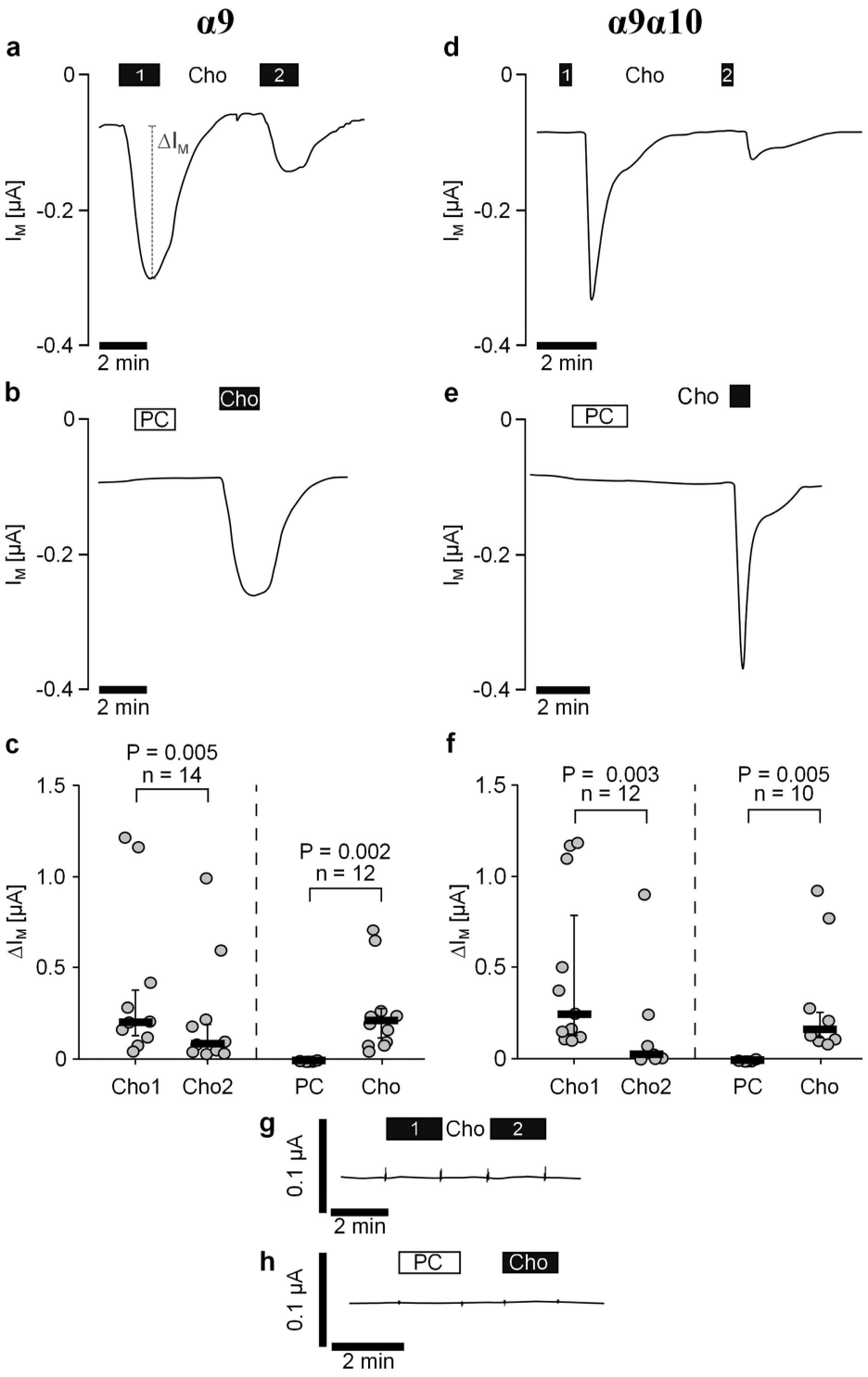

Figure 5. Phosphocholine does not induce ion channel functions in Xenopus laevis oocytes that heterologously express α9 or α9α10 nicotinic acetylcholine receptors. Two-electrode voltage-clamp (TEVC) measurements were performed on oocytes that heterologously expressed human α9 alone **(a–c)** or a combination of α9 and α10 **(d–f)** nicotinic acetylcholine receptor (nAChR) subunits. (**a,d**) Choline (Cho, 1 mM, black bars) induced repetitive stimulations of the transmembrane ion current (IM) in oocytes transfected with α9 **(a)** and in oocytes co-expressing α9α10 nAChR subunits (**d**). The second Cho-induced effect (Cho2)

was smaller compared to the first one (Cho1) indicating receptor desensitization. (**b,e**) Initial application of phosphocholine (PC, 1 mM, white bars) had no impact on IM, whereas application of Cho thereafter induced a current stimulation. Again, oocytes expressing only α9 and those co-expressing α9α10 nAChR subunits led to similar results. (**g,h**) Representative current traces of water injected control oocytes (no expression of human receptors). Neither repeated application of Cho (n = 17), nor PC (n = 11) induced any changes in I_M. Depicted are representative current curves (**a,b,d,e,g,h**). All changes of the transmembrane current (ΔI_M) induced by cholinergic stimulation are shown as individual data points, bars represent median, whiskers percentiles 25 and 75 (**c,f**). Statistical analyses were performed using the Wilcoxon signed-rank test.

steady-state baseline was achieved. Then, the perfusion solution was switched to one containing 1 mM PC and the Cho-evoked responses were monitored for changes in amplitude. Under these conditions, PC decreased the Cho-evoked responses to 13.1 ± 3.3% (n = 5) of control values after a 20 min perfusion (Fig. 6b). PC was then washed out and Cho-evoked responses were monitored for recovery from inhibition. The responses recovered to 98.2 ± 8.2% (n = 5) of control values after a 15 min perfusion with control solution.

Discussion

In the present study, we identify PC as a novel agonist of monocytic nAChR containing subunits α9 and α10. We provide evidence that α9 and α10 nAChR subunits are essential for the PC-mediated inhibition of ATP-induced ion channel functions at P2X7 receptor in monocytic cells and hence, for the inhibition of ATP-induced release of IL-1β. Cho, a well-known agonist of nAChR containing α9 subunits[21], and PC provoke similar metabotropic but no ionotropic effects in monocytic cells. As expected, Cho induces ion current responses at conventional ionotropic α9 nAChR homomers or α9α10 heteromers. In stark contrast, PC does not provoke any ion current changes at these canonical ionotropic receptors. To the best of our knowledge, we are the first to describe an agonist of nAChR containing α9 subunits that triggers metabotropic but no ionotropic receptor functions.

In the first part of this study, we demonstrated that PC and Cho are agonists of metabotropic nAChR composed of α9 and α10 subunits in monocytic cells. We showed previously that PC and Cho dose-dependently inhibit the ATP-induced release of IL-1β from LPS-primed human monocytic U937 cells via nAChR that are sensitive to Mec, α-bungarotoxin and strychnine[20]. Here, we clarified that PC and Cho act as ligands of monocytic α 9* nAChR by using the potent and selective antagonist RgIA4 which dose-dependently antagonized the inhibition of IL-1β release. These findings were further confirmed by gene-silencing experiments in U937 cells: silencing of *Chrna9* expression blunted the inhibitory effects of PC and Cho. The same observation was made, when *Chrna10* gene expression was silenced, suggesting that α9 and α10 nAChR subunits cooperate in monocytic cells.

The results of the gene-silencing experiments suggested but did not prove that α9 and α10 nAChR subunits are essential for the signalling of PC and Cho in monocytes. Therefore to corroborate the role of α9 nAChR subunit by an independent approach, we investigated freshly isolated PBMC from wild-type and gene-deficient mice. Although primary mouse PBMC were not intentionally primed to induce biosynthesis of IL-1β, they consistently released IL-1β in response to stimulation with BzATP. These observations are in accordance with previous findings on primary human PBMC[20,25]. We assume that freshly isolated PBMC became activated during cell isolation and culture[20]. As expected, PC and Cho efficiently inhibited the BzATP-induced release of IL-1β by PBMC obtained from wild-type mice but did not impair the IL-1β release from PBMC of *Chrna9* −/− and *Chrna10* −/− mice. From these data, we conclude that α9 and α10 subunits are essential for signalling of PC and Cho. Interactions of the α9 and α10 nAChR subunits were previously described. Subunits α10 do not assemble into functional ionotropic homomeric nAChR[26], while co-expression of α9 and α10 nAChR subunits results in formation of functional heteromeric α9α10 nAChR[26–28]. Transcripts of α9 and α10 nAChR subunits have been detected in the auditory system[26,29], dorsal root ganglion[30], skin[31], as well as in mononuclear leukocytes[20,32,33] including human monocytic cells[20].

Next, we confirmed our hypothesis that PC and Cho inhibit ATP-mediated ion current responses in monocytic cells. In accordance with our previous study[20], we detected BzATP-induced ion current responses in whole-cell patch-clamp measurements on LPS-primed U937 cells. These currents are most likely due to activation of P2X7 receptors, as BzATP is a specific agonist of this ATP-receptor[34], and due to a consecutive opening of pannexin hemichannels[35,36]. In line with our hypothesis, BzATP-mediated P2X7 receptor activation was completely abolished in presence of PC and Cho. This inhibitory effect was antagonized by the general nicotinic antagonist Mec and the α9*-specific conotoxin RgIA4. These results corroborate the involvement of α9 and/or α10 nAChR subunits in signalling of PC. A functional interaction of other subtypes of nAChR and P2X receptors was demonstrated previously in neurons[37] and in heterologous expression systems[38,39]. In these studies, co-application of ATP and nicotinic agonists evoked current responses that were smaller than the sum of the individual currents induced by ATP and ACh or nicotine[37,38]. In the present study, PC or Cho alone did not evoke any ion current responses in U937 cells, consistent with functional coupling of a non-canonical metabotropic nAChR to ionotropic P2X7 receptors. While in excitable cells such as neurons nAChR are ligand-gated ion channels, no ionotropic nAChR functions have been observed in leukocytes[20,32,33,40]. At present, we do not know how activation of monocytic nAChR by PC or Cho translates into the observed inhibition of P2X7 receptor function.

In the last part of the study, we investigated the effect of PC at canonical ionotropic nAChR using *Xenopus laevis* oocytes as a heterologous expression system for human homomeric α9 nAChR as well as heteromeric α9α10 nAChR. Cho was included in these experiments as a positive control and transmembrane ion currents were recorded in TEVC measurements. We detected Cho-induced current responses in oocytes expressing homomeric α9 and heteromeric α9α10 nAChR. Current responses to the first Cho application for 30 s varied from 50 nA to 1200 nA (see Fig. 5a,f). Subsequent application of Cho resulted in smaller current responses, indicating receptor

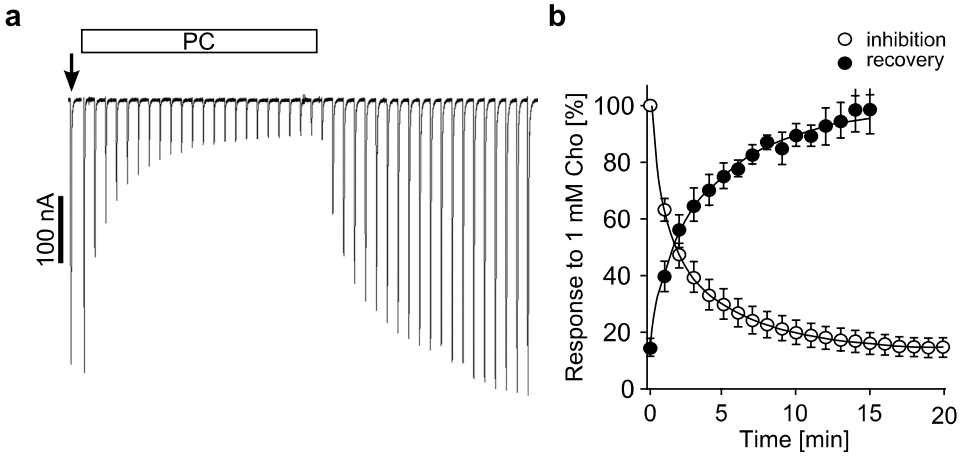

Figure 6. Phosphocholine inhibits choline-gated currents mediated by α9α10 nicotinic acetylcholine receptors heterologously expressed by Xenopus laevis oocytes. Two-electrode voltage-clamp experiments were performed on oocytes that heterologously expressed human α9α10 nicotinic acetylcholine receptors as described in Methods. (**a**) Representative current traces showing the inhibitory effects of phosphocholine (PC, 1 mM) on choline-gated currents (Cho, 1 mM). The current traces are each from 30 s recordings and are shown concatenated (omitting the 30 s gap between each individually recorded trace). Oocytes were continuously perfused with control solution and stimulated with 1 sec pulses of Cho once per min until steady-state baseline responses were observed (indicated by the arrow). Subsequently, the control solution was changed to one containing PC and the Cho-gated currents monitored for changes in amplitude for 20 min. Thereafter, PC was washed out and recovery from inhibition by PC monitored. (**b**) Analysis of inhibition and recovery from inhibition of Cho-gated currents by PC. The error bars denote the standard error of mean (SEM) from 5 oocytes.

desensitisation. This is in contrast to previous studies and to our own observations, where application of ACh or Cho in short 1 s pulses resulted in repeatable current responses without desensitisation[27,41]. We assume that receptor desensitisation is a consequence of extended exposure of nAChR to Cho.

In sharp contrast to Cho, PC did not evoke ion current responses in α9 and/or α9α10 nAChR expressing oocytes. We conclude that at least in our experimental settings, PC is an agonist of metabotropic nAChR containing α9* subunits, whereas it does not stimulate canonical ligand-gated nAChR functions. This finding suggests that PC acts as a potent regulator of innate immunity but does not activate current responses in neuronal or non-neuronal cells expressing canonical ionotropic nAChR containing α9 subunits. Hence, PC and other molecules containing a PC group may be promising therapeutics for the prevention and treatment of excessive inflammation involving IL-1β such as life-threatening SIRS, without entailing the risk of adverse effects involving excitable cells.

Although PC did not trigger ion channel functions at α9 and α9α10 subunit containing nAChR expressed by *Xenopus laevis* oocytes, we obtained evidence that PC interacts with these canonical receptors and hypothesised that PC might act as a silent agonist. Per definition, silent agonists desensitise receptors without activating their function[42,43]. To further asses this, we used α9α10 nAChR expressing oocytes in an experimental setup that enabled application of Cho in 1 s pulses every 60 s, where Cho induced repetitive current responses without receptor desensitisation. We found that responses to Cho at heteromeric α9α10 nAChR were blunted in the presence of PC. The observed slow kinetics of inhibition and recovery from inhibition by PC was consistent with silent desensitisation. However, we should point out that our results do not rule out that the observed inhibition by PC is due to simple antagonism. Nicotine is an example of a ligand, which acts as an antagonist at ionotropic receptors[44] containing α9 subunits, but acts as an agonist at monocytic metabotropic α9 nAChR that inhibit P2X7 function[20]. A silent agonist for homomeric nAChR was recently reported for homomeric α7 nAChR[43]. These authors showed that ACh-induced current responses were reduced after preincubation of the oocytes with compound NS6740[43].

In conclusion, we identified PC as a novel agonist of metabotropic nAChR containing α9 and α10 subunits. PC and Cho evoke no ion current responses at these receptors expressed by monocytes but efficiently inhibit ATP-mediated P2X7 receptor activation and release of IL-1β. In contrast to Cho, PC does not trigger ionotropic functions at canonical human α9 nAChR homomers and α9α10 nAChR heteromers. These findings suggest that PC may be a valuable active substance for the treatment of inflammatory diseases that targets nAChR of monocytes without disturbing ionotropic functions of excitable cells.

Methods

U937 cell culture and stimulation. U937 cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were cultured in RPMI 1640 (Gibco by Life Technologies GmbH, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS; Biochrome, Berlin, Germany) and 2 mM L-glutamine (Gibco by Life Technologies GmbH) under 5% CO_2 atmosphere at 37 °C.

To investigate IL-1β release cells were transferred to 24-well plates (1×10^6 cells/ml and per well). Cells were primed with 1 μg/ml LPS from *Escherichia coli* (L2654; 1 μg/ml; Sigma-Aldrich, Deisenhofen, Germany) for 5 h. After priming, the P2X7 receptor agonist BzATP (Sigma-Aldrich; 100 μM) was added for 30 min in presence or absence of different concentrations of cholinergic agonists and antagonists. Cho chloride (100 μM), PC chloride calcium salt tetrahydrate (100 μM), and Mec hydrochloride (100 μM) were purchased from Sigma-Aldrich. An analogue of α-conotoxin RgIA (RgIA4)[22] was used in concentrations from 0.2 to 200 nM. After cell treatment, cells were spun down (500 g, 8 min) the supernatants were collected and stored at −20 °C. IL-1β concentrations were measured using a human Quantikine Immunoassays (R&D Systems, Minneapolis, MN) and LDH was determined.

Silencing of α9 and α10 nAChR subunit expression. In some experiments the expression of nAChR containing the α9 and/or α10 subunits in U937 cells was reduced by using siRNA technology. U937 cells were transfected with ON-TARGETplus human *Chrna9* or *Chrna10* siRNA SMARTpool (Thermo Fisher Scientific, Schwerte, Germany). As a control for unspecific effects of siRNA transfection cells were transfected with negative control ON-TARGETplus Non-targeting Control Pool (Thermo Fisher Scientific). In accordance with the manufacturer's protocol, all cells were transfected with 30 pM siRNA/1×10^6 cells using Amaxa Cell Line Nucleofector Kit C and Nucleofector II Device (both from Lonza Cologne, Cologne, Germany). 48 h after siRNA transfection, IL-1β release experiments were performed.

Mononuclear leukocytes from *Chrna9* and *Chrna10* gene-deficient mice. Male and female gene-deficient *Chrna9* (129S-*Chrna9*^{tm1Bedv}/J)[45] and *Chrna10* (129S4-*Chrna10*^{tm1Bedv}/Mmucd)[46] as well as corresponding WT mice (for details see[45,46]) were used for isolation of PBMC. Experimental animals received humane care according to NIH "Guide for the Care and Use of Laboratory Animals". Animal experiments were approved by the local committee at the Regierungspräsidium Giessen, Hesse, Germany (permit No. Gi 20/23-A10/2011).

Mice were euthanized by neck dislocation, and blood was drawn from the caval vein into heparinized syringes. PBMC were separated by discontinuous Percoll (Ge Healthcare Bio-Sciences AB, Uppsala, Sweden; 1.082 g/ml) density gradient centrifugation and cultured for 2 h in RPMI 1640 (Gibco by Life Technologies GmbH) supplemented with 10% FCS (Biochrome) and 2 mM L-glutamine (Gibco by Life Technologies GmbH), at 5% CO_2 and 37 °C. For investigation of IL-1β release, BzATP (Sigma-Aldrich; 100 μM) was added for 30 min in the presence or absence of PC (100 μM) or Cho (100 μM). Subsequently, cell culture supernatants were collected and stored at −20 °C. Finally, IL-1β concentrations were measured by using mouse Quantikine IL-1β Immunoassay (R&D Systems).

LDH measurements. In order to test for cell viability, activity of the cytoplasmic enzyme LDH was assayed by the Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) according to the supplier's instructions. LDH in the cell culture supernatants of U937 cells and PBMC was measured at the end of the experiments. For calculating the proportion of dead cells, a maximum LDH release control was generated. For this purpose, U937 cells were lysed by freezing them twice (−80 °C). Subsequently, the samples were analysed according to the supplier's instructions and values determined in cell culture supernatants were compared with the total content of LDH in lysed cells.

Whole-cell patch-clamp recordings on U937 cells. For electrophysiological recordings, U937 cells were placed in poly-L-lysine coated cell culture dishes (Nunc, Roskilde, Denmark) containing bath solution (in mM: 5.4 KCl, 120 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 glucose and 10 HEPES (4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid); pH 7.4).

Patch pipettes with a resistance of 2–3 MΩ were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) using a dmz-puller (Zeitz, Augsburg, Germany) and filled with a pipette solution (in mM: 120 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 11 EGTA (ethylene glycol tetraacetic acid), and 20 glucose; pH 7.3). After 5 h incubation with LPS, whole-cell patch-clamp recordings were performed at room temperature. Cells were clamped to −60 mV and transmembrane currents were recorded using an EPC-9 amplifier (HEKA, Lambrecht, Germany) and acquired via an ITC-16 interface with Pulse software (HEKA). BzATP (100 μM), PC (1 mM), Cho (100 μM), RgIA4 (200 nM) and Mec (100 μM) were dissolved in bath solution and applied via a pressure-driven microperfusion system. Rg1A4 (sequence ID3) was prepared as previously described[22]. All chemicals used for preparation of bath and pipette solution were purchased from Fluka (Deisenhofen, Germany), except for HEPES and EGTA (Sigma-Aldrich).

At least once per measuring day respective control experiments were performed in which BzATP was applied twice and the BzATP-induced effect was tested for reversibility and repeatability.

Heterologous expression of human nAChR in oocytes and TEVC measurements. Oocytes were obtained from adult female South African Clawed Frogs (*Xenopus laevis*; *Xenopus*-Express, Le Bourg, France). Manipulations of animals were conducted in accordance to the guidelines of the German law of animal care and were authorized by the local committee at the Regierungspräsidium Giessen, Hesse, Germany (permit number 400_M and 478_M). Oocytes were separated by collagenase treatment (1.5 mg/ml; Biochem, Karlsruhe, Germany) for 90 min. The follicle layer was removed by incubation of cells in Ca^{2+}-free oocyte Ringer's solution (ORi) containing (in mM): 90 NaCl, 1 KCl, 5 HEPES, and 1 EGTA (pH 7.4) for 10 min. Defolliculated oocytes were stored in a low-Na^+ solution (in mM): 10 NaCl, 80 NMDG–Cl (N-methyl-d-glucamine), 1 KCl, 2 $CaCl_2$, 5 HEPES, 2.5 Na-pyruvate (Applichem, Darmstadt, Germany), 0.06 penicillin (Sigma-Aldrich), 0.02 streptomycin (Sigma-Aldrich) at 17 °C (pH 7.4).

Plasmid DNA encoding the human *Chrna9*, human *Chrna10* as well as the human 43 kDa receptor-associated protein of the synapse (*RAPSN*) were obtained from Eurofins Genomics (Ebersberg, Germany). Capped cRNA

was synthesized using an *in vitro* transcription kit (T7-RiboMAX™ Large Scale RNA Production System Kit, PROMEGA, Mannheim, Germany).

Oocytes of stages V and VI (Dumont 1972) were injected with cRNA encoding α9 nAChR subunits (20 ng per oocyte) or α9α10 nAChR subunits (each 20 ng per oocyte) using a microinjector (Nanoject, Drummond Scientific, Broomall, USA). In order to increase expression levels and obtain stable nAChR expression, cRNA encoding *RAPSN* (5 ng per oocyte) was co-injected in both cases[47,48]. All cRNA was dissolved in nuclease-free water. The injection volume was 50.6 nl. In all TEVC experiments representative controls were performed with oocytes that were injected with 50.6 nl of nuclease-free water.

After an incubation time of 3–5 days, the transmembrane currents (I_M) of water- or RNA-injected oocytes were recorded by the TEVC technique. Oocytes were placed in a perfusion chamber and perfused (gravity driven) with ORi containing (in mM): 90 NaCl, 1 KCl, 2 $CaCl_2$, and 5 HEPES (pH 7.4). Intracellular microelectrodes were pulled from borosilicate glass capillaries and filled with 1 M KCl solution. The membrane voltage was clamped to −60 mV using a TEVC amplifier (Warner Instruments, Hamden, USA), and transmembrane currents were low-pass filtered at 1000 Hertz (Frequency Devices 902, Haverhill, Massachusetts, USA) and recorded with a strip chart recorder (Kipp & Zonen, Delft, The Netherlands). In all experimental groups, measurements were performed on oocytes from at least two different *Xenopus laevis* individuals.

For experiments examining the effects of continuous exposure to PC on α9- and α10-mediated currents, oocytes were injected with a 1:1 ratio of cRNA for human α9 and α10 nAChR subunits and incubated at 17 °C for 3 days. To conduct TEVC experiments, the oocytes were placed in a 30 μl chamber and continuously perfused by gravity with a solution containing 96 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 1 mM $MgCl_2$. The pH of the solution was adjusted to 7.4 with NaOH. A stock solution of 100 mM PC was prepared in distilled water. A working solution of 1 mM PC was prepared in perfusion solution containing lower $CaCl_2$ (0.8 mM) such that the final concentration of Ca^{2+} in all solutions was 1.8 mM. The oocyte membranes were clamped at a holding potential of −70 mV and stimulated with 1 s pulses of 1 mM Cho once every 60 s until a steady-state baseline response was observed. The perfusion solution was then switched on one containing 1 mM PC and the oocytes stimulated with 1 s pulses of 1 mM Cho plus 1 mM PC and the Cho-evoked responses monitored for changes in amplitude. The data for inhibition of the Cho-evoked responses were normalized to 3 averaged control pulses and analysed with an exponential decay equation. Data for recovery from inhibition were analysed with an exponential association equation. The data for inhibition by PC were best fit with a double exponential and the data for recovery from inhibition with a single exponential.

Statistical analyses. Data were analysed with the SPSS software (Munich, Germany) or GraphPad Prism 6 software (Ja Lolla, CA, USA). Values derived from different cells were compared, where applicable, by the non-parametric Kruskal-Wallis test, followed by the Mann-Whitney rank-sum test. The Wilcoxon signed-rank test was used for analyses of dependent values. The number (n) of individual experiments is indicated in the Results section and the Figures. In TEVC measurements oocytes from at least two different *Xenopus laevis* individuals were used.

References

1. Crilly, K. S., Tomono, M. & Kiss, Z. The choline kinase inhibitor hemicholinium-3 can inhibit mitogen-induced DNA synthesis independent of its effect on phosphocholine formation, *Arch. Biochem. Biophys.* **352,** 137–143 (1998).
2. Li, Z. & Vance, D. E. Phosphatidylcholine and choline homeostasis, *J. Lipid Res.* **49,** 1187–1194 (2008).
3. Bobenchik, A. M., Augagneur, Y., Hao, B., Hoch, J. C. & Ben Mamoun, C. Phosphoethanolamine methyltransferases in phosphocholine biosynthesis: functions and potential for antiparasite therapy, *FEMS Microbiol. Rev.* **35,** 609–619 (2011).
4. Park, Y. I., Buszko, M. L. & Gander, J. E. Utilization of phosphocholine from extracellular complex polysaccharide as a source of cytoplasmic choline derivatives in *Penicillium fellutanum*, *J. Bacteriol.* **179,** 1186–1192 (1997).
5. Grabitzki, J. & Lochnit, G. Immunomodulation by phosphocholine - biosynthesis, structures and immunological implications of parasitic PC-epitopes, *Mol. Immunol.* **47,** 149–163 (2009).
6. Fischer, W. Phosphocholine of pneumococcal teichoic acids: role in bacterial physiology and pneumococcal infection, *Res. Microbiol.* **151,** 421–427 (2000).
7. Young, N. M., Foote, S. J. & Wakarchuk, W. W. Review of phosphocholine substituents on bacterial pathogen glycans: synthesis, structures and interactions with host proteins, *Mol. Immunol.* **56,** 563–573 (2013).
8. Thompson, D., Pepys, M. B. & Wood, S. P. The physiological structure of human C-reactive protein and its complex with phosphocholine, *Structure* **7,** 169–177 (1999).
9. Gang, T. B., Hanley, G. A. & Agrawal, A. C-reactive protein protects mice against pneumococcal infection via both phosphocholine-dependent and phosphocholine-independent mechanisms, *Infect. Immun.* **83,** 1845–1852 (2015).
10. Goldenberg, H. B., McCool, T. L. & Weiser, J. N. Cross-reactivity of human immunoglobulin G2 recognizing phosphorylcholine and evidence for protection against major bacterial pathogens of the human respiratory tract, *J. Infect. Dis.* **190,** 1254–1263 (2004).
11. Harnett, W. & Harnett, M. M. Modulation of the host immune system by phosphorylcholine-containing glycoproteins secreted by parasitic filarial nematodes, *Biochim. Biophys. Acta* **1539,** 7–15 (2001).
12. Clark, S. E., Snow, J., Li, J., Zola, T. A. & Weiser, J. N. Phosphorylcholine allows for evasion of bactericidal antibody by *Haemophilus influenz*ae, *PLos Pathog.* **8,** e1002521 (2012).
13. Percy, M. G. & Gründling, A. Lipoteichoic acid synthesis and function in gram-positive bacteria, *Annu. Rev. Microbiol.* **68,** 81–100 (2014).
14. Moran, A. P., Prendergast, M. M. & Appelmelk, B. J. Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease, *FEMS Immunol. Med. Microbiol.* **16,** 105–115 (1996).
15. Pang, B. *et al.* Lipooligosaccharides containing phosphorylcholine delay pulmonary clearance of nontypeable *Haemophilus influenzae*, *Infect. Immun.* **76,** 2037–2043 (2008).
16. Dinarello, C. A. Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist, *Int. Rev. Immunol.* **16,** 457–499 (1998).
17. Jaffer, U., Wade, R. G. & Gourlay, T. Cytokines in the systemic inflammatory response syndrome: a review, *HSR Proc. Intensive Care Cardiovasc. Anesth.* **2,** 161–175 (2010).
18. Alnemri, E. S. Sensing cytoplasmic danger signals by the inflammasome, *J. Clin. Immunol.* **30,** 512–519 (2010).
19. Lamkanfi, M. & Dixit, V. M. Mechanisms and functions of inflammasomes, *Cell* **157,** 1013–1022 (2014).

20. Hecker, A. *et al.* Phosphocholine-Modified Macromolecules and Canonical Nicotinic Agonists Inhibit ATP-Induced IL-1β Release, *J. Immunol.* **195,** 2325–2334 (2015).
21. Pereira, E. F. R. *et al.* Unconventional ligands and modulators of nicotinic receptors, *J. Neurobiol.* **53,** 479–500 (2002).
22. McIntosh, J. M. *Conotoxin peptides, pharmaceutical compositions and uses thereof* (Google Patents, 2014), https://www.google.com/patents/WO2014194284A1?cl=en.
23. Vincler, M. *et al.* Molecular mechanism for analgesia involving specific antagonism of alpha9alpha10 nicotinic acetylcholine receptors, *Proc. Natl. Acad. Sci. USA* **103,** 17880–17884 (2006).
24. North, R. A. Molecular physiology of P2X receptors, *Physiol. Rev.* **82,** 1013–1067 (2002).
25. Grahames, C. B., Michel, A. D., Chessell, I. P. & Humphrey, P. P. Pharmacological characterization of ATP- and LPS-induced IL-1 beta release in human monocytes, *Br. J. Pharmacol.* **127,** 1915–1921 (1999).
26. Elgoyhen, A. B. *et al.* alpha10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells, *Proc. Natl. Acad. Sci. USA* **98,** 3501–3506 (2001).
27. Azam, L. & McIntosh, J. M. Molecular basis for the differential sensitivity of rat and human α9α10 nAChRs to α-conotoxin RgIA, *J. Neurochem.* **122,** 1137–1144 (2012).
28. Filchakova, O. & McIntosh, J. M. Functional expression of human α9* nicotinic acetylcholine receptors in *X. laevis* oocytes is dependent on the α9 subunit 5′ UTR, *PLos ONE* **8,** e64655 (2013).
29. Elgoyhen, A. B., Johnson, D. S., Boulter, J., Vetter, D. E. & Heinemann, S. Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells, *Cell* **79,** 705–715 (1994).
30. Lips, K. S., Pfeil, U. & Kummer, W. Coexpression of alpha 9 and alpha 10 nicotinic acetylcholine receptors in rat dorsal root ganglion neurons, *Neuroscience* **115,** 1–5 (2002).
31. Kurzen, H. *et al.* Phenotypical and molecular profiling of the extraneuronal cholinergic system of the skin, *J. Invest. Dermatol.* **123,** 937–949 (2004).
32. Peng, H. *et al.* Characterization of the human nicotinic acetylcholine receptor subunit alpha (α) 9 (CHRNA9) and alpha (α) 10 (CHRNA10) in lymphocytes, *Life Sci.* **76,** 263–280 (2004).
33. Hecker, A. *et al.* Pivotal Advance: Up-regulation of acetylcholine synthesis and paracrine cholinergic signaling in intravascular transplant leukocytes during rejection of rat renal allografts, *J. Leukoc. Biol.* **86,** 13–22 (2009).
34. Zarghooni, S. *et al.* Expression of muscarinic and nicotinic acetylcholine receptors in the mouse urothelium, *Life Sci.* **80,** 2308–2313 (2007).
35. Pelegrin, P. & Surprenant, A. Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor, *EMBO J.* **25,** 5071–5082 (2006).
36. Pelegrin, P. & Surprenant, A. Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1beta release through a dye uptake-independent pathway, *J. Biol. Chem.* **282,** 2386–2394 (2007).
37. Searl, T. J., Redman, R. S. & Silinsky, E. M. Mutual occlusion of P2X ATP receptors and nicotinic receptors on sympathetic neurons of the guinea-pig, *J. Physiol.* **510,** 783–791 (1998).
38. Khakh, B. S., Fisher, J. A., Nashmi, R., Bowser, D. N. & Lester, H. A. An angstrom scale interaction between plasma membrane ATP-gated P2X2 and alpha4beta2 nicotinic channels measured with fluorescence resonance energy transfer and total internal reflection fluorescence microscopy, *J. Neurosci.* **25,** 6911–6920 (2005).
39. Limapichat, W., Dougherty, D. A. & Lester, H. A. Subtype-specific mechanisms for functional interaction between α6β4* nicotinic acetylcholine receptors and P2X receptors, *Mol. Pharmacol.* **86,** 263–274 (2014).
40. Razani-Boroujerdi, S. *et al.* T cells express alpha7-nicotinic acetylcholine receptor subunits that require a functional TCR and leukocyte-specific protein tyrosine kinase for nicotine-induced Ca^{2+} response, *J. Immunol.* **179,** 2889–2898 (2007).
41. Cartier, G. E. *et al.* A new alpha-conotoxin which targets alpha3beta2 nicotinic acetylcholine receptors, *J. Biol. Chem.* **271,** 7522–7528 (1996).
42. Chojnacka, K., Papke, R. L. & Horenstein, N. A. Synthesis and evaluation of a conditionally-silent agonist for the α7 nicotinic acetylcholine receptor, *Bioorg. Med. Chem. Lett.* **23,** 4145–4149 (2013).
43. Papke, R. L. *et al.* The analgesic-like properties of the alpha7 nAChR silent agonist NS6740 is associated with non-conducting conformations of the receptor, *Neuropharmacology* **91,** 34–42 (2015).
44. Verbitsky, M., Rothlin, C. V., Katz, E. & Elgoyhen, A. B. Mixed nicotinic-muscarinic properties of the alpha9 nicotinic cholinergic receptor, *Neuropharmacology* **39,** 2515–2524 (2000).
45. Vetter, D. E. *et al.* Role of alpha9 nicotinic ACh receptor subunits in the development and function of cochlear efferent innervation, *Neuron* **23,** 93–103 (1999).
46. Vetter, D. E. *et al.* The alpha10 nicotinic acetylcholine receptor subunit is required for normal synaptic function and integrity of the olivocochlear system, *Proc. Natl. Acad. Sci. USA* **104,** 20594–20599 (2007).
47. Froehner, S. C., Luetje, C. W., Scotland, P. B. & Patrick, J. The postsynaptic 43K protein clusters muscle nicotinic acetylcholine receptors in *Xenopus* oocytes, *Neuron* **5,** 403–410 (1990).
48. Ramarao, M. K. & Cohen, J. B. Mechanism of nicotinic acetylcholine receptor cluster formation by rapsyn, *Proc. Natl. Acad. Sci. USA* **95,** 4007–4012 (1998).

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Author Contributions

K.R., V.M., A.Z., G.K.-C. and A.J.H. performed research. M.F., M.A., A.H. and W.P. contributed to the design of the study. K.R., A.J.H., J.M.M. and V.G. designed research, analysed and interpreted data and wrote the manuscript.

Additional Information

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Surfactant inhibits ATP-induced release of interleukin-1β via nicotinic acetylcholine receptors[S]

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Abstract Interleukin (IL)-1β is a potent pro-inflammatory cytokine of innate immunity involved in host defense. High systemic IL-1β levels, however, cause life-threatening inflammatory diseases, including systemic inflammatory response syndrome. In response to various danger signals, the pro-form of IL-1β is synthesized and stays in the cytoplasm unless a second signal, such as extracellular ATP, activates the inflammasome, which enables processing and release of mature IL-1β. As pulmonary surfactant is known for its anti-inflammatory properties, we hypothesize that surfactant inhibits ATP-induced release of IL-1β. Lipopolysaccharide-primed monocytic U937 cells were stimulated with an ATP analog in the presence of natural or synthetic surfactant composed of recombinant surfactant protein (rSP)-C, palmitoylphosphatidylglycerol, and dipalmitoylphosphatidylcholine (DPPC). Both surfactant preparations dose-dependently inhibited IL-1β release from U937 cells. DPPC was the active constituent of surfactant, whereas rSP-C and palmitoylphosphatidylglycerol were inactive. DPPC was also effective in primary mononuclear leukocytes isolated from human blood. Experiments with nicotinic antagonists, siRNA technology, and patch-clamp experiments suggested that stimulation of nicotinic acetylcholine receptors (nAChRs) containing subunit α9 results in a complete inhibition of the ion channel function of ATP receptor, P2X7. In conclusion, the surfactant constituent, DPPC, efficiently inhibits ATP-induced inflammasome activation and maturation of IL-1β in human monocytes by a mechanism involving nAChRs.—Backhaus, S., A. Zakrzewicz, K. Richter, J. Damm, S. Wilker, G. Fuchs-Moll, M. Küllmar, A. Hecker, I. Manzini, C. Ruppert, J. M. McIntosh, W. Padberg, and V. Grau. **Surfactant inhibits ATP-induced release of interleukin-1β via nicotinic acetylcholine receptors. *J. Lipid Res.* 2017.** 58: **1055–1066.**

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In order to enable effective gas exchange, the blood-air barrier of the mammalian lung is extremely thin-walled and fragile. At the same time, lungs are exposed to a considerable intake of foreign matter, including allergens, particulates, and potentially pathogenic microorganisms. Hence, healthy lungs should efficiently clear invading pathogens without inducing excessive inflammation and impairment of the blood-air barrier. This is achieved by a whole arsenal of potent factors involved in host defense against infection and by numerous anti-inflammatory mediators, which are present in lung tissue and in the pulmonary immune system (1–4).

Interleukin (IL)-1β is a potent pro-inflammatory cytokine that is predominantly produced by activated monocytes/macrophages, but also by numerous other cells, such as pulmonary epithelia and endothelial cells (5–7). On the one hand, IL-1β plays a central role in host defense against viral and bacterial infections. On the other hand, IL-1β is an important pathogenic factor in acute lung injury, acute

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Abbreviations: ARDS, acute respiratory distress syndrome; BzATP, 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium salt; DPG, 1,2-dipalmitoyl-*sn*-glycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (dipalmitoylphosphatidylcholine); DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; IL, interleukin; LDH, lactate dehydrogenase; LLME, Leu-Leu methyl ester hydrobromide; LPS, lipopolysaccharide; nAChR, nicotinic acetylcholine receptor; NLRP3, Nod-like receptor protein 3; PBMC, peripheral blood mononuclear cell; PC, phosphocholine; POPG, palmitoyl-oleyl-phosphatidylglycerol; pro-, pro-form; PS, 1,2-diacyl-*sn*-glycero-3-phospho-L-serine (phosphatidylserine); rSP, recombinant surfactant protein; SP, surfactant protein; TLR4, Toll-like receptor 4.

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respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease, silicosis, and other pulmonary diseases (8–14). High systemic levels of IL-1β, which, among others, may originate from an inflamed lung, can cause a life-threatening systemic inflammatory response syndrome (6).

Synthesis and release of IL-1β are tightly controlled under steady-state conditions (15, 16). Lipopolysaccharide (LPS) originating from gram-negative bacteria is a classic stimulus inducing the biosynthesis of the inactive cytoplasmic pro-form of IL-1β (pro-IL-1β) in monocytes/macrophages. Release of mature IL-1β normally depends on a second danger signal, such as extracellular ATP, that induces the assembly of the Nod-like receptor protein 3 (NLRP3)-containing inflammasome via P2X7 receptors. Inflammasomes, in turn, activate the protease, caspase-1, that cleaves pro-IL-1β and enables release of bioactive mature IL-1β (15, 16). LPS and ATP are relevant stimuli to mimic sterile and infectious pulmonary inflammation. LPS is a cell wall component of gram-negative bacteria and serves as a model ligand of Toll-like receptor 4 (TLR4). Endogenous danger-associated molecular patterns, such as biglycan, HMGB1, or heat shock proteins, are also known agonists of TLR4 (17). An important source of extracellular ATP is traumatic cell injury. In the lung, this can be direct trauma caused by accidents, but can also be ventilator-induced lung injury and therapeutic alveolar recruitment maneuvers. In addition, during sterile and infectious inflammation, ATP can be released in significant amounts by different immune cells (18).

Recently, we discovered that activation of nicotinic acetylcholine receptors (nAChRs), composed of subunits α7, α9, and/or α10, efficiently control ATP-induced activation of P2X7 receptors and, thereby, release of IL-1β by human monocytes (19). These nAChR subunits are expressed by both human and rat monocytes under steady-state conditions and during severe inflammation (20–22). In this context, we identified free phosphocholine (PC), PC-modified protein, and PC-modified LPS as novel agonists of nAChR and as potent inhibitors of monocytic IL-1β release (19, 23). These findings suggested that other compounds bearing PC moieties might also trigger this noncanonical function of nAChR.

Pulmonary surfactant lines the surface of alveoli and is composed of a lipid fraction, which represents about 90%, and four surfactant proteins (SPs), named SP-A, SP-B, SP-C, and SP-D. The main constituents of the lipid fraction are phospholipids with phosphatidylcholine and phosphatidylglycerol as the main classes. Dipalmitoylated phosphatidylcholine [1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (dipalmitoylphosphatidylcholine) (DPPC)] is the most abundant single constituent, representing approximately 40% of all phospholipid molecules (24, 25). Surfactant exerts a dual function in the lung. First, it exerts a biophysical activity that lowers the surface tension within the alveoli and, thus, contributes to their stability and enables gas exchange. Lipids, in particular DPPC, and the hydrophobic proteins, SP-B and SP-C, are involved in this mechanical function. Second, surfactant is a core part of pulmonary innate immunity (26). A wide variety of pathogens are bound by SP-A and SP-D, microbial growth is inhibited, pathogens are opsonized, and phagocytosis by alveolar macrophages is mediated (26). In addition, a variety of anti-inflammatory effects have been described for SP-B and SP-C and for surfactant lipids (26, 27). For example, surfactant lipids suppress inflammation by interfering with sensing of LPS (28–30), by suppression of respiratory burst (31), and by regulating cyclooxygenase-2 expression (32).

Here, we test and confirm the hypothesis that pulmonary surfactant inhibits ATP-induced release of monocytic IL-1β. DPPC is identified as the active constituent of surfactant and we provide evidence that it signals via unconventional nAChR containing subunits α9, α7, and/or α10.

MATERIALS AND METHODS

U937 cells

U937 cells, a human histiocytic lymphoma cell line, were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and cultured in RPMI 1640 (Gibco by Life Technologies GmbH, Darmstadt, Germany) supplemented with 10% fetal calf serum (Biochrome, Berlin, Germany) and 2 mM L-glutamine (Gibco). In the exponential phase of cell growth, 1×10^6 U937 cells were seeded in 24-well plates and primed with 1 μg/ml LPS from *Escherichia coli* (L2654; Sigma-Aldrich, Steinheim, Germany) for 5 h. Thereafter, cells were stimulated with 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium salt (BzATP; Sigma-Aldrich) at a concentration of 100 μM. In some experiments Ac-YVAD-cmk, an inhibitor of caspase-1 (50 μM; Sigma-Aldrich), was applied together with BzATP. Cells were spun down 30 min thereafter, and cell culture supernatant was stored at −20°C until measurement of IL-1β and lactate dehydrogenase (LDH).

A natural bovine surfactant extract (Alveofact®; Lyomark Pharma, Oberhaching, Germay), synthetic surfactant (Venticute®; Nycomed, Konstanz, Germany), human recombinant SP-C (rSP-C; Nycomed), palmitoyl-oleyl-phosphatidylglycerol [POPG, 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phospho-rac-(1-glycerol) sodium salt; Sigma-Aldrich], 1,2-diacyl-*sn*-glycero-3-phospho-L-serine (PS; Sigma-Aldrich), DPPC (Sigma-Aldrich), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE; Sigma-Aldrich), or 1,2-dipalmitoyl-*sn*-glycerol (DPG; Sigma-Aldrich) were added at different concentrations shortly before stimulation with BzATP in the presence or absence of different nicotinic antagonists. All surfactant preparations and lipids were sonicated shortly before use. In some experiments, apyrase (0.5 U/ml, A6410; Sigma-Aldrich) was added to degrade endogenous extracellular ATP and cells were stimulated with the pore-forming bacterial toxin, nigericin (50 μM; Sigma-Aldrich), for 30 min. Leu-Leu methyl ester hydrobromide (LLME, 1 mM; Sigma-Aldrich) was also applied for 30 min as another ATP-independent stimulus for inflammasome activation. Nicotine hydrogen tartrate (100 μM; Sigma-Aldrich) served as a positive inhibitory control in most experiments. The following nicotinic antagonists were used: mecamylamine hydrochloride (100 μM; Sigma-Aldrich), α-bungarotoxin (1 μM; Tocris Bioscience, Bristol, UK), strychnine hydrochloride (10 μM; Sigma-Aldrich), as well as α-conotoxins, ArIB[V11L,V16D] (500 nM), and RgIA4 (200 nM).

Transfection of siRNA

As previously described (19, 23), the expression of nAChR subunits α7, α9, and α10 (*CHRNA*7, *CHRNA*9, *CHRNA*10) was

reduced in U937 cells using siRNA SMARTpool (Thermo Fisher Scientific, Schwerte, Germany). ON-TARGETplus nontargeting pool (Thermo Fisher Scientific) was used as a control for nonspecific effects. In addition to single knock-down, double knock-down experiments were performed.

Primary human mononuclear cells

Experiments on human blood cells were performed in accordance with the principles of the Declaration of Helsinki, as well as to Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, revised January 15, 2009, effective July 14, 2009. The ethics committee of the University of Giessen approved these studies (No. 81/13). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of healthy male nonsmoking volunteers by density gradient centrifugation using Leukosep gradients (Greiner Bio-One, Frickenhausen, Germany). In some experiments, heparinized blood was pulsed with 5 ng/ml LPS before separation. PBMCs were incubated in 24-well plates for 3 h in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Gibco) at a density of 5×10^5 PBMCs/0.5 ml per well. Nonadherent cells were removed, cell culture medium was replaced, and cells were stimulated for 30 min with BzATP (100 μM) in the presence or absence of DPPC (100 μM).

Immunocytochemistry

LPS-pulsed PBMCs were cultivated and stimulated as described in Cellview™ cell culture slides (Greiner Bio-One), fixed in Cytofix/Cytoperm™ (BD Biosciences, Heidelberg, Germany) for 20 min on ice, washed with Perm/Wash™ (BD Biosciences), air-dried, and stored at 4°C until staining. Endogenous peroxidase activity was inactivated by incubation in 1% H_2O_2, unspecific protein binding sites were blocked with 1% BSA (Serva, Heidelberg, Germany), primary rabbit anti-ASC antibodies (Santa Cruz sc-22514-R; Santa Cruz, CA) were diluted 1:50 in Perm/Wash™, 1% BSA, and 5% heat-inactivated human serum and incubated for 1 h at room temperature. Bound antibodies were detected by horseradish peroxidase-labeled goat anti-rabbit Ig antibodies (DAKO, Hamburg, Germany) and 3,3′-diaminobenzidine (Sigma-Aldrich). Absence of unspecific background staining caused by secondary antibodies was tested by omission of primary antibodies. Slides were lightly counterstained with hemalum, covered with Glycergel (Dako), and evaluated with an Olympus (Hamburg, Germany) BX51 microscope and the analySIS software (Olympus).

Measurement of cytokines

Human quantikine immunoassays (R&D Systems, Minneapolis, MN) were used to determine IL-1β, IL-6, and IL-18 concentrations in cell culture supernatants according to the instructions of the supplier. A high sensitivity kit for the measurement of IL-1β was mandatory because U937 cells release low amounts of IL-1β in response to BzATP (19, 23).

Cell viability

LDH was measured in cell culture supernatants and lysed cells by nonradioactive cytotoxicity assay (Promega, Madison, WI) according to the protocol indicated by the supplier. The total content of LDH measured in a batch of lysed untreated control cells was set to 100% and values obtained for individual cell culture supernatants were calculated accordingly.

Western blots

Western blot analysis of IL-1β and caspase-1, as well as their proforms, was performed as described before (19). In short, human PBMCs were stimulated with BzATP in serum-free medium and supernatants were concentrated by a factor of 10 using Amicon® Ultra centrifugal filters (UltracelTM 10K; Merck Millipore, Darmstadt, Germany). Cell lysates and concentrated supernatants were separated along with prestained molecular weight standards (Precision Plus protein standards, dual color; Bio Rad, Hercules, CA) on 15% reducing SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Mouse monoclonal antibodies to IL-1β (kindly supplied by the National Cancer Institute, Frederick, MD), polyclonal rabbit antibodies to caspase-1 (#2225; Cell Signaling Technology, Danvers, MA), and mouse monoclonal antibodies to β-actin (A2228; Sigma-Aldrich) were used as primary antibodies that were detected by horseradish peroxidase-conjugated secondary antibodies (Dako Cytomation, Glostrup, Denmark) and chemoluminescent substrates. Documentation and densitometry were performed using a digital gel documentation system (Biozym, Hessisch Oldendorf, Germany).

Real-time RT-PCR

Extraction of RNA, reverse transcription, and real-time RT-PCR for pro-IL-1β (*IL1B*), subunits α9 (*CHRNA9*) and α10 (*CHRNA10*), and the housekeeping gene, porphobilinogen deaminase (*HMBS*), were performed as described previously (19). In negative control experiments, where the cDNA was replaced by water, no DNA was amplified. Agarose gel electrophoresis and sequencing (SeqLab, Göttingen, Germany) of the PCR products confirmed the specificity of the PCR reaction. Gene expression was analyzed using the $2^{-\Delta Ct}$ method and the mean values of the controls were set to one arbitrary unit.

Whole-cell patch-clamp recordings on U937 cells

Electrophysiological recordings on LPS-primed U937 cells were essentially performed as described before (19). Briefly, U937 cells were transferred to poly-L-lysine-coated cell culture dishes containing bath solution (in millimoles: 5.4 KCl, 120 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 glucose, and 10 HEPES [4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (pH 7.4)] and primed with LPS for 5 h. Thereafter, whole-cell patch-clamp recordings were performed at room temperature. BzATP (100 μM) was always applied as a first stimulus via a pressure-driven microperfusion system followed by DPPC (100 μM) and a second BzATP stimulus. Control experiments in which BzATP was applied twice in the absence of DPPC were performed at least once per day to confirm reversibility and repeatability of the BzATP-induced changes in ion currents.

Statistical analyses

SPSS software (Munich, Germany) was used to analyze data by nonparametric Kruskal-Wallis test followed by Mann-Whitney rank sum test. Wilcoxon sign rank test was used to analyze data obtained from PBMCs. $P \leq 0.05$ was considered as statistically significant.

RESULTS

Surfactant inhibits the release of IL-1β

To test the hypothesis that pulmonary surfactant inhibits ATP-induced release of IL-1β, human monocytic U937 cells were primed with LPS for 5 h followed by stimulation with BzATP, a specific agonist of ATP receptor, P2X7. As expected, IL-1β was released into the cell culture

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supernatant (**Fig. 1A**, B), whereas IL-18 was not detected. Maturation and release of IL-1β depended on activated caspase-1 (supplemental Fig. S1). The natural bovine surfactant, Alveofact®, dose-dependently and efficiently inhibited BzATP-induced IL-1β release ($P \leq 0.00001$, n = 11 at a concentration of 90 ng/ml) with an IC_{50} of about 9 ng/ml (Fig. 1A). Nicotine (100 μM) that was included in each experiment as a positive control also significantly inhibited BzATP-induced IL-1β release ($P \leq 0.00001$, n = 25), as described before (19). Essentially the same results were obtained when the synthetic surfactant preparation, Venticute®, was used, which is composed of rSP-C, POPG, and DPPC (Fig. 1B). To estimate cell death, LDH was measured in the cell culture supernatant at the end of each experiment. Elevated LDH levels were not detected in any of the experimental settings (supplemental Fig. S2A, B).

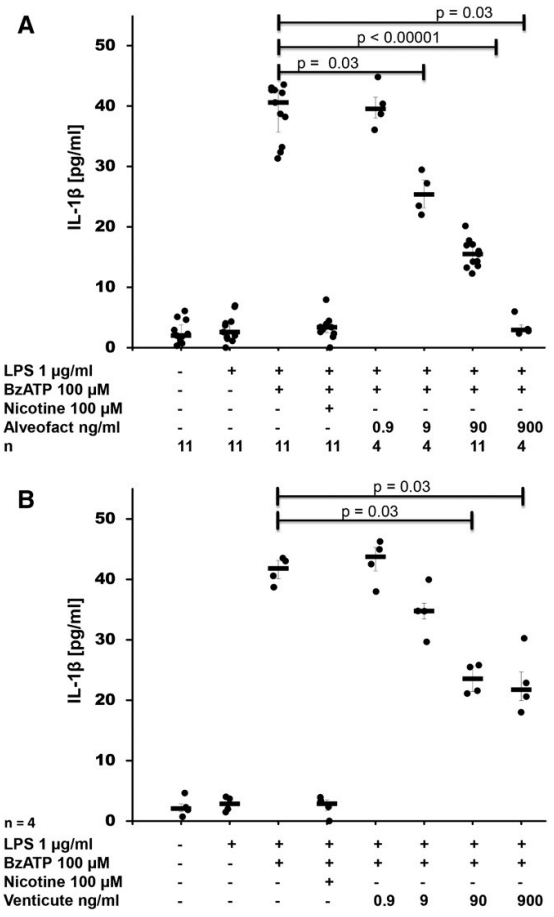

Fig. 1. Surfactant dose-dependently inhibits BzATP-mediated release of IL-1β. Different concentrations of the natural surfactant preparation, Alveofact® (A), and the synthetic surfactant, Venticute® (B), were added to LPS-primed U937 cells together with BzATP. IL-1β levels were measured 30 min thereafter in cell culture supernatants. Nicotine was included as a known inhibitor of BzATP-dependent IL-1β release. A Kruskal-Wallis test was followed by Mann-Whitney rank-sum test; data are presented as individual data points; bars represent median; whiskers represent percentiles 25 and 75.

The inhibitory function of surfactant is mediated by DPPC

To identify the active compound that inhibits BzATP-induced release of IL-1β by U937 cells, we investigated the effect of the different constituents of Venticute®, rSP-C (**Fig. 2A**), POPG (Fig. 2B), and DPPC (Fig. 2C) at concentrations that reflected their relative concentration in Venticute® (33). As an additional control, we included PS, a constituent of natural surfactant (Fig. 2D). rSP-C, POPG, and PS did not inhibit BzATP-induced release of IL-1β from LPS-primed U937 cells, although nicotine that served as positive control was effective in the same experiments. In contrast, application of DPPC resulted in a dose-dependent and efficient inhibition of IL-1β release at concentrations of 10, 100, and 1,000 μM ($P = 0.03$, n = 4, each) with an IC_{50} of about 10 μM (Fig. 2D), which was in line with the data obtained for surfactant. When DPPC was added to LPS-primed U937 cells in the absence of BzATP, virtually no IL-1β was detected in the cell culture supernatant (n = 25; Fig. 2C). To test whether other dipalmitoylated compounds devoid of a PC group also inhibit BzATP-induced release of IL-1β, we tested DPPE (100 μM) and DPG (100 μM). Both compounds did not impair IL-1β release (Fig. 2E). None of the compounds tested resulted in an increased LDH content of cell culture supernatants (supplemental Fig. S3A–E).

When DPPC was added together with LPS during priming of U937 cells, no change in the mRNA expression of pro-IL-1β was seen (supplemental Fig. S4). Of note, release of IL-6, an inflammasome-independent cytokine, was induced by priming with LPS, but remained unchanged irrespective of the presence of BzATP, DPPC, or nicotine (supplemental Fig. S5).

DPPC inhibits release of IL-1β by primary PBMCs

Next, we tested whether DPPC is also effective in primary human PBMCs isolated from the peripheral blood of healthy human donors. Unprimed PBMCs released a considerable amount of IL-1β upon stimulation with BzATP (**Fig. 3A**), whereas, at best, traces of IL-18 were detected. Priming of PBMCs with LPS led to even higher concentrations of IL-1β in cell culture supernatants (Fig. 3B). In both experimental settings, addition of 100 μM DPPC significantly ($P = 0.03$, n = 5, each) reduced the BzATP-induced release of IL-1β into the cell culture medium (Fig. 3A, B) without changing the release of LDH (supplemental Fig. S6).

Next, we used antibodies to ASC to detect ASC speck formation by immunohistochemistry. In unstimulated cells, a diffuse cytoplasmic and nuclear immunopositivity was seen in most cells and almost no ASC specks were detected. Upon stimulation with BzATP, the frequency of ASC specks per 100 nuclei increased and the presence of DPPC significantly ($P = 0.03$, n = 5) reduced speck formation (Fig. 3C, D). In controls, where the primary antibody

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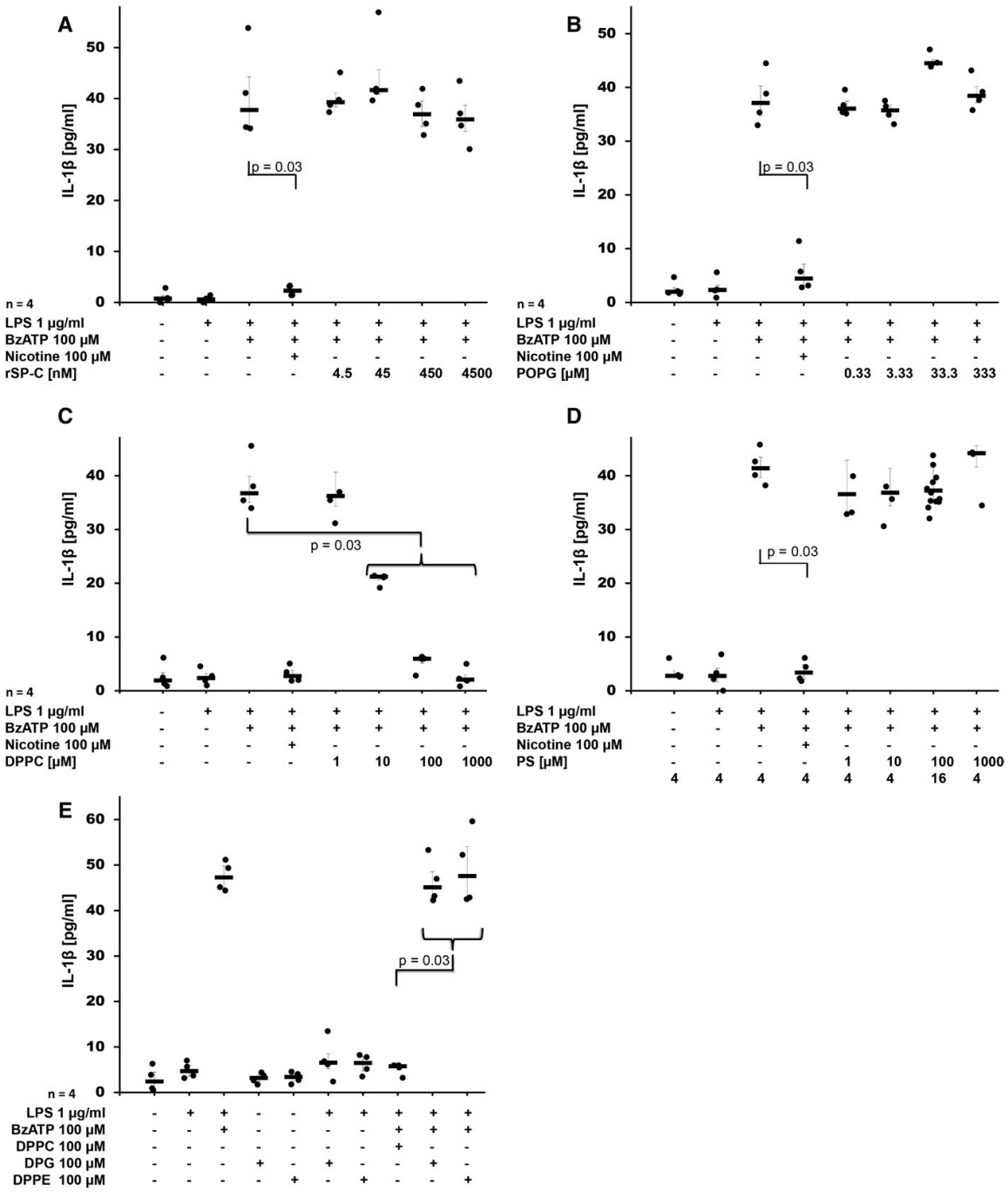

Fig. 2. DPPC is the active component of surfactant that dose-dependently inhibits BzATP-mediated release of IL-1β. Different concentrations of rSP-C (A), POPG (B), DPPC (C), PS (D), DPPE (E), or DPG (E) were added to LPS-primed U937 cells together with BzATP. IL-1β levels were measured 30 min thereafter in cell culture supernatants. Nicotine was included as a known inhibitor of BzATP-dependent IL-1β release. A Kruskal-Wallis test was followed by Mann-Whitney rank-sum test; data are presented as individual data points; bars represent median; whiskers represent percentiles 25 and 75.

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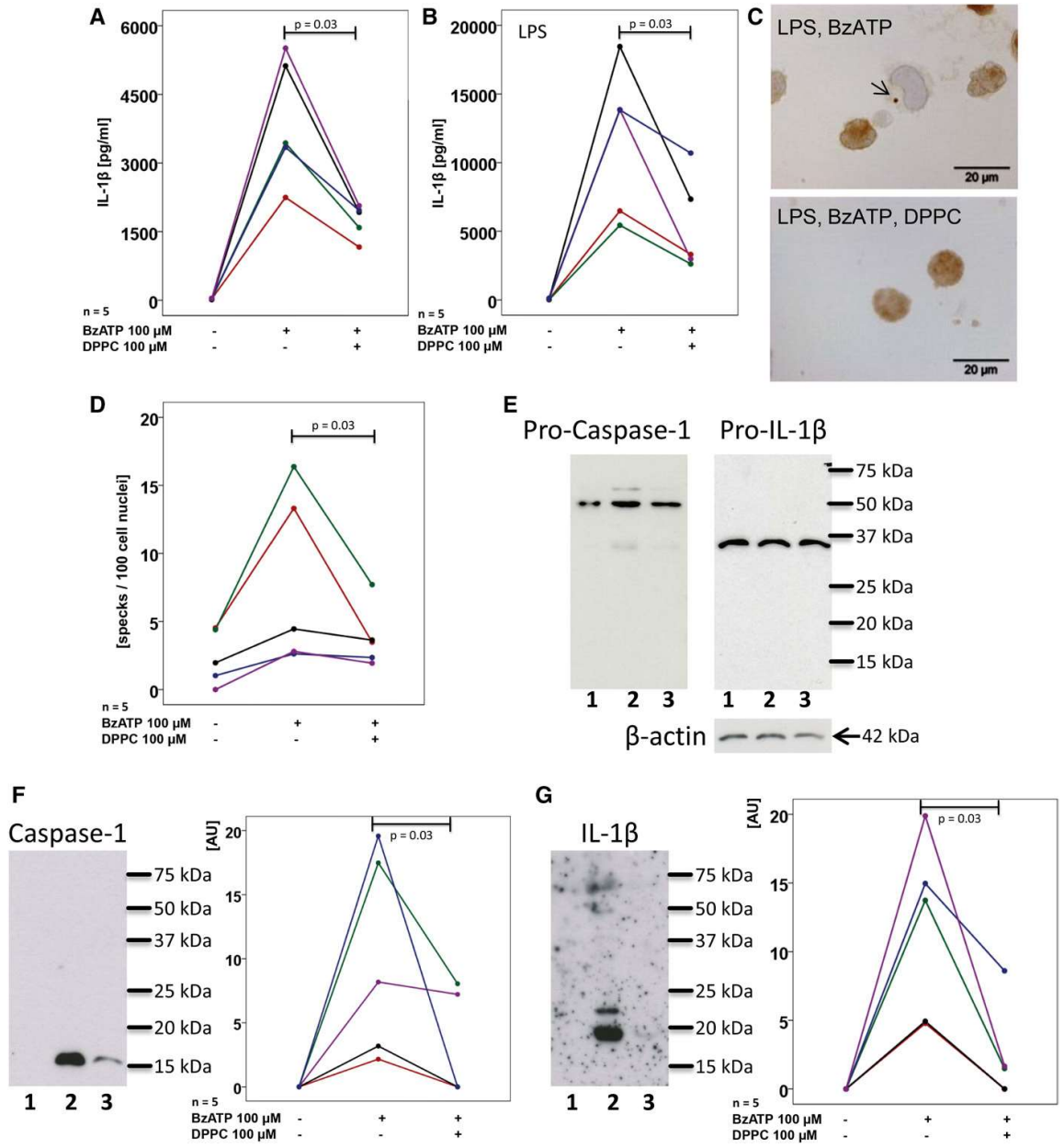

Fig. 3. DPPC inhibits BzATP-mediated inflammasome activation in human PBMCs. PBMCs were isolated from healthy human donors, left untreated (A) or pulsed with LPS (B), cultured for 3 h and stimulated with BzATP in the presence or absence of DPPC. IL-1β levels were measured 30 min thereafter in cell culture supernatants. C, D: ASC specks were detected by immunocytochemistry in LPS-pulsed PBMC and stimulated with BzATP in the presence or absence of DPPC. C: Micrographs of PBMC: ASC immunopositive material is stained in brown, cell nuclei were lightly counterstained with hemalum, and the arrow is pointing to an ASC speck. D: The number of specks per 100 cell nuclei is depicted. E–G: PBMCs were pulsed with LPS (lane 1) and stimulated with BzATP in the absence (lane 2) or presence (lane 3) of DPPC and Western blots were performed with antibodies directed to human caspase-1, IL-1β, and β-actin. E: In cell lysates, exclusively, the pro-forms, pro-caspase-1 and pro-IL-1β, were detected; whereas, in concentrated cell culture supernatants, mature caspase-1 (F) and IL-1β (G) were detected. F, G: Immunopositive signals were quantified by densitometry and expressed as arbitrary units (AU). Data points obtained from individual blood donors are depicted and connected by lines. Data from the same individuals are coded by the same color in (A) and (B) as well as in (F) and (G), but in (A) and (B) a cohort of volunteers was investigated that was different than in (F) and (G). Wilcoxon sign rank test.

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was omitted, virtually no immunopositivity was visible. The specificity of the antibody to ASC was controlled by Western blotting, where a single band of the expected molecular mass was detected (data not shown).

In Western blots of cell lysates only pro-IL-1β and procaspase-1 were detected, suggesting that DPPC does not inhibit the release of mature IL-1β (Fig. 3E). Western blots of concentrated cell culture supernatants confirmed that, in response to BzATP, activated caspase-1 is released to cell culture supernatants along with mature IL-1β (Fig. 3F, G). In the presence DPPC, this release was significantly reduced (Fig. 3F, G).

DPPC does not inhibit ATP-independent release of IL-1β

In order to investigate whether DPPC also affects ATP-independent IL-1β release, we added apyrase to degrade any ATP present in the cell culture medium and stimulated LPS-primed U937 cells with the bacterial pore-forming toxin, nigericin, which induced release of IL-1β (**Fig. 4A**), but not of LDH (supplemental Fig. S7A). DPPC did not inhibit nigericin-induced release of IL-1β (Fig. 4A). In the same experimental setting, nicotine was also ineffective, which is in line with published data (19). In LPS-primed U937 cells, LLME did not stimulate IL-1β secretion, which is in contrast to naïve and LPS-primed PBMCs. In these cells, nigericin and LLME triggered the release of IL-1β and LDH and, again, addition of DPPC did not change IL-1β levels in cell culture supernatants (Fig. 4B, C; supplemental Fig. S7B, C).

The effect of surfactant and DPPC is mediated via nAChRs

To test the hypothesis that the inhibitory effects of Alveofact®, Venticute®, and DPPC are mediated via nAChRs, we used the general nicotinic antagonist, mecamylamine (100 μM), which blocked the inhibition of BzATP-induced release of IL-1β in U937 cells ($P = 0.03$, n = 4) (**Fig. 5A–C**). The same effects were seen when α-bungarotoxin (1 μM) and strychnine (10 μM) were used ($P = 0.03$, n = 4, each), suggesting that nAChRs composed of subunits α7, α9, and/or α10 are involved (Fig. 5A–C). RgIA4 (200 nM), an α-conotoxin specific for nAChRs containing subunit α9 (23, 34), also completely blocked the effect of DPPC ($P = 0.03$, n = 4, each), whereas ArIB[V11L,V16D] (500 nM), an α-conotoxin specifically antagonizing α7 nAChRs (35, 36), was ineffective (Fig. 5C). In all experimental settings, no increase in LDH release was seen (supplemental Fig. S5A–C). Treatment of U937 cells with LPS and BzATP did not change the mRNA expression of pro-IL-1β and nAChR subunits α9 and α10 (supplemental Fig. S8A–C). The mRNA of nAChR subunit α7 is detectable in U937 cells, but expression levels are too low for quantification by real-time RT-PCR (19). We demonstrated before that addition of mecamylamine, α-bungarotoxin, and strychnine did not induce the release of IL-1β in the absence of BzATP (19). Similarly, α-conotoxins, ArIB[V11L,V16D] and RgIA4, neither induced nor impaired BzATP-induced mRNA levels or the release of IL-β from LPS-primed U937 cells (supplemental Fig. S8A, Fig. 5D). None of the nicotinic

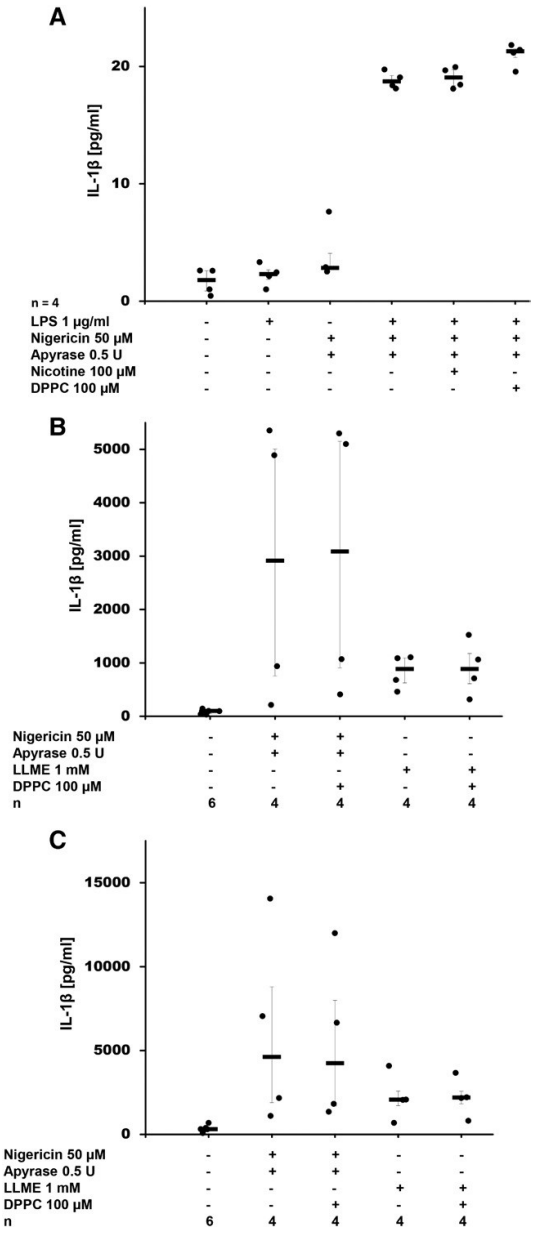

Fig. 4. DPPC does not inhibit nigericin- or LLME-induced release of IL-1β. A: DPPC was added to LPS-primed U937 cells together with nigericin. IL-1β levels were measured 30 min after application of nigericin in cell culture supernatants. Nicotine was included as a known inhibitor of BzATP-dependent IL-1β release. B, C: Human PBMCs were isolated from healthy human donors, left untreated (B) or pulsed with LPS (C), cultured for 3 h and stimulated with nigericin or LLME in the presence or absence of DPPC. IL-1β levels were measured 30 min thereafter in cell culture supernatants. Apyrase was included (A–C) to hydrolyze ATP that might have been released by U937 cells. A Kruskal-Wallis test was followed by Mann-Whitney rank-sum test; data are presented as individual data points; bars represent median; whiskers represent percentiles 25 and 75.

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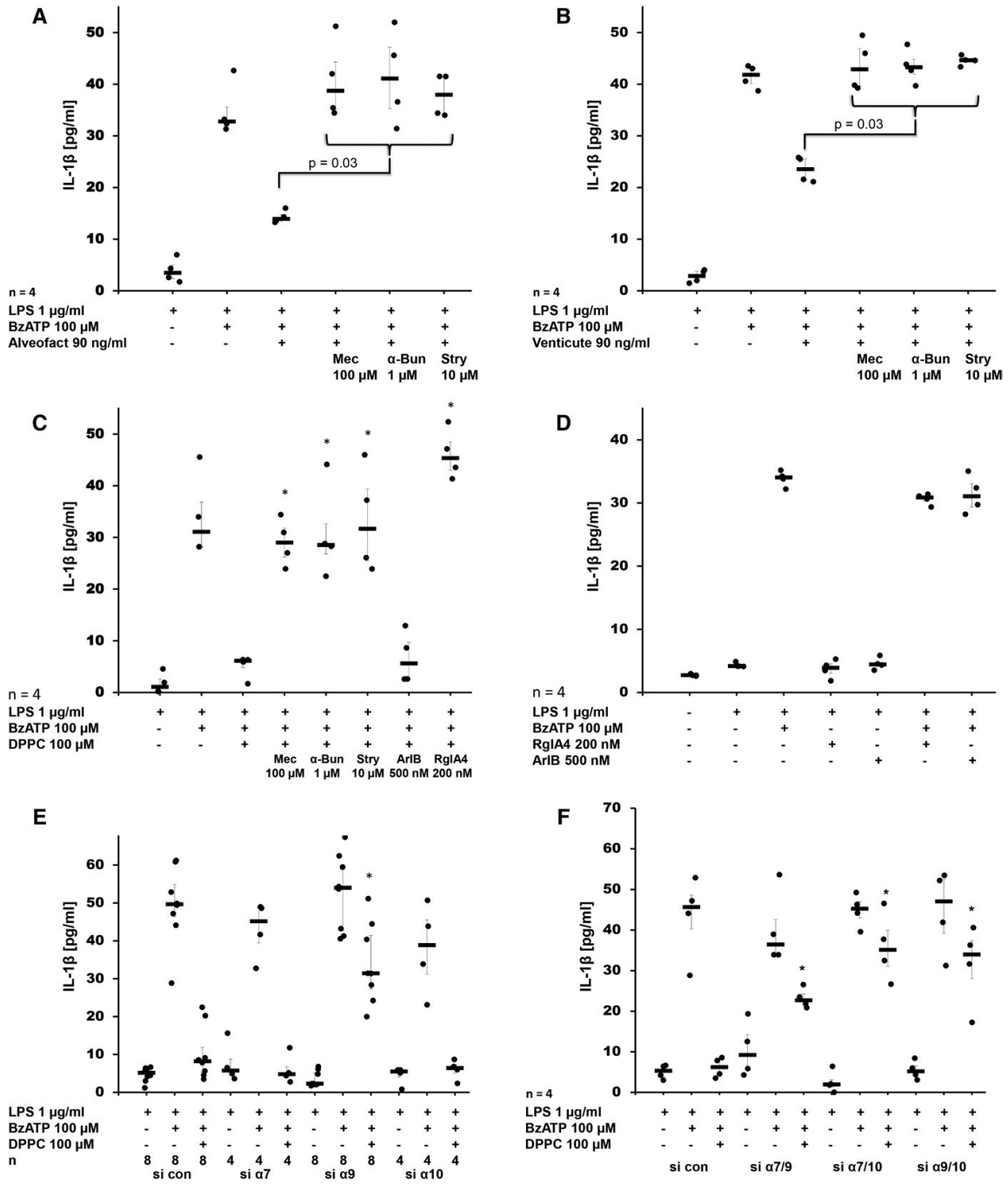

Fig. 5. The inhibitory effect of surfactant and DPPC on the BzATP-mediated release of IL-1β by LPS-primed U937 cells is mediated by nAChR subunits α7, α9, and α10. Natural surfactant Alveofact® (A), synthetic surfactant Venticute® (B), or DPPC (C) were added to LPS-primed U937 cells together with BzATP in the presence and absence of the nicotinic antagonists mecamylamine (Mec), α-bungarotoxin (α-Bun), strychnine (Stry), RgIA4, or ArIB[V11L,V16D] (ArIB). IL-1β levels were measured 30 min thereafter in cell culture supernatants. In LPS-primed U937 cells that were transfected with control siRNA (si con) the BzATP-stimulated IL-1β release was inhibited by DPPC Conotoxins RgIA4 or ArIB did neither induce the release of IL-1beta by LPS-primed U937 cells nor impair the effects of BzATP. (E, F). E: After transfection of siRNA to nAChR subunit α9, the effect of DPPC was blunted, whereas silencing of subunit α7 and α10 or the use of control siRNA (si con) did not provoke any effect. F: When double knock-down experiments were performed, the inhibitory effect of DPPC

antagonists caused a significant increase in LDH concentration in cell culture supernatants (supplemental Fig. S9A–C)

To further elucidate the role of nAChR subunits α7, α9, and α10 in signal transduction of DPPC, we used siRNA technology to silence the expression of each individual gene separately. The siRNA-mediated downregulation of the target gene mRNA of subunits α9 and α10 was shown by real-time RT-PCR, whereas the mRNA expression of subunit α7 was too low for quantification (supplemental Fig. S10A–D). It is, however, not yet possible to investigate nAChR expression on the protein level because no specific antibodies are available (37, 38). Silencing of α7 or α10 alone, as well as transfection of U937 cells with irrelevant control siRNA, did not impair the inhibitory effect of DPPC on BzATP-induced release of IL-1β (Fig. 5E). Only treatment of U937 cells with siRNA to the α9 nAChR subunit blunted the effect of DPPC ($P = 0.03$, n = 4). Next, we performed double knock-down experiments (α7/α9, α7/α10, and α9/α10) in U937 cells. Silencing of each gene combination blunted the response to DPPC ($P = 0.03$, n = 4, each) (Fig. 5F). Transfection of U937 cells with siRNA caused the release of variable amounts of LDH into the cell culture medium, but no differences were seen between control siRNA and siRNA targeting nAChR subunits (supplemental Fig. S9D, E).

DPPC inhibits BzATP-induced ion currents

Patch-clamp experiments were performed in LPS-primed U937 cells to investigate the effect of DPPC on BzATP-induced ion currents in LPS-primed U937 cells. Application of BzATP reproducibly induced ion currents (**Fig. 6A**), which were fully inhibited in the presence of 100 μM DPPC (Fig. 6B, C).

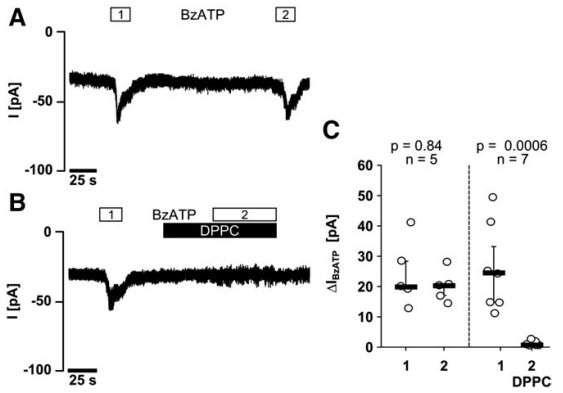

Fig. 6. DPPC inhibits BzATP-induced ion currents in LPS-primed U937 cells. Whole cell patch-clamp recordings of U937 cells are depicted in (A, B), and changes in ion currents in response to BzATP (ΔI_{BzATP}) are summarized in (C). Two consecutive BzATP applications (1 and 2) resulted in repeatable ion current changes that were abolished in the presence of DPPC (100 μM). Data are presented as individual data points; bars indicate median; whiskers represent percentiles 25 and 75.

DISCUSSION

The results of this study confirm our hypothesis that natural and artificial pulmonary surfactants inhibit ATP-mediated release of IL-1β by human monocytic cells. We investigated all constituents of artificial surfactant separately and identified DPPC as the active substance. Furthermore, we provide evidence that DPPC acts via noncanonical nAChRs.

DPPC is the main lipid constituent of natural and synthetic surfactant (24, 25). It is a prototypical phosphatidylcholine, a class of zwitterionic phospholipids that incorporate choline as a head group, glycerophosphoric acid, and a variety of fatty acids. Apart from DPPC, natural surfactant also contains high concentrations of unsaturated phosphatidylcholines (24, 25), which might also inhibit IL-1β release and deserve further investigation. Phosphatidylcholines are important constituents of biomembranes, but are also found in lining fluids of inner surfaces, such as the intestinal mucus (39, 40).

Patients suffering from acute lung injury or ARDS typically have reduced surfactant DPPC levels (41), together with increased activation of the NLRP3-inflammasome and increased levels of inflammasome-dependent cytokines, such as IL-1β and IL-18 (8, 10, 11). Animal studies suggest that NLRP3-inflammasome activation is further enhanced by mechanical damage caused by artificial ventilation (42). DPPC and other phosphatidylcholines have been shown before to inhibit the response of human macrophage cell lines to various pro-inflammatory stimuli (28, 30, 31, 43) and they also seem to play a protective role in diverse inflammatory diseases (44–47). These effects were shown to be, at least in part, due to incorporation of phosphatidylcholine into the cell membrane and to alterations of the functionally critical localization of TLR4 in lipid rafts (30, 31, 43, 47).

In this study, we provide evidence that DPPC triggers a different mechanism of action in human monocytic cells, which closely resembles a cholinergic mechanism recently discovered by our group (19, 23). DPPC, much like PC and conventional nicotinic agonists (19), inhibited ATP-dependent activation of IL-1β release, but DPPC was ineffective when the activation of the NLRP3 inflammasome was triggered by the pore-forming toxin, nigericin. We demonstrated before that nicotine and PC completely inhibit BzATP-induced ion channel functions at ATP receptor, P2X7, in human monocytic cells (19, 23). Our experiments revealed that DPPC also efficiently inhibits BzATP-induced ion currents and, consequently, inflammasome assembly, activation of caspase-1, and maturation of IL-1β. In contrast to DPPC, other related lipids devoid of a PC group did

was impaired in all siRNA combinations investigated (α7/9, α7/10, α9/10). A Kruskal-Wallis test was followed by a Mann-Whitney rank-sum test; data are presented as individual data points; bars represent median; whiskers represent percentiles 25 and 75; $*P \geq 0.03$ compared with respective experiments performed on cells treated with si con.

not impair IL-1β release, suggesting that the efficiency of DPPC depends on the presence of the PC head group.

The inhibitory effect of DPPC on ATP-mediated IL-1β release by U937 cells was sensitive to the nicotinic antagonists, mecamylamine, α-bungarotoxin, and strychnine, suggesting that one or more members of the evolutionarily conserved nAChR family comprising subunits α7, α9, and α10 are involved (48). α-Conotoxin RgIA4, which is a specific antagonist of human α9-containing nAChR, also reverted the effect of DPPC, whereas the α7-specific α-conotoxin, ArIB[V11L,V16D], was ineffective. These results prompted us to silence nAChR expression by siRNA technology. When subunits α7, α9, and α10 were silenced individually, only silencing of α9 blunted the effect of DPPC. In contrast, whenever two of these subunits were silenced concomitantly, the inhibitory effect of DPPC was impaired. We conclude from the pharmacological and the siRNA data that nAChR subunit α9 is essential for signaling of DPPC, whereas subunits α7 and α10 seem to be functionally interchangeable.

The functional combination of nAChR subunits α7 and α9 is unexpected, as subunits α7 and α9 are known to form homopentamers (49). In addition, α9 forms heteropentamers together with α10, a subunit that requires additional subunits to form a functional receptor (50, 51). Rather exotic combinations of α7 and α10 have also been described (52), but no functional combination of subunits α7 and α9. In monocytic cells and in leukocytes in general, nAChRs do not function as ligand-gated ion channels, but exert different metabotropic functions (19–22, 53, 54). Accordingly, DPPC also did not provoke ion current changes in U937 cells.

The structure of these noncanonical nAChRs is still enigmatic and we do not know if they form multimers at all. In contrast to classic nicotinic agonists, PC does not provoke ion channel functions at conventional nAChRs (19, 23). Considering the structural similarity between the head group of DPPC and PC, we speculate that DPPC also exclusively activates metabotropic functions at nAChRs expressed by immune cells, but does not activate canonical ionotropic functions. However, the physicochemical properties of the larger amphiphilic DPPC are strikingly distinct from PC and from classical agonists of nAChRs.

U937 cells, like every cell line, do not necessarily represent the properties of the cells from which they were derived. Therefore, we investigated freshly isolated PBMCs from healthy nonsmoking male donors. In these cells, the process of cell isolation and cell culturing was sufficient to induce the expression of pro-IL-1β and IL-1β was released in response to BzATP, as described previously (19, 55). As expected, treatment of these cells with LPS further increased IL-1β levels. In these primary cells, DPPC efficiently inhibited BzATP-induced inflammasome activation and maturation of caspase-1 and IL-1β, but not IL-1β secretion, as mature IL-1β was not detected in lysates of PBMCs that were stimulated with BzATP in the presence of DPPC.

DPPC or other phosphatidylcholines might be effective for the treatment of inflammatory diseases involving ATP-induced inflammasome activation. Numerous preclinical and clinical studies have investigated the use of various surfactant formulations in the context of ARDS. As the results were promising, but overall very diverse (56), this treatment never reached the clinical arena. Recently, however, a surfactant preparation rich in DPPC was highly protective in pediatric ARDS patients when combined with an alveolar recruitment maneuver (57). Hence, the use of surfactant containing high concentrations of DPPC deserves further investigation in a subpopulation of ARDS patients that are exposed to additional mechanical lung damage that results in ATP-dependent inflammasome activation. In addition, phosphatidylcholines were very efficient in a multi-center study for the treatment of ulcerative colitis, an inflammatory gut disease (58). We speculate that beneficial effects of phosphatidylcholines are at least in part due to the anti-inflammatory mechanism discovered in this study.

In conclusion, we describe a novel anti-inflammatory function of DPPC, the main lipid constituent of pulmonary surfactant that efficiently inhibits ATP receptor signaling and, hence, ATP-mediated release of IL-1β by monocytic cells. We discovered a novel anti-inflammatory mechanism of surfactant and suggest that DPPC might be effective for the treatment of various inflammatory diseases. In addition, we describe for the first time that the phosphatidylcholine, DPPC, functions as an agonist of noncanonical nAChRs expressed by immune cells.

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REFERENCES

1. Hiemstra, P. S. 2007. The role of epithelial beta-defensins and cathelicidins in host defense of the lung. *Exp. Lung Res.* **33:** 537–542.
2. Alkhouri, H., W. J. Poppinga, N. P. Tania, A. Ammit, and M. Schuliga. 2014. Regulation of pulmonary inflammation by mesenchymal cells. *Pulm. Pharmacol. Ther.* **29:** 156–165.
3. Bhattacharya, J., and K. Westphalen. 2016. Macrophage-epithelial interactions in pulmonary alveoli. *Semin. Immunopathol.* **38:** 461–469.
4. Robb, C. T., K. H. Regan, D. A. Dorward, and A. G. Rossi. 2016. Key mechanisms governing resolution of lung inflammation. *Semin. Immunopathol.* **38:** 425–448.
5. Chen, G. Y., and G. Nuñez. 2010. Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* **10:** 826–837.
6. Dinarello, C. A., A. Simon, and J. W. van der Meer. 2012. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat. Rev. Drug Discov.* **11:** 633–652.
7. Vladimer, G. I., R. Marty-Roix, S. Ghosh, D. Weng, and E. Lien. 2013. Inflammasomes and host defenses against bacterial infections. *Curr. Opin. Microbiol.* **16:** 23–31.
8. Meduri, G. U., S. Headley, G. Kohler, F. Stentz, E. Tolley, R. Umberger, and K. Leeper. 1995. Persistent elevation of inflammatory cytokines predicts a poor outcome in ARDS. Plasma IL-1 beta and IL-6 levels are consistent and efficient predictors of outcome over time. *Chest.* **107:** 1062–1073.
9. Ganter, M. T., J. Roux, B. Miyazawa, M. Howard, J. A. Frank, G. Su, D. Sheppard, S. M. Violette, P. H. Weinreb, G. S. Horan, et al. 2008. Interleukin-1beta causes acute lung injury via alphavbeta5 and alphavbeta6 integrin-dependent mechanisms. *Circ. Res.* **102:** 804–812.
10. Dolinay, T., Y. S. Kim, J. Howrylak, G. M. Hunninghake, C. H. An, L. Fredenburgh, A. F. Massaro, A. Rogers, L. Gazourian, K.

Nakahira, et al. 2012. Inflammasome-regulated cytokines are critical mediators of acute lung injury. *Am. J. Respir. Crit. Care Med.* **185:** 1225–1234.
11. Grailer, J. J., B. A. Canning, M. Kalbitz, M. D. Haggadone, R. M. Dhond, A. V. Andjelkovic, F. S. Zetoune, and P. A. Ward. 2014. Critical role for the NLRP3 inflammasome during acute lung injury. *J. Immunol.* **192:** 5974–5983.
12. Luna-Gomes, T., P. T. Santana, and R. Coutinho-Silva. 2015. Silica-induced inflammasome activation in macrophages: role of ATP and P2X7 receptor. *Immunobiology.* **220:** 1101–1106.
13. Borthwick, L. A. 2016. The IL-1 cytokine family and its role in inflammation and fibrosis in the lung. *Semin. Immunopathol.* **38:** 517–534.
14. Lee, S., G. Y. Suh, S. W. Ryter, and A. M. Choi. 2016. Regulation and function of the nucleotide binding domain leucine-rich repeat-containing receptor, pyrin domain-containing-3 inflammasome in lung disease. *Am. J. Respir. Cell Mol. Biol.* **54:** 151–160.
15. Gross, O., C. J. Thomas, G. Guarda, and J. Tschopp. 2011. The inflammasome: an integrated view. *Immunol. Rev.* **243:** 136–151.
16. Rathinam, V. A., S. K. Vanaja, and K. A. Fitzgerald. 2012. Regulation of inflammasome signaling. *Nat. Immunol.* **13:** 333–342.
17. Schaefer, L. 2014. Complexity of danger: the diverse nature of damage-associated molecular patterns. *J. Biol. Chem.* **289:** 35237–35245.
18. Cekic, C., and J. Linden. 2016. Purinergic regulation of the immune system. *Nat. Rev. Immunol.* **16:** 177–192.
19. Hecker, A., M. Küllmar, S. Wilker, K. Richter, A. Zakrzewicz, S. Atanasova, V. Mathes, T. Timm, S. Lerner, J. Klein, et al. 2015. Phosphocholine-modified macromolecules and canonical nicotinic agonists inhibit ATP-induced IL-1β release. *J. Immunol.* **195:** 2325–2334.
20. Hecker, A., K. S. Lips, U. Pfeil, A. Zakrzewicz, S. Wilker, W. Padberg, I. Wessler, W. Kummer, and V. Grau. 2009. Pivotal Advance: up-regulation of acetylcholine synthesis and paracrine cholinergic signaling in intravascular transplant leukocytes during rejection of rat renal allografts. *J. Leukoc. Biol.* **86:** 13–22.
21. Mikulski, Z., P. Hartmann, G. Jositsch, Z. Zasłona, K. S. Lips, U. Pfeil, H. Kurzen, J. Lohmeyer, W. G. Clauss, V. Grau, et al. 2010. Nicotinic receptors on rat alveolar macrophages dampen ATP-induced increase in cytosolic calcium concentration. *Respir. Res.* **11:** 133.
22. Kawashima, K., T. Fujii, Y. Moriwaki, H. Misawa, and K. Horiguchi. 2015. Non-neuronal cholinergic system in regulation of immune function with a focus on α7 nAChRs. *Int. Immunopharmacol.* **29:** 127–134.
23. Richter, K., V. Mathes, M. Fronius, M. Althaus, A. Hecker, G. Krasteva-Christ, W. Padberg, A. J. Hone, J. M. McIntosh, A. Zakrzewicz, et al. 2016. Phosphocholine – an agonist of metabotropic but not of ionotropic functions of α9-containing nicotinic acetylcholine receptors. *Sci. Rep.* **6:** 28660.
24. Lopez-Rodriguez, E., and J. Pérez-Gil. 2014. Structure-function relationships in pulmonary surfactant membranes: from biophysics to therapy. *Biochim. Biophys. Acta.* **1838:** 1568–1585.
25. Lang, C. J., A. D. Postle, S. Orgeig, F. Possmayer, W. Bernhard, A. K. Panda, K. D. Jürgens, W. K. Milsom, K. Nag, and C. B. Daniels. 2005. Dipalmitoylphosphatidylcholine is not the major surfactant phospholipid species in all mammals. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **289:** R1426–R1439.
26. Chroneos, Z. C., Z. Sever-Chroneos, and V. L. Shepherd. 2010. Pulmonary surfactant: an immunological perspective. *Cell. Physiol. Biochem.* **25:** 13–26.
27. Glasser, S. W., T. L. Witt, A. P. Senft, J. E. Baatz, D. Folger, M. D. Maxfield, H. T. Akinbi, D. A. Newton, D. R. Prows, and T. R. Korfhagen. 2009. Surfactant protein C-deficient mice are susceptible to respiratory syncytial virus infection. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **297:** L64–L72.
28. Gille, C., B. Spring, W. Bernhard, C. Gebhard, D. Basile, K. Lauber, C. F. Poets, and T. W. Orlikowsky. 2007. Differential effect of surfactant and its saturated phosphatidylcholines on human blood macrophages. *J. Lipid Res.* **48:** 307–317.
29. Kuronuma, K., H. Mitsuzawa, K. Takeda, C. Nishitani, E. D. Chan, Y. Kuroki, M. Nakamura, and D. R. Voelker. 2009. Anionic pulmonary surfactant phospholipids inhibit inflammatory responses from alveolar macrophages and U937 cells by binding the lipopolysaccharide-interacting proteins CD14 and MD-2. *J. Biol. Chem.* **284:** 25488–25500.
30. Abate, W., A. A. Alghaithy, J. Parton, K. P. Jones, and S. K. Jackson. 2010. Surfactant lipids regulate LPS-induced interleukin-8 production in A549 lung epithelial cells by inhibiting translocation of TLR4 into lipid raft domains. *J. Lipid Res.* **51:** 334–344.
31. Tonks, A., J. Parton, A. J. Tonks, R. H. Morris, A. Finall, K. P. Jones, and S. K. Jackson. 2005. Surfactant phospholipid DPPC downregulates monocyte respiratory burst via modulation of PKC. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **288:** L1070–L1080.
32. Morris, R. H., A. J. Tonks, K. P. Jones, M. K. Ahluwalia, A. W. Thomas, A. Tonks, and S. K. Jackson. 2008. DPPC regulates COX-2 expression in monocytes via phosphorylation of CREB. *Biochem. Biophys. Res. Commun.* **370:** 174–178.
33. Spragg, R. G., J. F. Lewis, W. Wurst, D. Häfner, R. P. Baughman, M. D. Wewers, and J. J. Marsh. 2003. Treatment of acute respiratory distress syndrome with recombinant surfactant protein C surfactant. *Am. J. Respir. Crit. Care Med.* **167:** 1562–1566.
34. McIntosh, J. M., inventor. Conotoxin peptides, pharmaceutical compositions and uses thereof. Patent WO2014194284A1. December 4, 2014. (Google Patents, 2014). https://www.google.com/patents/WO2014194284A1?cl=en.
35. Whiteaker, P., S. Christensen, D. Yoshikami, C. Dowell, M. Watkins, J. Gulyas, J. Rivier, B. M. Olivera, and J. M. McIntosh. 2007. Discovery, synthesis, and structure activity of a highly selective alpha7 nicotinic acetylcholine receptor antagonist. *Biochemistry.* **46:** 6628–6638.
36. Innocent, N., P. D. Livingstone, A. Hone, A. Kimura, T. Young, P. Whiteaker, J. M. McIntosh, and S. Wonnacott. 2008. Alpha-conotoxin arenatus IB[V11L,V16D] [corrected] is a potent and selective antagonist at rat and human native alpha7 nicotinic acetylcholine receptors. *J. Pharmacol. Exp. Ther.* **327:** 529–537.
37. Moser, N., N. Mechawar, I. Jones, A. Gochberg-Sarver, A. Orr-Urtreger, M. Plomann, R. Salas, B. Molles, L. Marubio, U. Roth, et al. 2007. Evaluating the suitability of nicotinic acetylcholine receptor antibodies for standard immunodetection procedures. *J. Neurochem.* **102:** 479–492.
38. Rommel, F. R., B. Raghavan, R. Paddenberg, W. Kummer, S. Tumala, G. Lochnit, U. Gieler, and E. M. Peters. 2015. Suitability of nicotinic acetylcholine receptor α7 and muscarinic acetylcholine receptor 3 antibodies for immune detection: evaluation in murine skin. *J. Histochem. Cytochem.* **63:** 329–339.
39. Kao, Y. C., and L. M. Lichtenberger. 1991. Phospholipid- and neutral lipid-containing organelles of rat gastroduodenal mucous cells. Possible origin of the hydrophobic mucosal lining. *Gastroenterology.* **101:** 7–21.
40. Lichtenberger, L. M. 1995. The hydrophobic barrier properties of gastrointestinal mucus. *Annu. Rev. Physiol.* **57:** 565–583.
41. Dushianthan, A., R. Cusack, V. Goss, A. D. Postle, and M. P. Grocott. 2012. Clinical review: exogenous surfactant therapy for acute lung injury/acute respiratory distress syndrome–where do we go from here? *Crit. Care.* **16:** 238.
42. Jones, H. D., T. R. Crother, R. A. Gonzalez-Villalobos, M. Jupelli, S. Chen, J. Dagvadorj, M. Arditi, and K. Shimada. 2014. The NLRP3 inflammasome is required for the development of hypoxemia in LPS/mechanical ventilation acute lung injury. *Am. J. Respir. Cell Mol. Biol.* **50:** 270–280.
43. Treede, I., A. Braun, R. Sparla, M. Kühnel, T. Giese, J. R. Turner, E. Anes, H. Kulaksiz, J. Füllekrug, W. Stremmel, et al. 2007. Anti-inflammatory effects of phosphatidylcholine. *J. Biol. Chem.* **282:** 27155–27164.
44. Erös, G., S. Ibrahim, N. Siebert, M. Boros, and B. Vollmar. 2009. Oral phosphatidylcholine pretreatment alleviates the signs of experimental rheumatoid arthritis. *Arthritis Res. Ther.* **11:** R43.
45. Schneider, H., A. Braun, J. Füllekrug, W. Stremmel, and R. Ehehalt. 2010. Lipid based therapy for ulcerative colitis—modulation of intestinal mucus membrane phospholipids as a tool to influence inflammation. *Int. J. Mol. Sci.* **11:** 4149–4164.
46. Rajaie, S., and A. Esmaillzadeh. 2011. Dietary choline and betaine intakes and risk of cardiovascular diseases: review of epidemiological evidence. *ARYA Atheroscler.* **7:** 78–86.
47. Stremmel, W., R. Ehehalt, S. Staffer, S. Stoffels, A. Mohr, M. Karner, and A. Braun. 2012. Mucosal protection by phosphatidylcholine. *Dig. Dis.* **30 (Suppl. 3):** 85–91.
48. Baker, E. R., R. Zwart, E. Sher, and N. S. Millar. 2004. Pharmacological properties of alpha 9 alpha 10 nicotinic acetylcholine receptors revealed by heterologous expression of subunit chimeras. *Mol. Pharmacol.* **65:** 453–460.
49. Elgoyhen, A. B., D. S. Johnson, J. Boulter, D. E. Vetter, and S. F. Heinemann. 1994. Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell.* **79:** 705–715.

50. Elgoyhen, A. B., D. E. Vetter, E. Katz, C. V. Rothlin, S. F. Heinemann, and J. Boulter. 2001. Alpha10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proc. Natl. Acad. Sci. USA.* **98:** 3501–3506.
51. Vincler, M., S. Wittenauer, R. Parker, M. Ellison, B. M. Olivera, and J. M. McIntosh. 2006. Molecular mechanism for analgesia involving specific antagonism of alpha9alpha10 nicotinic acetylcholine receptors. *Proc. Natl. Acad. Sci. USA.* **103:** 17880–17884.
52. Lips, K. S., P. König, K. Schätzle, U. Pfeil, G. Krasteva, M. Spies, R. V. Haberberger, S. A. Grando, and W. Kummer. 2006. Coexpression and spatial association of nicotinic acetylcholine receptor subunits alpha7 and alpha10 in rat sympathetic neurons. *J. Mol. Neurosci.* **30:** 15–16.
53. Peng, H., R. L. Ferris, T. Matthews, H. Hiel, A. Lopez-Albaitero, and L. R. Lustig. 2004. Characterization of the human nicotinic acetylcholine receptor subunit alpha (α) 9 (CHRNA9) and alpha (α) 10 (CHRNA10) in lymphocytes. *Life Sci.* **76:** 263–280.
54. Razani-Boroujerdi, S., R. T. Boyd, M. I. Dávila-García, J. S. Nandi, N. C. Mishra, S. P. Singh, J. C. Pena-Philippides, R. Langley, and M. L. Sopori. 2007. T cells express alpha7-nicotinic acetylcholine receptor subunits that require a functional TCR and leukocyte-specific protein tyrosine kinase for nicotine-induced Ca2+ response. *J. Immunol.* **179:** 2889–2898.
55. Grahames, C. B., A. D. Michel, I. P. Chessell, and P. P. Humphrey. 1999. Pharmacological characterization of ATP- and LPS-induced IL-1beta release in human monocytes. *Br. J. Pharmacol.* **127:** 1915–1921.
56. Davidson, W. J., D. Dorscheid, R. Spragg, M. Schulzer, E. Mak, and N. T. Ayas. 2006. Exogenous pulmonary surfactant for the treatment of adult patients with acute respiratory distress syndrome: results of a meta-analysis. *Crit. Care.* **10:** R41.
57. Rodríguez-Moya, V. S., C. M. Gallo-Borrero, D. Santos-Áreas, I. A. Prince-Martínez, E. Díaz-Casañas, and J. López-Herce Cid. Exogenous surfactant and alveolar recruitment in the treatment of the acute respiratory distress syndrome. *Clin. Respir. J.* Epub ahead of print. February 16, 2016; doi:10.1111/crj.12462.
58. Karner, M., A. Kocjan, J. Stein, S. Schreiber, G. von Boyen, P. Uebel, C. Schmidt, L. Kupcinskas, I. Dina, F. Zuelch, et al. 2014. First multicenter study of modified release phosphatidylcholine "LT-02" in ulcerative colitis: a randomized, placebo-controlled trial in mesalazine-refractory courses. *Am. J. Gastroenterol.* **109:** 1041–1051.

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Phosphocholine-Modified Lipooligosaccharides of *Haemophilus influenzae* Inhibit ATP-Induced IL-1β Release by Pulmonary Epithelial Cells

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Abstract: Phosphocholine-modified bacterial cell wall components are virulence factors enabling immune evasion and permanent colonization of the mammalian host, by mechanisms that are poorly understood. Recently, we demonstrated that free phosphocholine (PC) and PC-modified lipooligosaccharides (PC-LOS) from *Haemophilus influenzae*, an opportunistic pathogen of the upper and lower airways, function as unconventional nicotinic agonists and efficiently inhibit the ATP-induced release of monocytic IL-1β. We hypothesize that *H. influenzae* PC-LOS exert similar effects on pulmonary epithelial cells and on the complex lung tissue. The human lung carcinoma-derived epithelial cell lines A549 and Calu-3 were primed with lipopolysaccharide from *Escherichia coli* followed by stimulation with ATP in the presence or absence of PC or PC-LOS or LOS devoid of PC. The involvement of nicotinic acetylcholine receptors was tested using specific antagonists. We demonstrate that PC and PC-LOS efficiently inhibit ATP-mediated IL-1β release by A549 and Calu-3 cells via nicotinic acetylcholine receptors containing subunits α7, α9, and/or α10. Primed precision-cut lung slices behaved similarly. We conclude that *H. influenzae* hijacked an endogenous anti-inflammatory cholinergic control mechanism of the lung to evade innate immune responses of the host. These findings may pave the way towards a host-centered antibiotic treatment of chronic airway infections with *H. influenzae*.

Keywords: A549; Calu-3; CHRNA7; CHRNA9; CHRNA10; immune evasion; inflammasome; lung; nicotinic acetylcholine receptor; phosphocholine-modification

1. Introduction

Strains of *Haemophilus influenzae* can be divided into two categories: the encapsulated, typeable strains and the genetically highly variable non-encapsulated, non-typeable strains (NTHi) [1,2]. A well-described virulence factor of most wild-type NTHi is the *lic1* operon that encodes enzymes needed for the synthesis of phosphocholine-modified lipooligosaccharides (PC-LOS) [3–9]. *H. influenzae* strains carrying a non-functional mutant *lic1*-operon are rapidly cleared from the airways of experimental mice and rats, whereas PC-LOS-positive wild-type strains are selected and can cause severe pulmonary infections [6,10]. Accordingly, most human respiratory *H. influenzae* infections are due to strains with a functional *lic1*-operon [8]. Like *H. influenzae*, numerous Gram-negative and Gram-positive bacteria as well as eukaryotic parasites produce PC-modified cell surfaces or secretory products that are generally regarded as immunomodulatory mediators enabling pathogen survival [7,8].

PC-LOS from wild-type *H. influenzae* strains is at best a weak inducer of costimulatory molecules CD40 and CD58 as well as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) mRNA in human monocytic THP-1 cells, whereas PC-free LOS from a *lic1*-mutant is as efficient as lipopolysaccharide (LPS) from *Escherichia coli* [11]. It is, however, unclear if PC-LOS only weakly activates Toll-like receptor 4 or if other mechanisms are involved that control the expression and release of pro-inflammatory cytokines including IL-1β. A better understanding of immune evasion strategies is needed for the development of novel anti-infective therapies to treat *H. influenzae* infections.

IL-1β is a highly potent pro-inflammatory cytokine of innate immunity that plays an essential role in host defense against infections [12,13]. As excessive systemic IL-1β levels can cause fever, shock and multiple organ failure, including acute respiratory distress syndrome [13–15], a tight regulation of its release is vital. The production of IL-1β often requires two consecutive so-called "danger signals" [13,16,17]. The pathogen-associated molecular pattern LPS is a typical first signal inducing the biosynthesis of cytoplasmic pro-IL-1β, an inactive cytoplasmic pro-form of IL-1β. Extracellular ATP is an indicator of severe cellular damage and a prototypical second danger signal that initiates ion currents at P2X7 receptors, including efflux of potassium ions. Reduced cytoplasmic potassium levels leads to the assembly of the NACHT, LRR and PYD domains-containing protein 3 (NLRP3)-containing inflammasome and to caspase-1 activation [13,16,17]. Caspase-1 enables the rapid maturation and release of cytokines of the IL-1 family including IL-1β [13,16,17].

We recently reported that agonists of nicotinic acetylcholine receptors (nAChRs) containing subunits α7, α9 and/or α10 efficiently inhibit the ATP-induced release of IL-1β by human monocytic cells [18–20]. Apart from classical nicotinic agonists such as acetylcholine (ACh), choline or nicotine, free PC and PC-LOS from bacterial walls of wild-type *H. influenzae* function as unconventional nAChR agonists that also inhibit the ATP-mediated IL-1β release [18–20]. In contrast, PC-free LOS isolated from *H. influenzae lic1*-mutants is ineffective [18]. Interestingly, the covalent binding of PC to LOS enhances the efficiency of PC by a factor of about 400 [18].

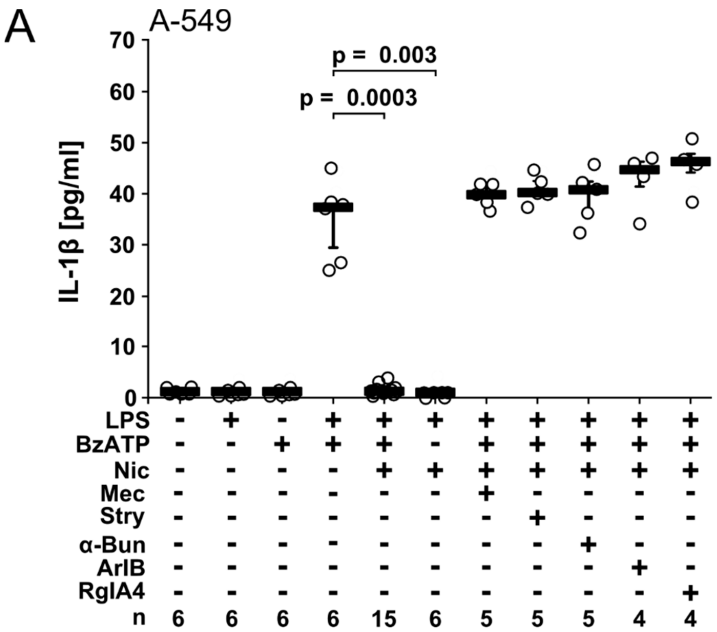
Up to now, we investigated the inhibition of ATP-induced IL-1β by nicotine, PC and PC-LOS in human monocytic blood cells [18]. In addition to mononuclear phagocytes of the lung, respiratory and alveolar epithelial cells contribute to host defense against respiratory infections by NLRP3 inflammasome activation and IL-1β release [15,21–26]. As those epithelial cells are among the first cells that come into contact with pathogens, they are expected to be of particular importance during the early phase of infection. Here, we confirm our hypothesis that nicotine, PC and PC-LOS inhibit the ATP-induced release of IL-β by human cancer cell lines A549, resembling alveolar epithelial cells type II [27,28], and Calu-3 cells, an established model for bronchial epithelial cells [29]. PC-LOS is also active in mouse lung tissue. Moreover, we provide evidence that nicotine, PC and PC-LOS signal via epithelial nAChR subunits α7 and α9, while PC-free LOS isolated from *lic*-mutants is inactive.

2. Results

2.1. Nicotine and PC Inhibit IL-1β Release from A549 Cells via nAChRs

We reported before that nicotine and PC inhibit the ATP-induced IL-1β release by human monocytic cells via nAChRs containing subunits α7, α9 and α10 [18–20]. To test whether this applies to lung epithelial cells, we primed A549 cells with LPS from *E. coli* (100 ng/mL) for 24 h followed by stimulation with the P2X7 receptor agonist 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate trieethylammonium salt (BzATP, 100 μM) for another 30 min. IL-1β released into the cell culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA) (Figure 1A,B). The concentration of IL-1β in the cell culture supernatant ranged between 25 and 50 pg/mL. When either priming with LPS or stimulation with BzATP was omitted, virtually no IL-1β was detected (Figure 1A,B). Nicotine (100 μM; $p = 0.000$, $n = 15$; Figure 1A) and PC (100 μM, $p = 0.0001$, $n = 15$; Figure 1B) fully inhibited the BzATP-induced release of IL-1β from LPS-primed A549 cells. In control experiments, in which nicotine or PC were added to LPS-primed cells in the absence of BzATP, virtually no IL-1β was released (Figure 1A,B). Cell viability as estimated by the measurement of the cytoplasmic enzyme lactate dehydrogenase (LDH) in cell culture supernatants was unimpaired in these and in all following experiments.

To analyze if nicotine and PC signal via nAChRs in A549 cells, a panel of different nAChR antagonists was used: (1) mecamylamine (100 μM), a general nAChR antagonist, (2) α-bungarotoxin (1 μM), an antagonist targeting nAChRs containing subunits α7 or α9, (3) strychnine (10 μM) that preferentially antagonizes nAChRs containing subunit α9, (4) α-conotoxin [V11L, V16D]ArIB (500 nM), a specific antagonist of nAChR α7, and 5) α-conotoxin RgIA4 (200 nM) that antagonizes nAChRs containing subunits α9/α10 [19,30–34]. When applied together with BzATP, all nAChR antagonists abolished the inhibitory effect of nicotine and PC and enabled the full release of IL-1β (Figure 1A,B). In control experiments, where BzATP was omitted, none of the nAChR antagonists induced the release of IL-1β by LPS-primed A549 cells (data not shown). We conclude from these data that nicotine and PC are efficient inhibitors of BzATP-induced IL-1β release by pulmonary epithelial cells, and that nAChR subunits α7, α9 and/or α10 are involved in signaling of nicotine and PC.



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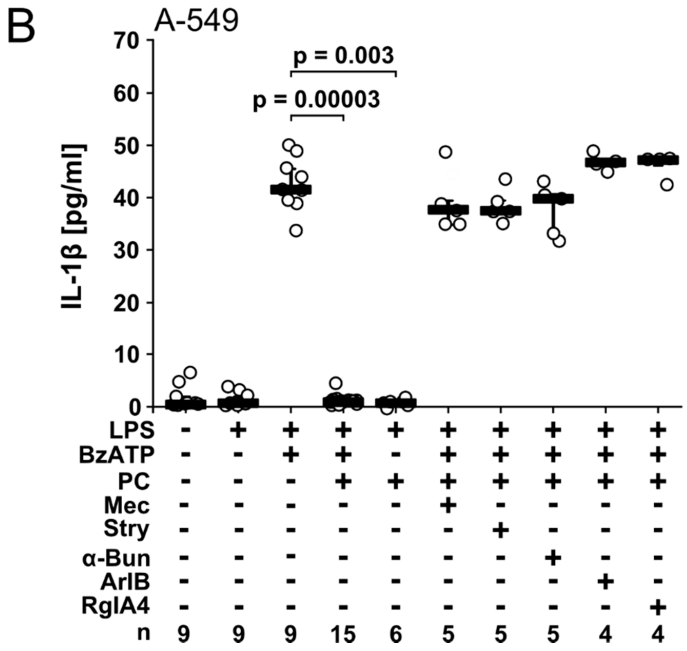

Figure 1. Nicotine (Nic) and phosphocholine (PC) inhibit the release of IL-1β by A549 cells. Human LPS-primed A549 cells were stimulated with 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP, 100 μM) in the presence or absence of Nic (100 μM) (**A**) or PC (100 μM) (**B**) and the IL-1β released to the supernatant was measured 30 min later. The inhibitory effects of Nic and PC were sensitive to nicotinic antagonists mecamylamine (Mec; 100 μM), strychnine (Stry; 10 μM), α-bungarotoxin (α-Bun; 1 μM), [V11L, V16D]ArIB (500 nM), or RgIA4 (200 nM). Data are presented as individual data points, bars represent median, whiskers encompass the 25th to 75th percentile; n-numbers of independent experiments are indicated in the figure. Experimental groups were compared by Kruskal Wallis test followed by Mann Whitney rank sum test.

2.2. Nicotine and PC Inhibit IL-1β Release from Calu-3 Cells via nAChRs

In the next set of experiments, we demonstrated that Calu-3 cells essentially reacted like A549 cells (Figure 2). Application of BzATP (100 μM) to LPS-primed Calu-3 cells resulted in the release of IL-1β into the cell culture supernatant with concentrations ranging between 28 and 58 pg/mL (Figure 2) that was fully inhibited in the presence of nicotine (100 μM; $p = 0.029$, $n = 4$; Figure 2) or PC (100 μM, $p = 0.029$, $n = 4$; Figure 2). These inhibitory effects were fully reversed by addition of [V11L, V16D] ArIB (500 nM) or RgIA4 (200 nM; Figure 2), suggesting that also in Calu-3 cells nAChR subunits α7, α9 and/or α10 are involved in signaling of nicotine and PC [18].

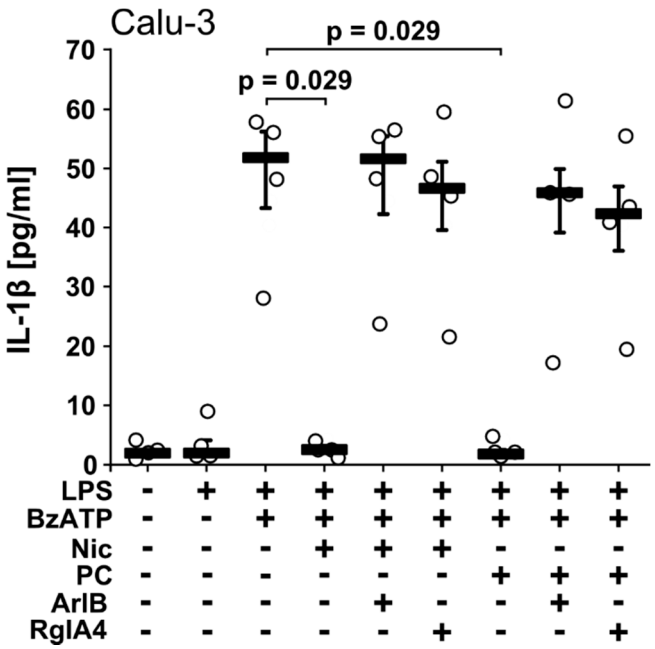

Figure 2. Nicotine (Nic) and phosphocholine (PC) inhibit the release of IL-1β by Calu-3 cells. Human LPS-primed Calu-3 cells were stimulated with BzATP (100 μM) in the presence or absence of Nic (100 μM) or PC (100 μM). [V11L, V16D]ArIB (500 nM) or RgIA4 (200 nM) was added together with BzATP and IL-1β released to the supernatant was measured after 30 min. In one of the experiments, IL-1β levels released in response to BzATP was low throughout. Data are presented as individual data points, bars represent median, whiskers encompass the 25th to 75th percentile, $n = 4$ per experiment. Experimental groups were compared by Kruskal Wallis test followed by Mann Whitney rank sum test.

2.3. PC-LOS Inhibit BzATP-Mediated IL-1β Release from A549 and Calu-3 Cells

We showed before that nicotine, PC and PC-LOS inhibit the ATP-induced release of IL-1β from monocytic cells via nAChR subunits α7 and α9, while PC-free LOS isolated from *lic1*-mutants is inactive [18]. Here, we tested the effects of PC-LOS from two independent *H. influenzae* strains, RM118 and NTHi123323 in the epithelial cell lines A549 and Calu-3. PC-LOS (1 μg/mL) purified from both strains efficiently inhibited the BzATP-induced release of IL-1β from LPS-primed A549 and Calu-3 cells (Figure 3A,B). The concentration of PC-LOS used in this study has been determined before [18]. In contrast, PC-free LOS from the corresponding mutant strains lacking the PC-modification, RM7004-*lic1* and NTHi1233-*lic1* [4], was ineffective (Figure 3A,B). We selected A549 cells to test the hypothesis that PC-LOS signals via the same nAChR subunits like free PC. The inhibitory effects of PC-LOS were sensitive to mecamylamine (100 μM), α-bungarotoxin (1 μM), strychnine (10 μM), [V11L, V16D]ArIB (500 nM), and RgIA4 (200 nM) (Figure 3C), suggesting that PC-LOS signals via nAChR subunits α7, α9 and/or α10 similar to free PC.

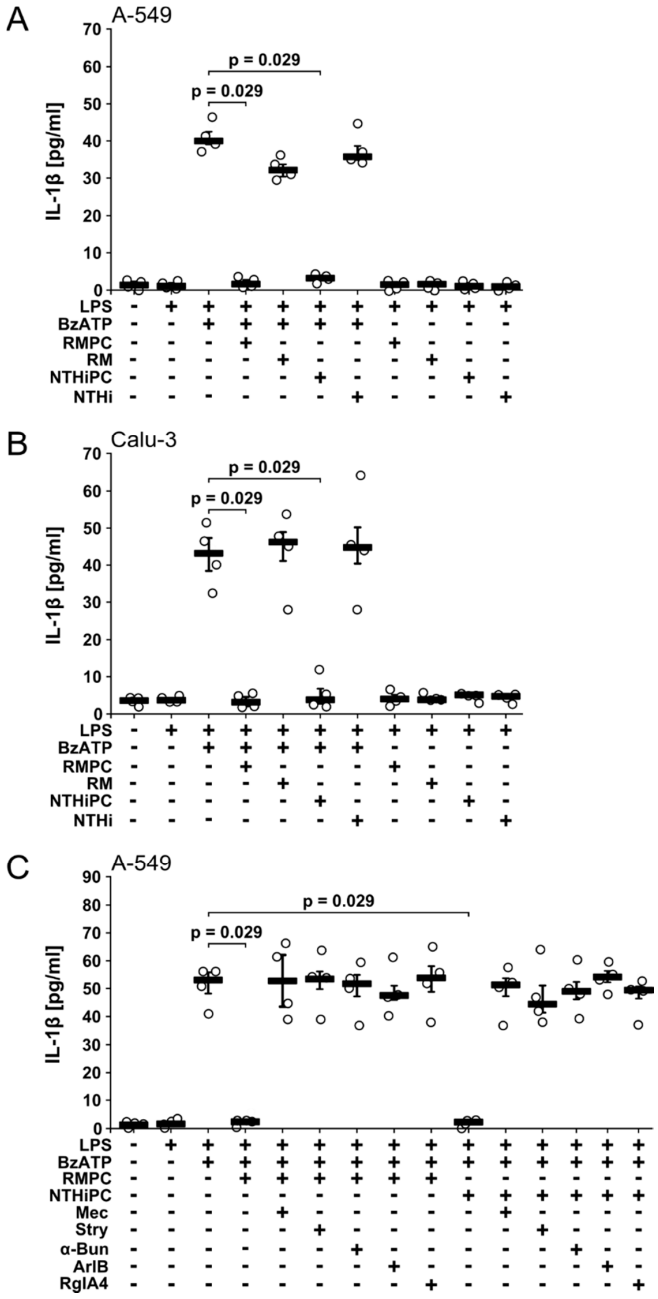

Figure 3. Phosphocholine-modified lipooligosaccharides (PC-LOS) isolated from *H. influenzae* strains inhibit the release of IL-1β by A549 and Calu-3 cells. Human LPS-primed A549 (**A**,**C**) or Calu-3 (**B**) cells were stimulated with BzATP (100 μM) in the presence or absence of PC-LOS isolated from the *H. influenzae* strains RM118 (RMPC; 1 μg/mL) and NTHi 1233 (NTHiPC; 1 μg/mL) and IL-1β released to the supernatant was measured after 30 min. PC-free LOS from the corresponding *lic1*-mutant strains RM7004-*lic1* (RM; 1 μg/mL) and NTHi1233-*lic1* (NTHi; 1 μg/mL) lacking the PC-modification were included as a negative control. (**C**) The inhibitory effects of RMPC and NTHiPC were reversed by nicotinic antagonists mecamylamine (Mec; 100 μM)), strychnine (Stry; 10 μM), α-bungarotoxin (α-Bun; 1 μM), [V11L, V16D]ArIB (500 nM), or RgIA4 (200 nM). Data are presented as individual data points, bars represent median, whiskers encompass the 25th to 75th percentile, $n = 4$ per experiment. Experimental groups were compared by Kruskal Wallis test followed by Mann Whitney rank sum test.

2.4. PC-LOS Inhibit the BzATP-Mediated IL-1β Release from Mouse Precision Cut Lung Slices (PCLS)

To test, if the inhibitory potential of PC-LOS on the BzATP-induced release of IL-1β also applies to lung tissue, we investigated PCLS from healthy wild-type mice, an established ex vivo model to investigate pulmonary inflammation [35]. PCLS were cultured for 48 h. The lung tissue retained normal morphology (Figure 4A) and viability as estimated by the release of LDH (Figure 4B).

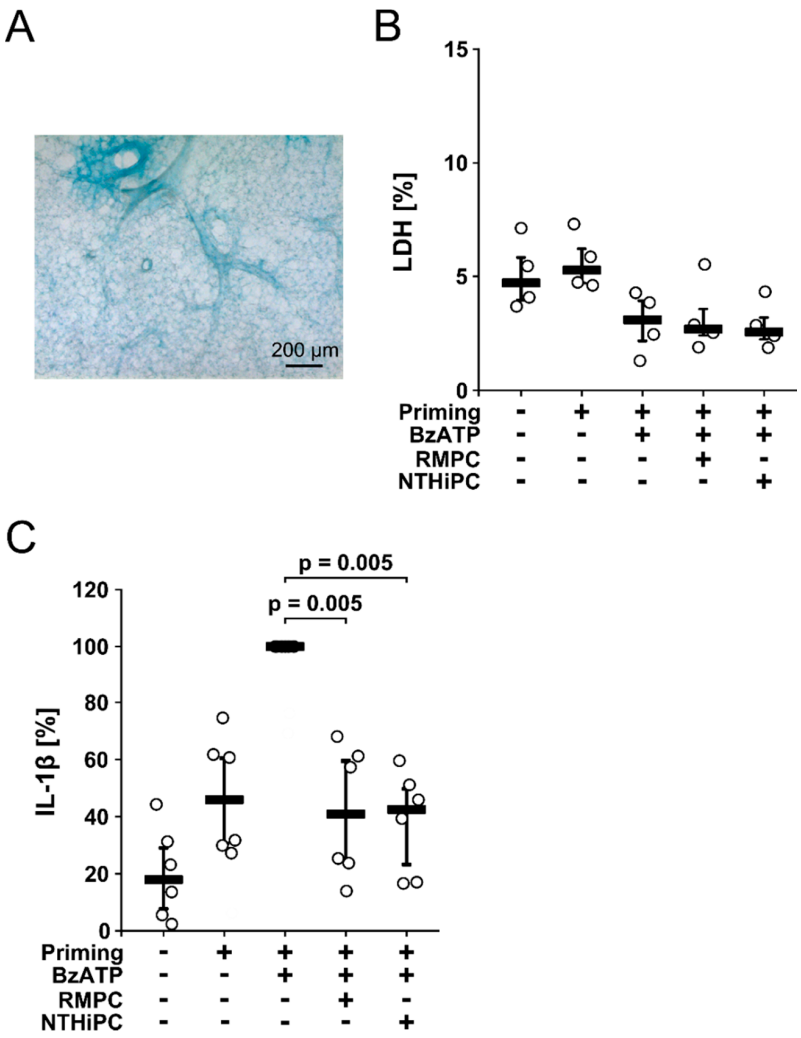

Figure 4. Phosphocholine-modified lipooligosaccharides (PC-LOS) isolated from *H. influenzae* strains inhibit the release of IL-1β by wild-type mouse precision cut lung slices (PCLS, $n = 6$). (**A**) A fixed PCLS, lightly stained with Richardson's stain depicts normal pulmonary morphology; (**B**,**C**) PCLS were primed with LPS (100 ng/mL), IFN-γ (20 ng/mL) and TNF-α (10 ng/mL) for 24 h followed by application of BzATP (150 µM) in the presence or absence of PC-LOS isolated from the *H. influenzae* strains RM118 (RMPC; 1 µg/mL) and NTHi 1233 (NTHiPC; 1 µg/mL); (**B**) Lactate dehydrogenase (LDH) was measured in the cell culture supernatant 30 min later and expressed as % of the total LDH content of the individual PCLS ($n = 4$). Due to a technical problem, LDH release was not measured in two out of six experiments; (**C**) IL-1β was measured in cell culture supernatant at the same time point as the LDH ($n = 6$). The IL-1β concentration in experiments where primed PCLS were stimulated with BzATP was set to 100% and all other values were calculated accordingly. (**B**,**C**) Data are presented as individual data points, bars represent median, whiskers encompass the 25th to 75th percentile. Experimental groups were compared by the Wilcoxon sighed-rank test.

Thereafter, PCLS were primed with LPS (100 ng/mL), interferon-γ (IFN-γ; 20 ng/mL) and TNF-α (10 ng/mL) for 24 h and BzATP (100 μM) was added to the tissue culture supernatant for 30 min in the presence or absence of PC-LOS (1 μg/mL) purified from *H. influenzae* strains RM118 or NTHi123323. During priming, PCLS released IL-1β into the tissue culture supernatant (4–28 pg/mg, $n = 6$; Figure 4B), which was further increased by addition of BzATP (13–46 pg/mg, $n = 6$; Figure 4B). PC-LOS fully inhibited the BzATP-induced IL-1β release (RM118: 3–28 pg/mg, $p = 0.005$, $n = 6$; NTHi123323: 2–27 pg/mg, $p = 0.005$, $n = 6$; Figure 4B) suggesting that the inhibitory potential of PC-LOS also applies to the complex lung tissue.

3. Discussion

We demonstrate here that the ATP-induced release of IL-1β by the pulmonary epithelial cell lines A549 and Calu-3 is efficiently inhibited by nicotine, PC and PC-LOS isolated from wild-type *H. influenzae* bacterial cell walls, whereas PC-free LOS isolated from mutant stains is inactive. We further show that PC-LOS exerts similar effects in PCLS, an ex vivo model that is suited to investigate aspects of pulmonary inflammation [35–37]. In addition, we are the first to demonstrate that PC and PC-LOS function as unconventional nAChR agonists signaling via nAChR subunits α7, α9 and/or α10 in pulmonary epithelial cells. We suggest that this mechanism contributes to the known PC-LOS-mediated immune evasion of *H. influenzae* [6–9].

Several mechanisms have been suggested to be involved in the positive selection of PC-LOS-exposing *H. influenzae* strains on epithelial surfaces, where NTHi strains are predominantly found. These mechanisms include molecular mimicry, increased adherence of PC-LOS-exposing bacteria to respiratory epithelia by binding to the receptor of the platelet activating factor (PAF) [38,39] as well as PC-LOS-dependent biofilm formation that facilitates host colonization [40–42] and protection from the attack of host proteases [43].

The dominance of the PC-LOS-carrying NTHi variants in vivo is surprising in the light of known host effector mechanisms targeting PC-LOS. The acute-phase reactant C-reactive protein (CRP) binds to cell surface-exposed PC-LOS, and is expected to fix complement and to result in pathogen elimination [10,44–46]. In addition, high titers of natural antibodies to PC are ubiquitously found in humans [47,48]. They should also enable a quick eradication of PC-LOS exposing *H. influenzae* [49–52]. These anti-bacterial attack mechanisms, however, are expected to be predominantly active in the blood plasma, where *H. influenzae* strains lacking the PC-decoration on their cell wall might have a better chance of survival [10]. In contrast, PC-LOS-positive strains seem to have a survival benefit in the respiratory tract, the source of most PC-LOS-positive *H. influenzae* isolates from infected patients [8]. Accordingly, frequent spontaneous phase variations have been observed regarding the on-off switching in the exposition of PC-LOS on the bacterial surface [53], a mechanism that might enable a quick adaptation of *H. influenzae* to its environment within the host. These considerations prompted us to focus on respiratory and pulmonary epithelial cells.

Epithelial cells including lung epithelial cells form the first line of mechanical and immunological defense against infections. In response to numerous microbial noxes and sterile cell damage leading to the release of cytoplasm, inflammasomes assemble and exert vital functions regarding the preservation of the epithelial integrity and in the coordination of the immune response [15,21,24–26]. In vivo analyses and ex vivo experiments on intact tissues are difficult to interpret regarding the origin of released mediators, because they contain a multitude of different cell types including leukocytes. We used A549 cells resembling alveolar epithelial cells type II [27,28], and the bronchial epithelial cell line Calu-3 [29] to ascertain the purity of the epithelial cells. The capacity A549 cells to form NLRP3 inflammasomes and to secrete mature IL-1β has been shown before [54,55] and we demonstrate in this study that Calu-3 cells are also able to secrete IL-1β. These data must be interpreted with caution, because cell lines never fully reflect the properties of primary cells. In future experiments, A549 cells, Calu-3, or primary lung epithelial cells should be investigated in air liquid interface cultures that more closely mimic many of the features of the native polarized airway epithelium.

To approach reality, we investigated PCLS, thin slices of the highly complex lung tissue. In this setting, the cellular source of IL-1β released by PCLS is unclear because the lung tissue is composed of numerous cell types including monocytes and macrophages. However, PC-LOS was able to fully inhibit the IL-1β release caused by stimulation with BzATP, which is in line with our hypothesis. PCLS are widely used to study the lung ex vivo, to reduce the number of experiments on living animals (e.g., [35,56,57]). This approach is not without controversy because PCLS have large wounded surfaces and they are neither ventilated nor perfused with blood. However, this also applies to parts of severely damaged lungs and other studies have shown that PCLS are a good model for innate immunity of the inflamed lung [35–37]. More research using living experimental animals and living *H. influenzae* strains are certainly needed to confirm these initial results.

Immune evasion strategies are the result of million years of co-evolution of microorganisms with their respective hosts and frequently, endogenous anti-inflammatory mechanisms are hijacked by well adapted pathogens [58,59]. Recently, we reported that dipalmitoyl-phosphatidylcholine, the most abundant lipid present in the pulmonary surfactant, efficiently inhibits inflammasome assembly and release of IL-1β by a mechanism that resembles that of PC-LOS [60]. A similar pathway is induced by the pulmonary anti-protease alpha1-antitrypsin [37]. The content of pulmonary dipalmitoyl-phosphatidylcholine is, however, reduced upon severe acute lung injury or inflammation [61] and alpha1-antitrypsin is consumed by proteases that are mainly released by activated neutrophils [62]. Hence, local inflammasome assembly and IL-1β release should be enabled in damaged lungs to allow efficient host defense. We speculate that PC-LOS of wild-type *H. influenzae* subverts the immune response of the host by preventing ATP-induced inflammasome assembly despite of a local lack in phosphatidylcholines and alpha1-antitrypsin.

The effect of PC-LOS is sensitive to nAChR antagonists targeting subunits α7, α9 and α10 that form an evolutionary conserved family of nAChR subunits [63]. Human bronchial epithelial cells express nAChR subunits α7 and α9 and respond with ion currents to nicotine or choline, known agonists of nAChRs [64]. We demonstrated before for human monocytic cells, that nicotine and free PC inhibit the BzATP-induced release of IL-1β via nAChR subunits α7, α9 and α10 [18,19]. Also, PC-LOS seems to signal via the same nAChR family. The data presented in this study are not unequivocal regarding the involvement of nAChR subunits α9 and/or α10. RgIA4 is an antagonist of nAChRs containing subunits α9 and/or α10 but cannot differentiate between both subunits [34]. However, as we demonstrated before, that nAChR subunits α7, α9 and α10 are needed for the PC-mediated metabotropic inhibition of monocytic P2X7 receptors [18–20] this might also hold true for pulmonary epithelial cells.

People carrying *H. influenzae* in their respiratory tract usually do not exhibit clinical signs of disease. Reduced mucociliary clearance, however, causes the propagation of *H. influenzae* resulting in otitis media, pharyngitis, bronchitis and pneumonia [65–70]. In patients suffering from chronic obstructive pulmonary disease (COPD), 50% of all exacerbations are caused by bacterial pathogens; among them, *H. influenzae* is most frequently isolated [70,71]. Treatment of COPD exacerbations includes antibiotics, corticosteroids and bronchodilators. Earl and colleagues showed that these regimens promote the persistence of *H. influenzae* and antibiotic resistance [72]. First antibiotic resistances to ampicillin and amoxicillin were described for *H. influenzae* as early as 1972. Later on, co-trimoxazole, combination of trimethoprim and sulfamethoxazole, became ineffective in the treatment of NTHi [73,74].

The solution to this problem could be a host centered anti-infective therapy beyond the classical antibiotic regimens that is less prone to the development of resistances. More precisely, we suggest that a short-term treatment of *H. influenzae*-colonized patients with antagonists of nAChRs might enable a vigorous inflammasome-dependent immune reaction and eradication of the infection.

In conclusion, we demonstrate that the ATP-induced release of IL-1β by pulmonary epithelial cells and by lung tissue (PCLS) is efficiently inhibited by PC-LOS from wild-type *H. influenzae* strains. PC-LOS signaling via archaic non-neuronal nAChRs seems to contribute to the immune evasion of *H. influenzae* and might be a promising therapeutic target.

4. Materials and Methods

4.1. Reagents

Mecamylamine hydrochloride, (−)-nicotine hydrogen tartrate salt (N5260), phosphocholine chloride calcium salt tetrahydrate, strychnine hydrochloride, and LPS (L2654 from *E. coli*) were obtained from Sigma-Aldrich (Taufkirchen, Germany). BzATP was provided by Jena Bioscience (Jena, Germany) and α-bungarotoxin by Tocris Bioscience (Bristol, UK). [V11L, V16D]ArIB and RgIA4 were produced and characterized as described previously [32–34]. Concentrations of all compounds used in this study were optimized in previous experiments on human monocytic U937 cells [18,19].

4.2. Purification and Characterization of PC-LOS

PC-LOS and PC-free LOS were isolated from various *H. influenzae* strains as described before [75]. In short, LOS was extracted from bacteria using a phenol/chloroform/light-petroleum method and further purified by ultracentrifugation. The structure of LOS was analyzed by high-field NMR and ESI-MS techniques. In addition, composition and linkage analyses were performed on *O*-deacylated LOS and oligosaccharide samples.

4.3. Pulmonary Epithelial Cell Lines

All cells were cultivated in a standard incubator at 37 °C, 5% CO_2. The adherent human epithelial lung carcinoma cell line Calu-3 was obtained from the American Type Culture Collection (ATTC®, Manassas, VA, USA) and cultivated in ATCC-formulated Eagle's MEM (No. 30-2003) supplemented with 10% fetal bovine serum (FBS, S-EUR30-I, Cell Concepts, Umkirch, Germany). A549 cells, adherent epithelial cells derived from a lung tumor, were provided by the Leibniz-Institute DSMZ (Braunschweig, Germany) and cultivated in Dulbecco's MEM with GlutaMAX™ (Gibco/Life Technologies, Carlsbad, CA, USA) containing 10% FBS. Epithelial cells were seeded at a density of 1×10^5 cell in 1 ml medium in 12 well cell culture plates, cultivated for 24 h, followed by another 24 h stimulation with LPS (100 ng/mL). Epithelial cell cultures reached about 50% confluency. BzATP (100 µM) was added to the LPS-primed cells in the presence of different concentrations of nicotine, PC, PC-LOS or PC-free LOS. Thirty min later, cell-free cell culture supernatant was harvested and stored at −20 °C until measurement of IL-1β and LDH.

4.4. Mouse PCLS

Specified pathogen-free mice were obtained via Janvier Labs, Le Genest-Saint-Isle, France and were housed in our animal facility for about 2 weeks under a 12 h light/dark cycle and access to standard chow and water ad libitum. Experimental animals received humane care according to NIH "Guide for the Care and Use of Laboratory Animals". The protocol was registered and approved by the local authorities (Regierungspräsidium Giessen, Germany; reference no. 571_M). Mouse PCLS were prepared using a modified protocol described previously [56,57,76]. Briefly, lungs were taken from 8 to 12 weeks old female C57BL/6NRj mice. The animals were sacrificed after anesthesia with isoflurane (5%); (Abbott, Wiesbaden, Germany) by cervical dislocation. The airways were filled via the cannulated trachea with 1.5% low melting agarose (Bio-Rad Medical Diagnostics, Dreieich, Germany) dissolved in HEPES-Ringer buffer. Lungs were removed and transferred to ice-cold HEPES-Ringer buffer to solidify the agarose. The lung lobes were cut into 350 µm thick slices using a vibratome (VT10000S, Leica, Bensheim, Germany). Slices were incubated in HEPES-Ringer buffer, supplemented with penicillin (100 U/mL, PAA, Etobicoke, Canada) and streptomycin (0.1 mg/mL, PAA) for at least 1.5 h at 37 °C to remove the agarose.

PCLS were washed once and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 with L-glutamine and without HEPES (No. DF-042-B; Merck, Darmstadt, Germany) supplemented with 100 U/mL Pen Strep (Gibco) at 37 °C, 5% CO_2, and 100% air humidity in 12-well tissue culture plates, using 2 slices per well. After 24 h of incubation, PCLS were treated with LPS (100 ng/mL),

recombinant human IFN-γ (20 ng/mL; R&D Systems, Minneapolis, MN, USA) and recombinant human TNF-α (10 ng/mL; R&D Systems) for additional 24 h. As a control PCLS were left untreated for the same period of time. The functional integrity of PCLS produced according to the same protocol in the same laboratory was published recently [76]. Some of the untreated PCLS were fixed for 24 h in 4% paraformaldehyde (Carl Roth, Karlsruhe, Germany) and lightly stained with Richardson's blue as described before [77]. Thereafter, PCLS were stimulated for 30 min with BzATP (150 μM) in presence and absence of RMPC (1 μg/mL) or NTHiPC (1 μg/mL). Tissue culture supernatants were harvested and stored at −20 °C until measurement of IL-1β and LDH

4.5. Cytokine Measurement

IL-1β was measured in cell-free cell culture supernatants by the human IL-1 beta/IL-1F2 DuoSet®ELISA (R&D Systems, Minneapolis, MN, USA) or mouse Quantikine®IL-1β Immunoassay (R&D Systems) according to the instructions of the manufacturer. IL-1β values of PCLS supernatants were normalized to the total protein content of the tissue slices that was assessed using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

4.6. LDH Measurement

LDH was measured in cell culture supernatants by the Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. The proportion of dead cells was estimated by including a maximum LDH release control. For this purpose, an equivalent number of A459 or Calu-3 cells were lyzed by two cycles of freezing (−80 °C) and thawing. The amount of LDH in this control sample was set to 100% and the relative cell death in all other experiments was calculated accordingly.

4.7. Statistical Analyses

Data were analyzed with the IBM SPSS Statistics software Version 25 (IBM, Munich, Germany). Values derived from different cells were compared, where applicable, by the non-parametric Kruskal-Wallis test, followed by the Mann-Whitney rank-sum test. The Wilcoxon signed-rank test was used for analyses of dependent values. Data were visualized using program Inkscape version 0.92 (Free and Open Source Software licensed under the GPL). The number (n) of individual experiments is indicated in the Results section and the Figures.

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Conflicts of Interest: [V11L, V16D]ArIB and RgIA4 have been patented by the University of Utah. J.M.M. is an inventor on these patents. The other authors declare no conflict of interest.

References

1. King, P.T.; Sharma, R. The lung immune response to nontypeable *Haemophilus influenzae* (lung immunity to NTHi). *J. Immunol. Res.* **2015**, *2015*, 706376. [CrossRef] [PubMed]
2. Duell, B.L.; Su, Y.-C.; Riesbeck, K. Host-pathogen interactions of nontypeable *Haemophilus influenzae*: From commensal to pathogen. *FEBS Lett.* **2016**, *590*, 3840–3853. [CrossRef] [PubMed]

3. Risberg, A.; Masoud, H.; Martin, A.; Richards, J.C.; Moxon, E.R.; Schweda, E.K.H. Structural analysis of the lipopolysaccharide oligosaccharide epitopes expressed by a capsule-deficient strain of *Haemophilus influenzae* Rd. *Eur. J. Biochem.* **1999**, *261*, 171–180. [CrossRef] [PubMed]
4. Schweda, E.K.H.; Brisson, J.-R.; Alvelius, G.; Martin, A.; Weiser, J.N.; Hood, D.W.; Moxon, E.R.; Richards, J.C. Characterization of the phosphocholine-substituted oligosaccharide in lipopolysaccharides of type b *Haemophilus influenzae*. *Eur. J. Biochem.* **2000**, *267*, 3902–3913. [CrossRef] [PubMed]
5. Månsson, M.; Hood, D.W.; Moxon, E.R.; Schweda, E.K.H. Structural diversity in lipopolysaccharide expression in nontypeable *Haemophilus influenzae*. *Eur. J. Biochem.* **2003**, *270*, 610–624. [CrossRef] [PubMed]
6. Pang, B.; Winn, D.; Johnson, R.; Hong, W.; West-Barnette, S.; Kock, N.; Swords, W.E. Lipooligosaccharides containing phosphorylcholine delay pulmonary clearance of nontypeable *Haemophilus influenzae*. *Infect. Immun.* **2008**, *76*, 2037–2043. [CrossRef] [PubMed]
7. Grabitzki, J.; Lochnit, G. Immunomodulation by phosphocholine—Biosynthesis, structures and immunological implications of parasitic PC-epitopes. *Mol. Immunol.* **2009**, *47*, 149–163. [CrossRef] [PubMed]
8. Clark, S.E.; Weiser, J.N. Microbial modulation of host immunity with the small molecule phosphorylcholine. *Infect. Immun.* **2013**, *81*, 392–401. [CrossRef] [PubMed]
9. McSorley, H.J.; Hewitson, J.P.; Maizels, R.M. Immunomodulation by helminth parasites: Defining mechanisms and mediators. *Int. J. Parasitol.* **2013**, *43*, 301–310. [CrossRef] [PubMed]
10. Weiser, J.N.; Pan, N.; McGowan, K.L.; Musher, D.; Martin, A.; Richards, J. Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. *J. Exp. Med.* **1998**, *187*, 631–640. [CrossRef] [PubMed]
11. Choi, J.; Cox, A.D.; Li, J.; McCready, W.; Ulanova, M. Activation of innate immune responses by *Haemophilus influenzae* lipooligosaccharide. *Clin. Vaccine Immunol.* **2014**, *21*, 769–776. [CrossRef] [PubMed]
12. Vladimer, G.I.; Marty-Roix, R.; Ghosh, S.; Weng, D.; Lien, E. Inflammasomes and host defenses against bacterial infections. *Curr. Opin. Microbiol.* **2013**, *16*, 23–31. [CrossRef] [PubMed]
13. Dinarello, C.A. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol. Rev.* **2018**, *281*, 8–27. [CrossRef] [PubMed]
14. Dinarello, C.A.; Simon, A.; van der Meer, J.W.M. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat. Rev. Drug Discov.* **2012**, *11*, 633–652. [CrossRef] [PubMed]
15. Pinkerton, J.W.; Kim, R.Y.; Robertson, A.A.B.; Hirota, J.A.; Wood, L.G.; Knight, D.A.; Cooper, M.A.; O'Neill, L.A.J.; Horvat, J.C.; Hansbro, P.M. Inflammasomes in the lung. *Mol. Immunol.* **2017**, *86*, 44–55. [CrossRef] [PubMed]
16. Gross, O.; Thomas, C.J.; Guarda, G.; Tschopp, J. The inflammasome: An integrated view. *Immunol. Rev.* **2011**, *243*, 136–151. [CrossRef] [PubMed]
17. Lamkanfi, M.; Dixit, V.M. A new lead to NLRP3 inhibition. *J. Exp. Med.* **2017**, *214*, 3147–3149. [CrossRef] [PubMed]
18. Hecker, A.; Küllmar, M.; Wilker, S.; Richter, K.; Zakrzewicz, A.; Atanasova, S.; Mathes, V.; Timm, T.; Lerner, S.; Klein, J.; et al. Phosphocholine-modified macromolecules and canonical nicotinic agonists inhibit ATP-induced IL-1β release. *J. Immunol.* **2015**, *195*, 2325–2334. [CrossRef] [PubMed]
19. Richter, K.; Mathes, V.; Fronius, M.; Althaus, M.; Hecker, A.; Krasteva-Christ, G.; Padberg, W.; Hone, A.J.; McIntosh, J.M.; Zakrzewicz, A.; et al. Phosphocholine—An agonist of metabotropic but not of ionotropic functions of α9-containing nicotinic acetylcholine receptors. *Sci. Rep.* **2016**, *6*, 28660. [CrossRef] [PubMed]
20. Zakrzewicz, A.; Richter, K.; Agné, A.; Wilker, S.; Siebers, K.; Fink, B.; Krasteva-Christ, G.; Althaus, M.; Padberg, W.; Hone, A.J.; et al. Canonical and novel non-canonical cholinergic agonists inhibit ATP-induced release of monocytic interleukin-1β via different combinations of nicotinic acetylcholine receptor subunits α7, α9 and α10. *Front. Cell. Neurosci.* **2017**, *11*, 491. [CrossRef] [PubMed]
21. Message, S.D.; Johnston, S.L. Host defense function of the airway epithelium in health and disease: Clinical background. *J. Leukoc. Biol.* **2004**, *75*, 5–17. [CrossRef] [PubMed]
22. Brusselle, G.G.; Provoost, S.; Bracke, K.R.; Kuchmiy, A.; Lamkanfi, M. Inflammasomes in respiratory disease: From bench to bedside. *Chest* **2014**, *145*, 1121–1133. [CrossRef] [PubMed]
23. Peeters, P.M.; Wouters, E.F.; Reynaert, N.L. Immune homeostasis in epithelial cells: Evidence and role of inflammasome signaling reviewed. *J. Immunol. Res.* **2015**, *2015*, 828264. [CrossRef] [PubMed]

24. Kostadinova, E.; Chaput, C.; Gutbier, B.; Lippmann, J.; Sander, L.E.; Mitchell, T.J.; Suttorp, N.; Witzenrath, M.; Opitz, B. NLRP3 protects alveolar barrier integrity by an inflammasome-independent increase of epithelial cell adherence. *Sci. Rep.* **2016**, *6*, 30943. [CrossRef] [PubMed]
25. Santana, P.T.; Martel, J.; Lai, H.-C.; Perfettini, J.-L.; Kanellopoulos, J.M.; Young, J.D.; Coutinho-Silva, R.; Ojcius, D.M. Is the inflammasome relevant for epithelial cell function? *Microbes Infect.* **2016**, *18*, 93–101. [CrossRef] [PubMed]
26. Tian, X.; Sun, H.; Casbon, A.-J.; Lim, E.; Francis, K.P.; Hellman, J.; Prakash, A. NLRP3 inflammasome mediates dormant neutrophil recruitment following sterile lung injury and protects against subsequent bacterial pneumonia in mice. *Front. Immunol.* **2017**, *8*, 1337. [CrossRef] [PubMed]
27. Giard, D.J.; Aaronson, S.A.; Todaro, G.J.; Arnstein, P.; Kersey, J.H.; Dosik, H.; Parks, W.P. In vitro cultivation of human tumors: Establishment of cell lines derived from a series of solid tumors2. *J. Natl. Cancer Inst.* **1973**, *51*, 1417–1423. [CrossRef] [PubMed]
28. Smith, B.T. Cell line A549: A model system for the study of alveolar type II cell function. *Am. Rev. Respir. Dis.* **1977**, *115*, 285–293. [CrossRef] [PubMed]
29. Shen, B.Q.; Finkbeiner, W.E.; Wine, J.J.; Mrsny, R.J.; Widdicombe, J.H. Calu-3: A human airway epithelial cell line that shows cAMP-dependent Cl-secretion. *Am. J. Physiol.* **1994**, *266*, L493–L501. [CrossRef] [PubMed]
30. Orr-Urtreger, A.; Göldner, F.M.; Saeki, M.; Lorenzo, I.; Goldberg, L.; de Biasi, M.; Dani, J.A.; Patrick, J.W.; Beaudet, A.L. Mice deficient in the α7 neuronal nicotinic acetylcholine receptor lack α-bungarotoxin binding sites and hippocampal fast nicotinic currents. *J. Neurosci.* **1997**, *17*, 9165–9171. [CrossRef] [PubMed]
31. Baker, E.R.; Zwart, R.; Sher, E.; Millar, N.S. Pharmacological properties of α9α10 nicotinic acetylcholine receptors revealed by heterologous expression of subunit chimeras. *Mol. Pharmacol.* **2004**, *65*, 453–460. [CrossRef] [PubMed]
32. Whiteaker, P.; Christensen, S.; Yoshikami, D.; Dowell, C.; Watkins, M.; Gulyas, J.; Rivier, J.; Olivera, B.M.; McIntosh, J.M. Discovery, synthesis, and structure activity of a highly selective α7 nicotinic acetylcholine receptor antagonist. *Biochemistry* **2007**, *46*, 6628–6638. [CrossRef] [PubMed]
33. Innocent, N.; Livingstone, P.D.; Hone, A.; Kimura, A.; Young, T.; Whiteaker, P.; McIntosh, J.M.; Wonnacott, S. Alpha-conotoxin Arenatus IBV11L, V16D corrected is a potent and selective antagonist at rat and human native α7 nicotinic acetylcholine receptors. *J. Pharmacol. Exp. Ther.* **2008**, *327*, 529–537. [CrossRef] [PubMed]
34. Romero, H.K.; Christensen, S.B.; Di Cesare Mannelli, L.; Gajewiak, J.; Ramachandra, R.; Elmslie, K.S.; Vetter, D.E.; Ghelardini, C.; Iadonato, S.P.; Mercado, J.L.; et al. Inhibition of α9α10 nicotinic acetylcholine receptors prevents chemotherapy-induced neuropathic pain. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E1825–E1832. [CrossRef] [PubMed]
35. Switalla, S.; Lauenstein, L.; Prenzler, F.; Knothe, S.; Förster, C.; Fieguth, H.-G.; Pfennig, O.; Schaumann, F.; Martin, C.; Guzman, C.A.; et al. Natural innate cytokine response to immunomodulators and adjuvants in human precision-cut lung slices. *Toxicol. Appl. Pharmacol.* **2010**, *246*, 107–115. [CrossRef] [PubMed]
36. Henjakovic, M.; Sewald, K.; Switalla, S.; Kaiser, D.; Müller, M.; Veres, T.Z.; Martin, C.; Uhlig, S.; Krug, N.; Braun, A. Ex vivo testing of immune responses in precision-cut lung slices. *Toxicol. Appl. Pharmacol.* **2008**, *231*, 68–76. [CrossRef] [PubMed]
37. Siebers, K.; Fink, B.; Zakrzewicz, A.; Agné, A.; Richter, K.; Konzok, S.; Hecker, A.; Zukunft, S.; Küllmar, M.; Klein, J.; et al. Alpha-1 antitrypsin inhibits ATP-mediated release of interleukin-1β via CD36 and nicotinic acetylcholine receptors. *Front. Immunol.* **2018**, *9*, 633. [CrossRef] [PubMed]
38. Swords, W.E.; Ketterer, M.R.; Shao, J.; Campbell, C.A.; Weiser, J.N.; Apicella, M.A. Binding of the non-typeable *Haemophilus influenzae* lipooligosaccharide to the PAF receptor initiates host cell signalling. *Cell. Microbiol.* **2001**, *3*, 525–536. [CrossRef] [PubMed]
39. Branger, J.; Wieland, C.W.; Florquin, S.; Maris, N.A.; Pater, J.M.; Speelman, P.; Shimizu, T.; Ishii, S.; van der Poll, T. Plattelet-activating factor receptor-deficient mice show an unaltered clearance of nontypeable *Haemophilus influenzae* from their respiratory tract. *Shock* **2004**, *22*, 543–547. [CrossRef] [PubMed]
40. West-Barnette, S.; Rockel, A.; Swords, W.E. Biofilm growth increases phosphorylcholine content and decreases potency of nontypeable *Haemophilus influenzae* endotoxins. *Infect. Immun.* **2006**, *74*, 1828–1836. [CrossRef] [PubMed]
41. Swords, W.E. Nontypeable *Haemophilus influenzae* biofilms: Role in chronic airway infections. *Front. Cell. Infect. Microbiol.* **2012**, *2*, 97. [CrossRef] [PubMed]

42. Morey, P.; Viadas, C.; Euba, B.; Hood, D.W.; Barberán, M.; Gil, C.; Grilló, M.J.; Bengoechea, J.A.; Garmendia, J. Relative contributions of lipooligosaccharide inner and outer core modifications to nontypeable *Haemophilus influenzae* pathogenesis. *Infect. Immun.* **2013**, *81*, 4100–4111. [CrossRef] [PubMed]
43. Clark, S.E.; Snow, J.; Li, J.; Zola, T.A.; Weiser, J.N. Phosphorylcholine allows for evasion of bactericidal antibody by *Haemophilus influenzae*. *PLoS Pathog.* **2012**, *8*, e1002521. [CrossRef] [PubMed]
44. Humphries, H.E.; High, N.J. The role of licA phase variation in the pathogenesis of invasive disease by *Haemophilus influenzae* type b. *FEMS Immunol. Med. Microbiol.* **2002**, *34*, 221–230. [CrossRef] [PubMed]
45. Pepys, M.B.; Hirschfield, G.M. C-reactive protein: A critical update. *J. Clin. Investig.* **2003**, *111*, 1805–1812. [CrossRef] [PubMed]
46. Mantovani, A.; Garlanda, C.; Doni, A.; Bottazzi, B. Pentraxins in innate immunity: From C-reactive protein to the long pentraxin PTX3. *J. Clin. Immunol.* **2008**, *28*, 1–13. [CrossRef] [PubMed]
47. De Faire, U.; Frostegård, J. Natural antibodies against phosphorylcholine in cardiovascular disease. *Ann. N. Y. Acad. Sci.* **2009**, *1173*, 292–300. [CrossRef] [PubMed]
48. Frostegård, J. Low level natural antibodies against phosphorylcholine: A novel risk marker and potential mechanism in atherosclerosis and cardiovascular disease. *Clin. Immunol.* **2010**, *134*, 47–54. [CrossRef] [PubMed]
49. Scott, M.G.; Briles, D.E.; Shackelford, P.G.; Smith, D.S.; Nahm, M.H. Human antibodies to phosphocholine. IgG anti-PC antibodies express restricted numbers of V and C regions. *J. Immunol.* **1987**, *138*, 3325–3331. [PubMed]
50. Nishinarita, S.; Sawada, S.; Horie, T. Phosphorylcholine antibodies in pulmonary infection. *Med. Microbiol. Immunol.* **1990**, *179*, 205–214. [CrossRef] [PubMed]
51. Shaw, P.X.; Hörkkö, S.; Chang, M.K.; Curtiss, L.K.; Palinski, W.; Silverman, G.J.; Witztum, J.L. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J. Clin. Investig.* **2000**, *105*, 1731–1740. [CrossRef] [PubMed]
52. Fiskesund, R.; Steen, J.; Amara, K.; Murray, F.; Szwajda, A.; Liu, A.; Douagi, I.; Malmström, V.; Frostegård, J. Naturally occurring human phosphorylcholine antibodies are predominantly products of affinity-matured B cells in the adult. *J. Immunol.* **2014**, *192*, 4551–4559. [CrossRef] [PubMed]
53. Weiser, J.N.; Shchepetov, M.; Chong, S.T. Decoration of lipopolysaccharide with phosphorylcholine: A phase-variable characteristic of *Haemophilus influenzae*. *Infect. Immun.* **1997**, *65*, 943–950. [PubMed]
54. Liang, X.; Zhang, D.; Liu, W.; Yan, Y.; Zhou, F.; Wu, W.; Yan, Z. Reactive oxygen species trigger NF-κB-mediated NLRP3 inflammasome activation induced by zinc oxide nanoparticles in A549 cells. *Toxicol. Ind. Health* **2017**, *33*, 737–745. [CrossRef] [PubMed]
55. Zhang, Z.; Liang, Z.; Li, H.; Li, C.; Yang, Z.; Li, Y.; She, D.; Cao, L.; Wang, W.; Liu, C.; et al. Perfluorocarbon reduces cell damage from blast injury by inhibiting signal paths of NF-κB, MAPK and Bcl-2/Bax signaling pathway in A549 cells. *PLoS ONE* **2017**, *12*, e0173884. [CrossRef] [PubMed]
56. Martin, C.; Uhlig, S.; Ullrich, V. Videomicroscopy of methacholine-induced contraction of individual airways in precision-cut lung slices. *Eur. Respir. J.* **1996**, *9*, 2479–2487. [CrossRef] [PubMed]
57. Paddenberg, R.; König, P.; Faulhammer, P.; Goldenberg, A.; Pfeil, U.; Kummer, W. Hypoxic vasoconstriction of partial muscular intra-acinar pulmonary arteries in murine precision cut lung slices. *Respir. Res.* **2006**, *7*, 93. [CrossRef] [PubMed]
58. Sorci, G.; Cornet, S.; Faivre, B. Immune evasion, immunopathology and the regulation of the immune system. *Pathogens* **2013**, *2*, 71–91. [CrossRef] [PubMed]
59. Van Avondt, K.; van Sorge, N.M.; van Sorge, N.M.; Meyaard, L. Bacterial immune evasion through manipulation of host inhibitory immune signaling. *PLoS Pathog.* **2015**, *11*, e1004644. [CrossRef] [PubMed]
60. Backhaus, S.; Zakrzewicz, A.; Richter, K.; Damm, J.; Wilker, S.; Fuchs-Moll, G.; Küllmar, M.; Hecker, A.; Manzini, I.; Ruppert, C.; et al. Surfactant inhibits ATP-induced release of interleukin-1β via nicotinic acetylcholine receptors. *J. Lipid Res.* **2017**, *58*, 1055–1066. [CrossRef] [PubMed]
61. Dushianthan, A.; Cusack, R.; Goss, V.; Postle, A.D.; Grocott, M.P.W. Clinical review: Exogenous surfactant therapy for acute lung injury/acute respiratory distress syndrome—Where do we go from here? *Crit. Care* **2012**, *16*, 238. [CrossRef] [PubMed]
62. Janciauskiene, S.M.; Bals, R.; Koczulla, R.; Vogelmeier, C.; Köhnlein, T.; Welte, T. The discovery of α1-antitrypsin and its role in health and disease. *Respir. Med.* **2011**, *105*, 1129–1139. [CrossRef] [PubMed]

63. Franchini, L.F.; Elgoyhen, A.B. Adaptive evolution in mammalian proteins involved in cochlear outer hair cell electromotility. *Mol. Phylogenet. Evol.* **2006**, *41*, 622–635. [CrossRef] [PubMed]
64. Wang, Y.; Pereira, E.F.; Maus, A.D.; Ostlie, N.S.; Navaneetham, D.; Lei, S.; Albuquerque, E.X.; Conti-Fine, B.M. Human bronchial epithelial and endothelial cells express $\alpha 7$ nicotinic acetylcholine receptors. *Mol. Pharmacol.* **2001**, *60*, 1201–1209. [CrossRef] [PubMed]
65. Sethi, S.; Murphy, T.F. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. *N. Engl. J. Med.* **2008**, *359*, 2355–2365. [CrossRef] [PubMed]
66. Essilfie, A.-T.; Simpson, J.L.; Dunkley, M.L.; Morgan, L.C.; Oliver, B.G.; Gibson, P.G.; Foster, P.S.; Hansbro, P.M. Combined *Haemophilus influenzae* respiratory infection and allergic airways disease drives chronic infection and features of neutrophilic asthma. *Thorax* **2012**, *67*, 588–599. [CrossRef] [PubMed]
67. Jalalvand, F.; Riesbeck, K. Haemophilus influenzae: Recent advances in the understanding of molecular pathogenesis and polymicrobial infections. *Curr. Opin. Infect. Dis.* **2014**, *27*, 268–274. [CrossRef] [PubMed]
68. Ratjen, F.; Waters, V.; Klingel, M.; McDonald, N.; Dell, S.; Leahy, T.R.; Yau, Y.; Grasemann, H. Changes in airway inflammation during pulmonary exacerbations in patients with cystic fibrosis and primary ciliary dyskinesia. *Eur. Respir. J.* **2016**, *47*, 829–836. [CrossRef] [PubMed]
69. Simpson, J.L.; Daly, J.; Baines, K.J.; Yang, I.A.; Upham, J.W.; Reynolds, P.N.; Hodge, S.; James, A.L.; Hugenholtz, P.; Willner, D.; et al. Airway dysbiosis: *Haemophilus influenzae* and Tropheryma in poorly controlled asthma. *Eur. Respir. J.* **2016**, *47*, 792–800. [CrossRef] [PubMed]
70. Simpson, J.L.; Baines, K.J.; Horvat, J.C.; Essilfie, A.-T.; Brown, A.C.; Tooze, M.; McDonald, V.M.; Gibson, P.G.; Hansbro, P.M. COPD is characterized by increased detection of *Haemophilus influenzae*, *Streptococcus pneumoniae* and a deficiency of *Bacillus* species. *Respirology* **2016**, *21*, 697–704. [CrossRef] [PubMed]
71. Lugade, A.A.; Bogner, P.N.; Thatcher, T.H.; Sime, P.J.; Phipps, R.P.; Thanavala, Y. Cigarette smoke exposure exacerbates lung inflammation and compromises immunity to bacterial infection. *J. Immunol.* **2014**, *192*, 5226–5235. [CrossRef] [PubMed]
72. Earl, C.S.; Keong, T.W.; An, S.-Q.; Murdoch, S.; McCarthy, Y.; Garmendia, J.; Ward, J.; Dow, J.M.; Yang, L.; O'Toole, G.A.; et al. *Haemophilus influenzae* responds to glucocorticoids used in asthma therapy by modulation of biofilm formation and antibiotic resistance. *EMBO Mol. Med.* **2015**, *7*, 1018–1033. [CrossRef] [PubMed]
73. Ulanova, M.; Tsang, R.S.W. *Haemophilus influenzae* serotype a as a cause of serious invasive infections. *Lancet Infect. Dis.* **2014**, *14*, 70–82. [CrossRef]
74. MacVane, S.H. Antimicrobial resistance in the intensive care unit: A focus on Gram-negative bacterial infections. *J. Intensive Care Med.* **2017**, *32*, 25–37. [CrossRef] [PubMed]
75. Schweda, E.K.H.; Richards, J.C. Structural profiling of short-chain lipopolysaccharides from *Haemophilus influenzae*. *Methods Mol. Med.* **2003**, *71*, 161–183. [PubMed]
76. Paddenberg, R.; Mermer, P.; Goldenberg, A.; Kummer, W. Videomorphometric analysis of hypoxic pulmonary vasoconstriction of intra-pulmonary arteries using murine precision cut lung slices. *J. Vis. Exp.* **2014**, *83*, e50970. [CrossRef] [PubMed]
77. Richardson, K.C.; Jarett, L.; Finke, E.H. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.* **1960**, *35*, 313–323. [CrossRef] [PubMed]

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C-Reactive Protein Stimulates Nicotinic Acetylcholine Receptors to Control ATP-Mediated Monocytic Inflammasome Activation

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Blood levels of the acute phase reactant C-reactive protein (CRP) are frequently measured as a clinical marker for inflammation, but the biological functions of CRP are still controversial. CRP is a phosphocholine (PC)-binding pentraxin, mainly produced in the liver in response to elevated levels of interleukin-1β (IL-1β) and of the IL-1β-dependent cytokine IL-6. While both cytokines play important roles in host defense, excessive systemic IL-1β levels can cause life-threatening diseases such as trauma-associated systemic inflammation. We hypothesized that CRP acts as a negative feedback regulator of monocytic IL-1β maturation and secretion. Here, we demonstrate that CRP, in association with PC, efficiently reduces ATP-induced inflammasome activation and IL-1β release from human peripheral blood mononuclear leukocytes and monocytic U937 cells. Effective concentrations are in the range of marginally pathologic CRP levels (IC_{50} = 4.9 μg/ml). CRP elicits metabotropic functions at nicotinic acetylcholine (ACh) receptors (nAChRs) containing subunits α7, α9, and α10 and suppresses the function of ATP-sensitive P2X7 receptors in monocytic cells. Of note, CRP does not induce ion currents at conventional nAChRs, suggesting that CRP is a potent nicotinic agonist controlling innate immunity without entailing the risk of adverse effects in the nervous system. In a prospective study on multiple trauma patients, IL-1β plasma concentrations negatively correlated with preceding CRP levels, whereas inflammasome-independent cytokines IL-6, IL-18, and TNF-α positively correlated. In conclusion, PC-laden CRP is an unconventional nicotinic agonist that potently inhibits ATP-induced inflammasome activation and might protect against trauma-associated sterile inflammation.

Keywords: C-reactive protein, interleukin-1β, NLRP3 inflammasome, monocytes, nicotinic acetylcholine receptors, sterile inflammation

INTRODUCTION

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine of innate immunity that plays a seminal role in host defense (1). Secretion of monocytic IL-1β into the circulation in response to severe multiple trauma, however, may be more harmful than beneficial, as IL-1β is swept away from the site of inflammation, and high blood plasma levels can cause systemic inflammatory response syndrome (SIRS) and multi organ dysfunction syndrome (MODS) (2–5). Despite decades of intensive research, treatment of SIRS and MODS is mainly supportive, and mortality remains unacceptably high (5, 6).

IL-1β secretion normally requires two consecutive danger signals (5, 7–9). Ligands of toll-like receptors are typical first signals inducing pro-IL-1β synthesis, and extracellular ATP is a typical second signal that activates the ATP-sensitive P2X7 receptor (P2X7R), induces NLRP3 (NACHT, LRR, and PYD domains-containing protein 3) inflammasome assembly, caspase-1 activation, pro-IL-1β cleavage, and secretion of mature IL-1β (7–11). The secretion of IL-18 and high mobility group box 1 protein (HMGB1) also depends on inflammasome activation (7–13). Apart from the ATP-induced pathway typical for trauma-associated sterile inflammation, several alternative mechanisms of IL-1β maturation are activated during infection (1).

C-reactive protein (CRP) is a pentraxin (14, 15), mainly produced in the liver in response to elevated systemic levels of IL-1β and IL-6 (14, 15). Blood concentrations of this acute phase protein are a frequently used sensitive clinical marker for inflammation. In addition, slightly raised CRP levels correlate with cardiovascular disease and some disorders of the central nervous system (16–18). The biological functions of CRP are, however, highly disputed. CRP seems to play a vital role in humans, as its gene was conserved during evolution and there are no reports on CRP-deficient individuals (14). It has been proposed to be involved in the clearance of pathogens or apoptotic cells (14, 15), to induce pro-inflammatory cytokines (19, 20), and to play a pathogenic role in cardiovascular diseases (21). At least some of the pro-inflammatory effects are mediated by activated CRP that exposes binding sites for complement and immunoglobulins and is mainly found within inflamed tissues (22, 23). In contrast to the more pro-inflammatory functions, CRP induces high levels of the anti-inflammatory IL-1 receptor antagonist (24), and transgenic animals overexpressing human CRP are protected from inflammatory diseases including sepsis (25), alveolitis (26), arthritis (27), and atherosclerosis (28).

In the presence of Ca^{2+}, CRP associates with phosphocholine (PC) and a range of more complex molecules containing a PC group at a 1:1 M ratio per CRP monomer (14, 15, 29). Our group recently demonstrated that phosphatidylcholines and their metabolites including free PC are efficient inhibitors of ATP-induced release of IL-1β from human monocytes by a mechanism that involves non-canonical metabotropic functions of nicotinic acetylcholine (ACh) receptors (nAChRs) (30–33).

Here, we demonstrate that purified human endogenous CRP (eCRP) efficiently inhibits the ATP-induced release of IL-1β from monocytic cells. We provide evidence that CRP presents PC to nAChRs and thus potentiates the effect of free PC. PC-laden CRP is hence a novel agonist of unconventional nAChRs that controls inflammasome activation by inhibiting the function of P2X7R.

MATERIALS AND METHODS

U937 Cells

U937 cells (DSMZ, Braunschweig, Germany) were maintained in RPMI 1640 (Gibco by Life Technologies, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS, Biochrome, Berlin, Germany) and 2 mM L-glutamine (Gibco by Life Technologies) under 5% CO_2 atmosphere at 37°C. Cells (1×10^6 cells/ml) were seeded in 24-well plates, primed for 5 h with 1 μg/ml lipopolysaccharide (LPS) from *Escherichia coli* (L2654, Sigma-Aldrich, Deisenhofen, Germany) (30). BzATP [2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium salt; 100 μM, Sigma-Aldrich] or nigericin (50 μM, Sigma-Aldrich) were added for another 30 min in the presence or absence of different concentrations of eCRP from human pleural fluid (Millipore, AG732), recombinant CRP (rCRP) produced in *E. coli* (Millipore, 236608), serum amyloid P (SAP; Millipore, 565190), or PC chloride calcium salt tetrahydrate (Sigma-Aldrich). Nicotinic antagonists mecamylamine hydrochloride (Sigma-Aldrich), strychnine hydrochloride (Sigma-Aldrich), α-bungarotoxin (Tocris Bioscience, Bristol, UK), ArIB [V11L, V16D] (500 nM) (34, 35) and RgIA4 (200 nM) (31, 36) were also applied together with BzATP. Supernatants were stored at 20°C until cytokine and lactate dehydrogenase (LDH) measurement.

Human Peripheral Blood Mononuclear Cells (PBMC)

Peripheral blood mononuclear cells were obtained from healthy (self-reported) male non-smoking adult volunteers. The local ethics committee at the University of Giessen approved all studies on primary human cells (approval No. 81/13). Blood was drawn into sterile syringes containing 17.5 IU heparin (Ratiopharm, Ulm, Germany) per ml blood and PBMC were separated on Leucosep gradients (Greiner Bio-One, Frickenhausen, Germany). LPS (5 ng/ml) was added to blood samples before gradient centrifugation (30). PBMC were cultured in 24-well plates at a density of 5×10^5 cells/0.5 ml in RPMI 1640, 10% FCS, 2 mM L-glutamine for 3 h. Non-adherent cells were removed, and cell culture medium was replaced by medium devoid of FCS. Stimulation with BzATP in the presence or absence of eCRP was done as described for U937 cells.

Cell Viability

Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) was used to measure LDH concentrations in cell free supernatants as indicated by the supplier. LDH values are given as percentage of the total LDH content of lysed control cells. Cell viability was unimpaired in all experimental settings.

Cytokine Measurement

Blood concentrations of IL-1β, IL-18, and tumor necrosis factor-α (TNF-α) were measured by the Human Quantikine® Immunoassays (R&D Systems, Minneapolis, MN, USA). IL-6 was measured on the Siemens 150.

Immulite 2000 XPI system using the Siemens IL-6 reagent (Siemens, Erlangen, Germany). HMGB1 was measured by an ELISA obtained from IBL International (Hamburg, Germany). To detect low cytokine levels in cell culture supernatants, for IL-1β the Human IL-1 beta/IL-1F2 DuoSet ELISA (R&D Systems) was used, whereas IL6 and TNF-α were measured by the Human Quantikine® Immunoassays (R&D Systems, Minneapolis, MN, USA).

Dissociation and Formation of CRP/PC Complexes

Endogenous CRP was dissolved at a concentration of 5 μg/ml in PBS devoid of Ca^{2+} and Mg^{2+} (Gibco) containing 1.1 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich), incubated at 37°C for 15 min followed by ultrafiltration using Amicon® Ultra centrifugal filters. The high molecular weight fraction was diluted in PBS/EDTA, ultrafiltrated, and transferred to PBS, 5 mM Ca^{2+}, without EDTA by two additional ultrafiltration steps. In control, the same procedure was performed in the absence of EDTA. CRP purified by ultrafiltration and rCRP were incubated at a 1:1 and 1:3 M ratio per monomer, respectively, with PC at 37°C for 30 min and tested in IL-1β release assays at a concentration of 5 μg/ml CRP and 1 μM PC.

Gene Silencing

The expression of nAChR subunits α7 (*CHRNA7*), α9 (*CHRNA9*), and α10 (*CHRNA10*) in U937 cells was silenced by transfection of small interfering RNA (siRNA; 30 pmol per 1×10^6 cells, ON-TARGETplus human *CHRNA7*, *CHRNA9*, or *CHRNA10* siRNA SMARTpool, Thermo Fisher Scientific, Schwerte, Germany) using the Amaxa® Cell line Nucleofector® Kit C (Lonza Cologne AG, Cologne, Germany) and the Nucleofector® device II (Lonza Cologne AG). Negative control ON-TARGETplus non-targeting pool (Thermo Fisher Scientific) was included to control for non-specific effects of transfection. A reduction of the mRNA expression of subunits α9 and α10 to about 50% of control-transfected cells was recently shown by our group in the same experimental setting *via* real-time RT-PCR 6 h after transfection (30). The basal expression of α7 mRNA, however, was too low to be quantified. IL-1β release experiments were performed 2 days after transfection.

Immunocytochemistry

Lipopolysaccharide-primed PBMC were cultured in CELLview™ slides (Greiner Bio-One) at a density of 2×10^5 cells per well in 200 μl medium and stimulated with BzATP (100 μM) in the presence or absence of eCRP (5 μg/ml). Cells were fixed and permeabilized with ice-cold Cytofix/Cytoperm™ (BD Biosciences, Heidelberg, Germany) for 20 min, washed with Perm/Wash™ buffer (BD Biosciences), and air-dried before storage at 4°C. Slides were rehydrated with Perm/Wash™ buffer, endogenous peroxidase activity was inhibited by treatment with 1% H_2O_2 in Perm/Wash™ buffer, followed by 1% bovine serum albumin in Perm/Wash™ buffer for 30 min at ambient temperature. Polyclonal rabbit antibodies to human ASC (1:50, SC-22514-R, Santa Cruz Biotechnology, Dallas, TX, USA) or monoclonal mouse antibodies to human CD14 (1:100, HCD14, BioLegend *via* Biozol, Eching, Germany) were diluted in Perm/Wash™ buffer containing 1% bovine serum albumin and 5% human heat-inactivated serum. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit Ig (1:50) and rabbit anti-mouse Ig (1:70) antibodies (both from Dako Cytomation, Glostrup, Denmark) and 0.5 mg/ml 3,3′-diaminobenzidine (Sigma-Aldrich), 1% H_2O_2, 0.3 M Tris-buffered saline, pH 7.6, for 10 min at room temperature. Slides were slightly counterstained with hemalumn and cover-slipped in Glycergel mounting medium (Dako Cytomation). Slides were evaluated blinded for the experimental groups at a 200-fold magnification using an Olympus BX51 microscope and the analySIS software (Olympus, Hamburg, Germany). At least 150 cells in 12 fields of vision were counted per experiment. Total numbers of cells and specks were converted into mean numbers of specks per 100 cells. In negative controls that resulted in no staining, primary antibodies were omitted. The specificity of the antibodies to ASC was verified by Western blotting of protein extracts from U937 cells that revealed a single band with the expected molecular mass (not shown).

Gel Electrophoresis and Western Blotting

SDS polyacrylamide gel electrophoresis was performed under reducing conditions according to Laemmli (37). PBMC were lysed, the protein concentration of the lysate was determined (Micro BCA protein assay kit, Pierce Biotechnology, Rockford, IL, USA) and adjusted to 15 μg/10 μl. Cell culture supernatants were harvested at the end of the experiments, concentrated by a factor of 10 using Amicon® Ultra centrifugal filters (Ultracel™ 10K, Merck Millipore, Darmstadt, Germany) and mixed with 2× sample buffer. Samples (10 μl each) were loaded onto 15% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and stained with Brilliant Blue G (Sigma-Aldrich). Prestained molecular weight standards (Precision Plus Protein Standards, dual color, Bio Rad, Hercules, CA, USA) were separated in each gel. Mouse monoclonal antibodies to IL-1β that detect both pro-IL-1β and mature IL-1β (1:10,000, 3ZD, kindly supplied by the National Cancer Institute, Frederick, MD, USA), polyclonal rabbit anti-caspase-1 antibodies (1:1,000, #2225, Cell Signaling Technology, Danvers, MA, USA) and mouse monoclonal antibodies to β-actin (1:500,000, A2228, Sigma-Aldrich) were applied and detected with horseradish peroxidase-conjugated rabbit anti-mouse Ig (1:5,000) and goat anti-rabbit Ig (1:5,000) antibodies (both from Dako Cytomation). SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA) was used to detect IL-1β and Lumi-Light substrate (Roche, Mannheim, Germany) to detect β-actin. Documentation and densitometry of the blots were performed using a digital gel documentation system (Biozym, Hessisch Oldendorf, Germany).

Whole-Cell Patch-Clamp Recordings

U937 cells were incubated in poly-L-lysine-coated culture dishes (Nunc, Roskilde, Denmark) in bath solution [5.4 mM

KCl, 120 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid), 25 mM glucose, pH 7.4] for 5 h with LPS (1 μg/ml) at 37°C. Thereafter, whole-cell recordings were performed at ambient temperature on an inverted microscope (Axiovert, Zeiss, Göttingen, Germany). Patch pipettes were pulled from borosilicate glass capillaries (outer diameter 1.6 mm, Hilgenberg, Malsfeld, Germany) to a resistance of 2–4 MΩ using an automated puller (Zeitz, Augsburg, Germany). Pipettes were filled with pipette solution (120 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 11 mM ethylene glycol tetraacetic acid, 20 mM glucose, pH 7.3). The membrane potential of LPS-primed U937 cells was voltage-clamped to −60 mV and transmembrane currents in response to BzATP (100 μM) were amplified with an EPC 9 amplifier (HEKA, Lambrecht, Germany) and acquired *via* an ITC-16 interface with the Pulse software (HEKA). A pressure-driven microperfusion system was used to apply BzATP, eCRP (5 μg/ml) and RgIA4 (200 nM).

Measurements of Intracellular Ca^{2+}

To measure intracellular $[Ca^{2+}]_i$, U937 cells were incubated in poly-L-lysine-coated glass bottom culture dishes (CELLview™, Greiner Bio-One) for 5 h with LPS (1 μg/ml) at 37°C in the same bath solution as described for the whole-cell patch-clamp recordings. Thereafter, cells were loaded with 3.3 μM Fura-2/AM (Thermo Fisher Scientific) for 25 min at 37°C. Fura-2/AM was excited at 340 and 380 nm wavelengths and the fluorescence emission 510 nm was measured. Four independent batches of U937 cells were used in this experiment and a total number of 243 cells were tracked individually, and the fluorescence intensity ratio of 340:380 nm was recorded. Experiments were run at room temperature. After a calibration time of 100 s, cells were exposed to eCRP (5 μg/ml) for 300 s. At the end of the experiments, a positive control for the Ca^{2+} imaging setup was included. Forskolin (40 μM; Biozol), an activator of adenylyl cyclase that elevates cyclic adenosine monophosphate (cAMP) levels, was applied to induce a cAMP-triggered rise in $[Ca^{2+}]_i$ (38).

Two-Electrode Voltage-Clamp (TEVC) Measurements on *Xenopus laevis* Oocytes Expressing Human nAChR

Defolliculated *Xenopus laevis* oocytes were obtained from Ecocyte Bioscience (Castrop-Rauxel, Germany). Oocytes from at least two different *Xenopus laevis* individuals were used in all experimental groups. Oocytes were stored in Ringer's solution (ORi) containing (in mM) 90 NaCl, 1 KCl, 2 $CaCl_2$, 5 HEPES, 2.5 pyruvate, 20 mg/ml penicillin, and 25 mg/ml streptomycin (pH 7.4) at 16°C. All chemicals used for ORi preparation were purchased from Fluka (Deisenhofen, Germany), except for HEPES, penicillin, and streptomycin (Sigma-Aldrich). Plasmid DNAs encoding the human *CHRNA9* and *CHRNA10* as well as the 43 kDa receptor-associated protein of the synapse (*RAPSN*) were obtained from Eurofins Genomics (Ebersberg, Germany) and capped cRNA was synthesized as described before (31). Human *CHRNA7* encoding cRNA was kindly provided by G. Schmalzing (Department of Molecular Pharmacology, RWTH Aachen University, Aachen, Germany) and synthesized as described before (33). cRNA was dissolved in nuclease-free water and injected into oocytes in a volume of 50.6 nl using a microinjector (Nanoject, Drummond Scientific, Broomall, PA, USA). Oocytes were injected with cRNA encoding *CHRNA7*, *CHRNA9*, and *CHRNA10* nAChR subunits (16, 19, and 19 ng/oocyte, respectively) along with 5 ng cRNA encoding *RAPSN*, and oocytes were incubated at 16°C for 3–5 days. In control experiments, 50.6 nl of nuclease-free water was injected.

In TEVC measurements, oocytes were perfused (gravity driven) with ORi without pyruvate and antibiotics (pH 7.4). Intracellular borosilicate microelectrodes were filled with 1 M KCl solution and the membrane voltage was clamped to −60 mV using a TEVC amplifier (Warner Instruments, Hamden, CT, USA). Low-pass filtered transmembrane currents (1,000 Hz, Frequency Devices 902, Haverhill, MA, USA) were recorded using a strip chart recorder (Kipp & Zonen, Delft, The Netherlands). For experiments examining the inhibition and recovery from inhibition of choline-gated currents in presence and absence of eCRP (5 μg/ml), oocytes were injected with a 1:1 ratio of cRNA encoding human *CHRNA9* and *CHRNA10* in a 1:1 ratio, incubated at 17°C for 2–3 days and measured as described before (31).

Clinical Study

A single center prospective observational study (trial registration: DRKS00010991) was approved by the ethics committee of the medical faculty Giessen, Germany (No. 164/14) and performed in accordance with the Helsinki Declaration. Written informed consent was given by each patient or patient's legal representative. Patients were recruited at the surgical intensive care unit (ICU) of the University Hospital of Giessen, Germany from January 2015 until February 2016. Only patients with severe trauma as defined by an injury severity score (ISS) above 16 (39) were included. Patients younger than 18 years or with a history of HIV or hepatitis B/C infection were excluded. Detailed patient characteristics are listed in **Table 1**.

The first venous blood sample (day 0) was drawn within 15 h after admission to the hospital followed by daily blood collection in the morning. The levels of IL-1β, IL-18, TNF-α, IL-6, and HMGB1 were determined in the plasma of heparinized blood. CRP levels were analyzed turbidometrically by Siemens ADVIA XPT system (Siemens) using the wrCRP reagent (Siemens).

Quantification and Statistical Analysis

SPPS® (Version 23, IBM®, Armonk, NC, USA) and GraphPad Prism® (Version 6, GaphPad Software, La Jolla, CA, USA) were used for statistical and linear regression analyses. The IC_{50} value of eCRP in human U937 cells was determined in GraphPad Prism® (Version 6, GaphPad Software) by fitting log-transformed concentration values and the original effect data. Multiple groups were first analyzed by non-parametric Kruskal–Wallis test. In case of $p \leq 0.05$, non-parametric Mann–Whitney rank sum test was performed to compare between individual groups and again, a $p \leq 0.05$ was considered as statistically significant. Paired data were analyzed by Wilcoxon sign rank test.

TABLE 1 | Patient characteristics.

Number of patients	*n* = 38
Gender male	28 (73.7%)
Age (years)	47.7 ± 20.9
Body mass index (kg/m²)	25.3 ± 3.3
Clinical history	
COPD	0
Myocardial infarction	1 (2.6%)
Congestive heart failure	0
Renal failure	0
Immunosuppression	0
ISS	25 ± 6
NISS	27 ± 6
Type of injury	
Head	24 (63.2%)
Thorax	34 (89.5%)
Abdomen	24 (63.2%)
Extremities	28 (73.7%)
External injuries	35 (92.1%)
Cause of injury	
Car accident	16 (42.1%)
Motorcycle accident	10 (26.3%)
Bicycle accident	3 (7.9%)
Fall	7 (18.4%)
Other reasons	2 (5.3%)
RISC	2.6 ± 1.3
APACHE II	16.8 (7.5%)
SOFA	7.2 (4.3%)
24 h mortality	1 (2.6%)
30 days mortality	2 (5.3%)
Length of ICU stay (days)	11.2 ± 11.8
Length of hospital stay (days)	23.5 ± 15.2

APACHE II, acute physiology and chronic health evaluation score; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; ISS, injury severity score; NISS, new injury severity score; RISC, revised injury severity classification score; SOFA, sequential organ failure assessment score.
Data are shown as mean ± SD, as absolute numbers or as percentage.

RESULTS

CRP Inhibits ATP-Mediated IL-1β Release From Human Monocytic U937 Cells

To test if CRP inhibits ATP-induced IL-1β release, we primed human monocytic U937 cells for 5 h with LPS (1 μg/ml). Thereafter, 100 μM BzATP, a P2X7R agonist (40), was applied for 30 min in presence and absence of eCRP and IL-1β concentrations were measured in cell culture supernatants. We found that in LPS-primed U937 cells the BzATP-induced release of IL-1β was dose-dependently and efficiently inhibited by eCRP (**Figure 1A**). The half maximal inhibitory concentration (IC_{50}) was 4.9 μg/ml corresponding to an about 40 nM concentration of pentameric CRP. Of note, plasma levels between 3 and 10 μg/ml are clinically regarded as a minor pathological CRP elevation (41). In contrast to eCRP, the acute phase protein (5 μg/ml), another pentraxin with high structural similarity to CRP (15), did not inhibit ATP-induced IL-1β release (**Figure 1B**).

CRP Potentiates the Inhibitory Effect of PC

C-reactive protein, but not SAP, Ca^{2+}-dependently associates with PC in a 1:1 M ratio per subunit (15, 29). We previously showed that PC efficiently inhibits BzATP-induced IL-1β secretion from human monocytes (30, 31). Thus, we hypothesized that the inhibitory effect of eCRP on the release of IL-1β depends on its association with molecules containing a PC group. Accordingly, human rCRP (5 μg/ml) produced in *E. coli* that is expected to be devoid of PC, did not inhibit the BzATP-triggered release of IL-1β from LPS-primed U937 cells (**Figure 1C**), whereas a mixture of 5 μg/ml rCRP, 1 μM PC, and 5 mM Ca^{2+} was fully effective (**Figure 1C**). The same concentration of free PC was ineffective (**Figure 1C**), as expected (30). In a similar approach, eCRP was treated with EDTA (1.1 mM) to detach ligands from the PC-binding sites and was separated from small molecules by ultrafiltration (**Figures 1D,E**). This resulted in an inactive CRP preparation, the activity of which was restored by adding PC (1 μM) in the presence of Ca^{2+} (**Figure 1D**). Hence, the inhibition of ATP-induced IL-1β release by eCRP depends on its association with PC. Moreover, as the IC_{50} eCRP is around 40 nM and that of free PC around 10 μM (30, 31), CRP potentiates the inhibitory effect of PC by at least two orders of magnitude.

eCRP Signals *via* nAChRs

Next, we tested if eCRP signals *via* nAChRs in human monocytic U937 cells. Indeed, the eCRP-dependent inhibition of ATP-induced IL-1β release was completely reversed by the non-selective nAChR antagonist mecamylamine (100 μM). Similar results were obtained by using α-bungarotoxin (1 μM) or strychnine (10 μM), both preferential antagonists of the α7 and α9 nAChRs (42–44). The α-conotoxins ArIB [V11L, V16D] (500 nM) (34, 35) and RgIA4 (200 nM) (31, 36) that are specific for nAChRs composed of subunits α7 and α9α10, respectively, also completely blocked the inhibitory effect of eCRP (**Figure 1F**). These results indicate that eCRP signals *via* nAChRs containing subunits α7, α9, and α10. To corroborate the involvement of these nAChR subunits, U937 cells were transfected with siRNA targeting *CHRNA7*, *CHRNA9*, and *CHRNA10*. Indeed, single-gene silencing of each nAChR subunit significantly blunted the inhibitory effect of eCRP (**Figure 1G**).

eCRP Does Not Trigger Ion Channel Functions at Conventional nAChRs

The canonical ionotropic function of nAChR can be monitored by using *Xenopus laevis* oocytes as heterologous expression systems (45). To investigate if eCRP elicits ionfluxes human nAChR subunits α7, α9, and α10 were heterologously expressed in oocytes to perform TEVC measurements. We showed that eCRP (5 μg/ml) did not induce ion currents, whereas the classical nAChR agonist choline did (**Figures 2A–C**). Nevertheless, eCRP interacted with canonical nAChRs, as it reduced choline-triggered currents in oocytes expressing α9α10 nAChRs (**Figures 2D,E**). Thus, eCRP does not trigger canonical ion channel functions of nAChRs but acts as a silent agonist or partial antagonist that modulates the responses to classical nicotinic agonists.

eCRP Inhibits BzATP-Induced Current Responses in Monocytic Cells

Next, we tested if eCRP induces ion fluxes in LPS-primed U937 cells. Application of eCRP (5 μg/ml) did not induce ion

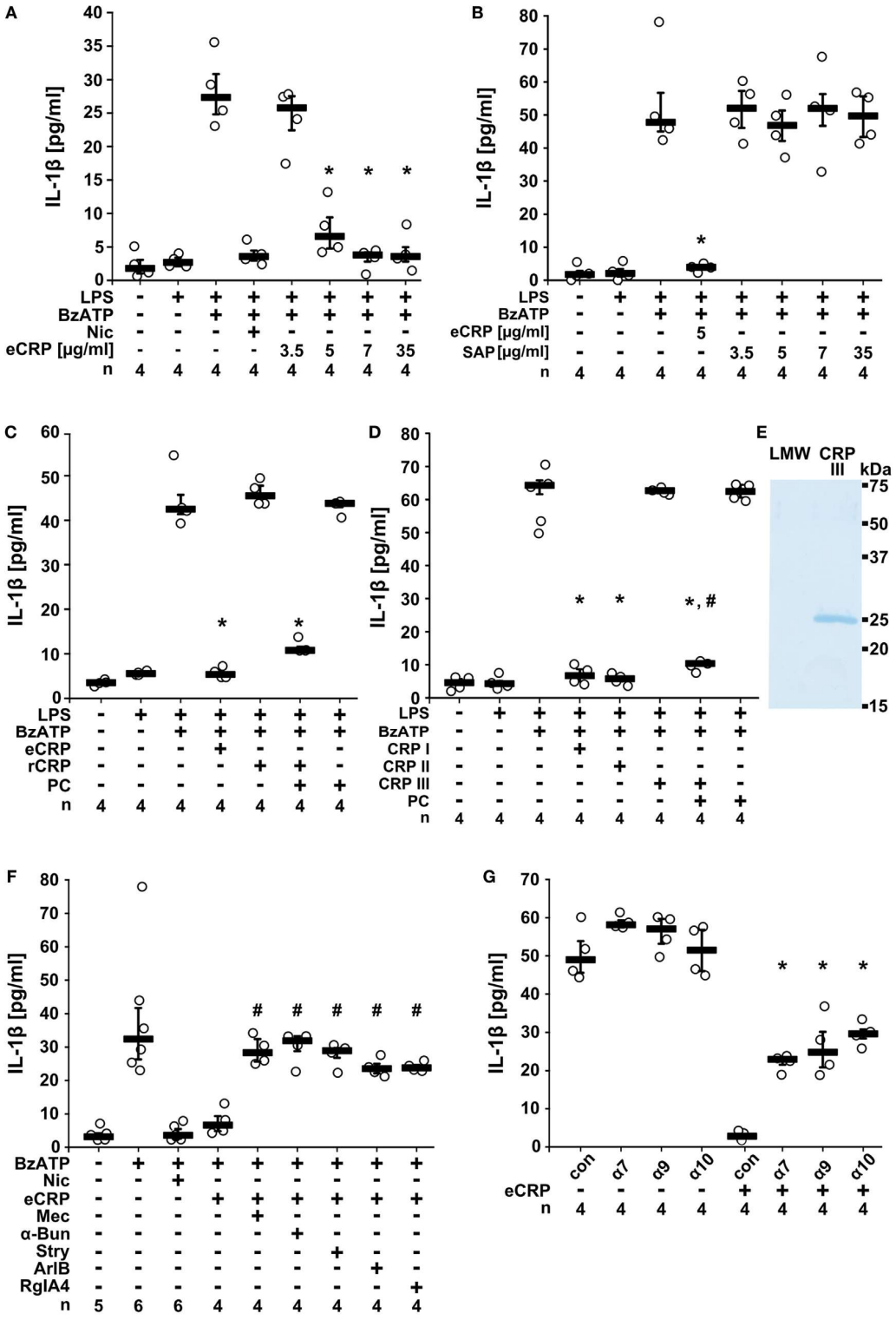

FIGURE 1 | Continued

FIGURE 1 | Purified human endogenous C-reactive protein (eCRP) inhibits BzATP-induced release of interleukin-1β (IL-1β) from U937 cells. Lipopolysaccharide (LPS)-primed (1 μg/ml, 5 h) U937 cells were stimulated with BzATP (2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium salt; 100 μM) and IL-1β was measured 30 min later in cell culture supernatants. **(A)** eCRP dose-dependently inhibited the BzATP-induced IL-1β release, nicotine (Nic; 100 μM) served as a positive control. **(B,C)** Serum amyloid P (5 μg/ml), human recombinant CRP (rCRP) (5 μg/ml), or low concentrations of free phosphocholine (PC) (1 μM) did not impair IL-1β release, but a combination of rCRP and PC (1 μM) did. **(D)** The inhibitory effect of eCRP (CRP I; 5 μg/ml) was preserved after ultrafiltration (cutoff 10 kDa; CRP II), but abolished by ultrafiltration in the presence of ethylenediaminetetraacetic acid (1.1 mM; CRP III). PC (1 μM) reconstituted the activity of CRP III, whereas 1 μM PC alone was ineffective. **(E)** CRP was retained in CRP III and absent from the low molecular weight fraction (LMW). SDS-PAGE followed by staining with Brilliant Blue. **(F)** The effect of eCRP was reversed by nicotinic acetylcholine receptor (nAChR) antagonists mecamylamine (Mec; 100 μM), α-bungarotoxin (α-Bun; 1 μM), strychnine (Stry; 10 μM), ArIB (500 nM), and RgIA4 (200 nM). **(G)** In experiments using small interfering RNA (siRNA), silencing of the nAChR subunits α7, α9, and α10, but not control siRNA (con) attenuated the inhibition by eCRP. Data are presented as individual data points, bar represents median, whiskers encompass the 25th to 75th percentile. $^{*}p \leq 0.05$, different from LPS-primed cells stimulated with BzATP alone. $^{\#}p \leq 0.05$, different LPS-primed cells were stimulated with BzATP and eCRP. Kruskal–Wallis followed by Mann–Whitney rank sum test.

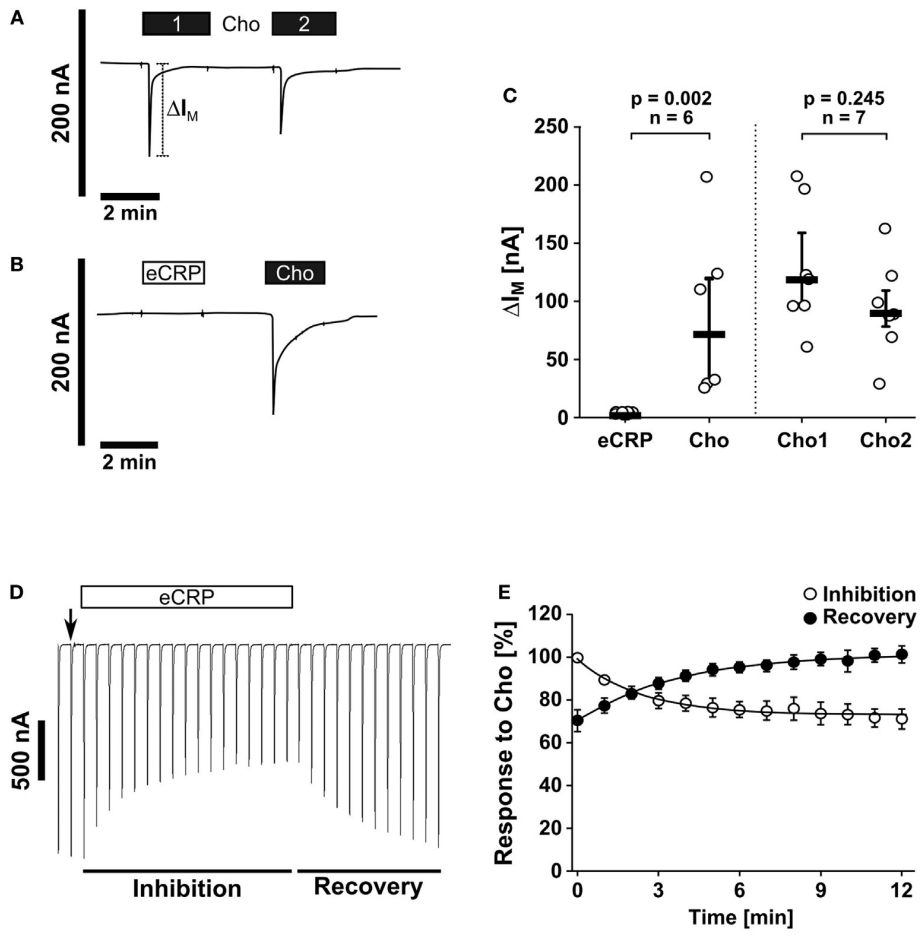

FIGURE 2 | Purified human endogenous C-reactive protein (eCRP) does not induce ion channel functions at canonical nAChRs. Two-electrode voltage-clamp (TEVC) measurements were performed on *Xenopus laevis* oocytes that heterologously expressed human nAChR subunits α7, α9, and α10. **(A,C)** The application of the known nAChR agonist choline (Cho1, 2 min, 1 mM, black bars) induced a stimulation of the transmembrane ion current (I_M) that could be repeated by a second Cho application (Cho2; $n = 7$). **(B,C)** By contrast, eCRP (5 μg/ml) did not provoke current responses, whereas a subsequent Cho application did ($n = 6$). **(C)** All changes of I_M (ΔI_M) induced by cholinergic stimulation are shown as individual data points, bars represent median, whiskers encompass the 25th to 75th percentile. Wilcoxon signed-rank test. **(D)** Representative current traces of Cho-gated currents in oocytes expressing α9α10 nAChRs illustrating the inhibitory effect of eCRP (5 μg/ml). The current traces represent 30 s recordings each and are shown concatenated omitting the 30 s gap between each individual trace. The oocytes were continuously perfused with saline solution and stimulated with Cho (1 s pulses, 1 mM) once per min until steady-state baseline responses were observed (indicated by the arrow). Subsequently, the Cho-gated currents were monitored in the presence of eCRP for changes in amplitude for 12 min. Thereafter, eCRP was washed out and the Cho-gated currents were monitored for recovery. **(E)** Graphical representation and analysis of the experimental results shown in **(D)**. Mean and SEM from five oocytes.

currents in whole-cell patch-clamp experiments (**Figures 3A,B**) and intracellular Ca^{2+} levels remained unchanged (**Figure 3C**). By contrast, BzATP (100 μM) induced a robust and repeatable current response in LPS-primed U937 cells (**Figures 3A,B**), as reported previously (30, 31). Remarkably, in the presence of eCRP, the BzATP-induced current responses were completely abrogated (**Figures 3A,B**). The inhibitory effect of eCRP was sensitive to the α-conotoxin RgIA4 (**Figures 3A,B**), confirming the involvement of nAChR subunits α9 and/or α10.

NLRP3 inflammasomes, in addition to extracellular ATP, assemble in response to other pro-inflammatory stimuli including pore-forming toxins (1). Here, the pore-forming bacterial toxin nigericin was used to investigate if eCRP also affects ATP-independent IL-1β release, which was clearly not the case (**Figure 3D**). Hence, stimulation of nAChRs with eCRP efficiently inhibits BzATP-induced ion currents in monocytic U937 cells but does not provoke canonical ion channel functions of nAChRs.

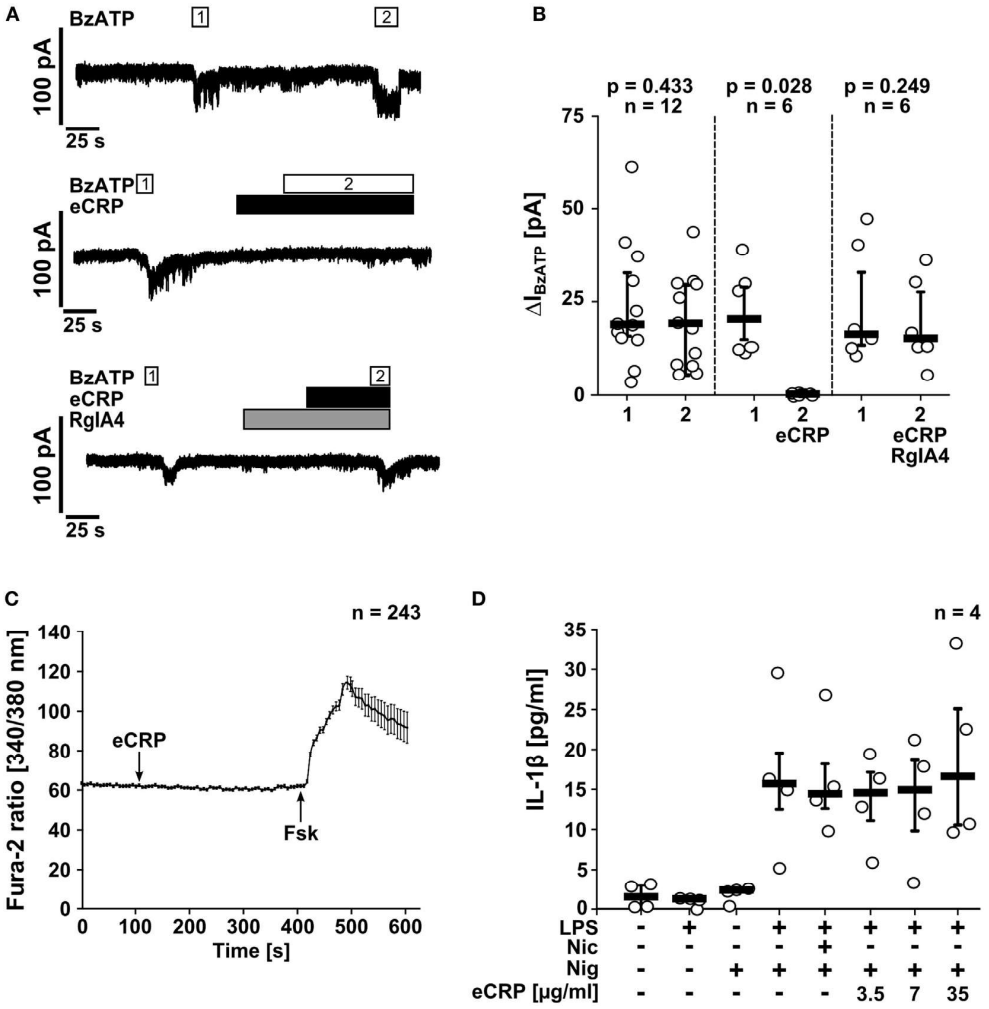

FIGURE 3 | Purified human endogenous C-reactive protein (eCRP) suppresses BzATP-induced whole-cell currents *via* nAChRs. **(A)** BzATP-induced ion currents were detected by whole-cell patch-clamp measurements in lipopolysaccharide (LPS)-primed (1 μg/ml, 5 h) U937 cells. Repetitive current changes were provoked by two (1, 2) consecutive BzATP (100 μM) applications (upper panel). Application of eCRP (5 μg/ml) alone did not provoke ion currents but fully inhibited the response to BzATP (middle panel). The inhibitory effect of eCRP was antagonized by addition of the α9α10 nAChR-specific α-conotoxin RgIA4 (200 nM; lower panel). **(B)** Graphical presentation of the two consecutive BzATP-induced ion current changes (1, 2, ΔI_{BzATP}). **(C)** $[Ca^{2+}]_i$ of LPS-primed U937 cells were recorded as Fura-2/AM (Fura-2) fluorescence intensity ratio of 340:380 nm excitation (mean ± SEM). Application of eCRP (5 μg/ml; indicated by arrow) did not cause significant alterations in $[Ca^{2+}]_i$ (values before eCRP compared to values obtained 300 s after eCRP application: $p = 0.726$). At the end of the experiments, a positive control for cell viability and the Ca^{2+} imaging setup was included: forskolin (Fsk, 40 μM, indicated by arrow) was applied to induce a cyclic adenosine monophosphate-triggered rise in $[Ca^{2+}]_i$. **(D)** The ATP-independent release of interleukin-1β (IL-1β) from LPS-primed monocytic U937 cells induced by nigericin (Nig; 50 μM) is neither inhibited by nicotine (Nic; 100 μM) nor by various concentrations of eCRP. Data are presented as individual data points, bar represents median, whiskers encompass the 25th to 75th percentile **(B,D)**. Wilcoxon signed-rank test **(B,C)** or Kruskal–Wallis followed by Mann–Whitney rank sum test **(D)**.

eCRP Inhibits Inflammasome Activation in Human PBMC

We performed experiments on the adherent fraction of freshly isolated PBMC from healthy human donors that were primed with a short pulse of LPS (5 ng/ml) during cell isolation. The spontaneous secretion of IL-1β by these cells was low, whereas a considerable amount of IL-1β was released in response to BzATP (100 μM). Indeed, eCRP (5 μg/ml) significantly attenuated the BzATP-induced release of IL-1β from these cells (**Figure 4A**), whereas the inflammasome-independent cytokines IL-6 and TNF-α (46) were neither induced by BzATP nor regulated by eCRP (**Figures 4B,C**). The concentrations of IL-6 and TNF-α released within 30 min after BzATP application are low and all apparent changes in response to BzATP or eCRP are not significant and probably random. We reported before, that almost no IL-18 is secreted by these cells in response to BzATP (30).

Inflammasome activation can lead to the formation of large aggregates, so-called specks or pyroptosomes, that can be detected with antibodies directed to ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain) (1). To investigate if CRP inhibits inflammasome and caspase-1 activation in primary monocytic cells LPS-pulsed PBMC were challenged with BzATP (100 μM) in the presence and absence of eCRP (5 μg/ml). A significant increase in ASC speck formation was detected in LPS-primed PBMC in response to BzATP, which was largely suppressed by concomitant application of eCRP (**Figure 5A**). Pro-IL-1β and pro-caspase-1, but not their mature forms, were detected by Western blotting of cell lysates of LPS-primed PBMC stimulated with BzATP in the absence or presence of eCRP (**Figure 5B**). In concentrated supernatants, however, mature caspase-1 and IL-1β were detectable upon BzATP-treatment, and eCRP significantly reduced their amount (**Figures 5C,D**).

We conclude that CRP suppresses inflammasome assembly, pyroptosome formation, activation of capase-1 and maturation of IL-1β.

CRP and IL-1β Levels Negatively Correlate in Multiple Trauma Patients

Our *in vitro* experiments led to the provocative hypothesis that elevated CRP levels protect against trauma-induced release of IL-1β into the circulation *in vivo*. We performed a prospective study on multiple trauma patients admitted to our hospital. Patient characteristics are summarized in **Table 1**. Plasma levels of IL-1β, IL-18, IL-6, TNF-α, and HMGB1 were measured in blood drawn at daily intervals until day 4 after admission. During the first 2 days, IL-1β levels negatively correlated with CRP values of the preceding day (**Figures 6A–D**), which is in line with our hypothesis. On day 4 after trauma, plasma levels of IL-6 (Figure S1 in Supplementary Material), IL-18 (Figure S2 in Supplementary Material), and TNF-α (Figure S3 in Supplementary Material) positively correlated with CRP values on day 3, whereas no correlation was seen for HMGB1 (Figure S4 in Supplementary Material). On days 0–2 after trauma, IL-1β levels did not correlate with disease severity acute physiology and chronic health evaluation score (APACHE II) and sequential organ failure assessment score (SOFA). In patients who remained on the ICU on days 3–4 after trauma a negative correlation of IL-1β levels with the SOFA score was seen (Table S1 in Supplementary Material). This subpopulation of patients typically suffers from a severe disease course. CRP levels did not correlate with APACHE II and SOFA score (Table S2 in Supplementary Material). Albeit statistically significant ($p \leq 0.05$), the correlation coefficients (r) and, accordingly, the coefficients of variation (CV) (**Figure 6**; Figures S1–S4 and Tables S1 and S2 in Supplementary Material), do not prove causality.

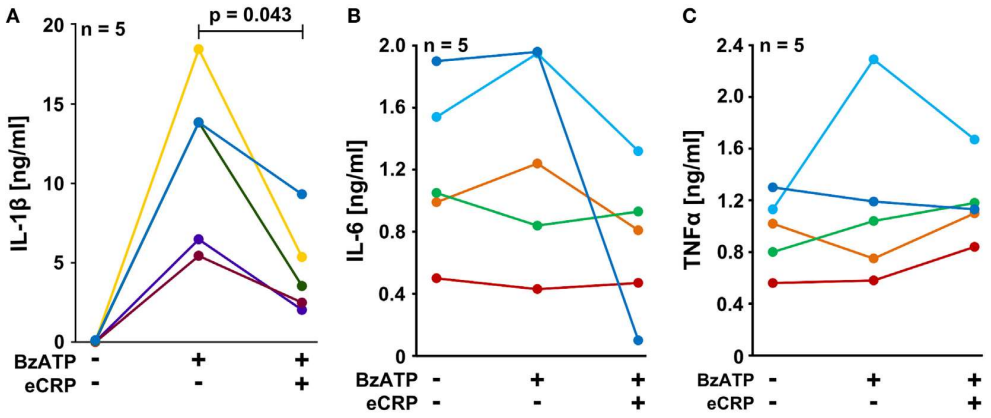

FIGURE 4 | Purified human endogenous C-reactive protein (eCRP) inhibits BzATP-induced release of interleukin-1β (IL-1β) from human peripheral blood mononuclear cells (PBMC). PBMC freshly isolated from healthy volunteers were pulsed with lipopolysaccharide (5 ng/ml) during the process of PBMC isolation and cultured for 3 h. The cells were stimulated with 100 μM BzATP in the presence or absence of eCRP (5 μg/ml) for 30 min. **(A)** IL-1β, **(B)** IL-6, and **(C)** TNF-α were measured by ELISA in cell culture supernatants ($n = 5$). Data points obtained from individual blood donors are coded by different colors and connected by lines. Wilcoxon signed-rank test.

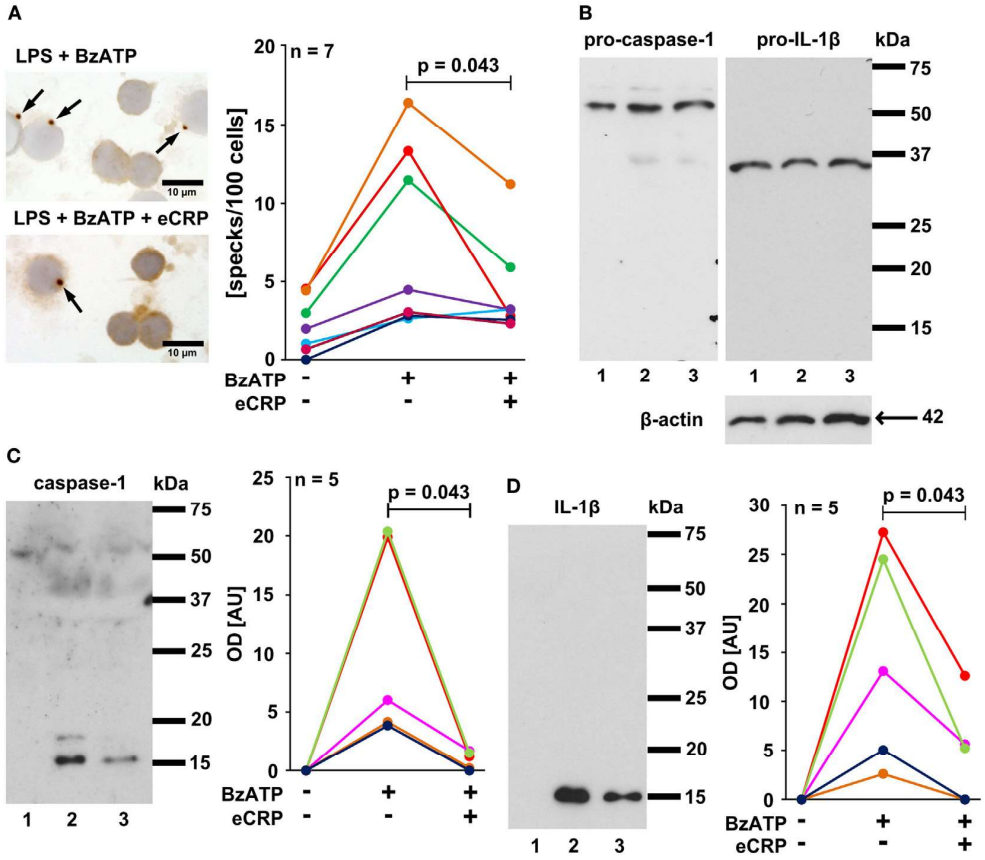

FIGURE 5 | Purified human endogenous C-reactive protein (eCRP) inhibits BzATP-induced inflammasome activation, caspase-1 activation, and maturation of interleukin-1β (IL-1β) in human peripheral blood mononuclear cells (PBMC). **(A)** ASC specks (arrows) were detected by immunocytochemistry using antibodies directed to ASC (brown staining). Cell nuclei were lightly counterstained with hemalum. Specks were induced by treatment with BzATP and occurred less frequently when eCRP was added concomitantly ($n = 7$). **(B–D)** Western blot experiments were performed using antibodies that detect both, the pro-forms and the mature forms of caspase-1 and IL-1β, respectively ($n = 5$). Cell lysates and cell culture supernatants were investigated from lipopolysaccharide (LPS)-primed PBMC (1), LPS-primed PBMC stimulated with BzATP (2), and LPS-primed PBMC stimulated with BzATP in the presence of eCRP (3). **(B)** Pro-caspase-1 and pro-IL-1β were detected in cell lysates at about equal amounts irrespective of stimulation with BzATP, whereas no mature forms were present, although the antibodies used detect both the pro-forms and the native forms. Detection of β-actin was included as a loading control. **(C)** Only mature caspase-1 and **(D)** IL-1β were present in concentrated cell culture supernatants. Stimulation with BzATP induced the release of both proteins and the release was reduced by eCRP. Immunoreactivity was measured by densitometry [optical density (OD)]. Data points obtained from individual blood donors are coded by different colors and connected by lines. Wilcoxon signed-rank test.

DISCUSSION

C-reactive protein is among the most frequently used clinical marker of inflammation, but its biological function is still a matter of debate. Here, we demonstrate that eCRP dose-dependently and efficiently inhibits the ATP-induced release of IL-1β from monocytic U937 cells at an IC_{50} of 4.9 µg/ml, corresponding to a marginally elevated blood CRP level (41). The activity of eCRP depends on the Ca^{2+}-dependent interaction with a small molecule, presumably PC (14, 15), and on nAChR subunits α7, α9, and α10. Stimulation of monocytic nAChRs in turn fully inhibits ATP-induced ion currents (**Figure 7**). Furthermore, eCRP is also active in primary human adherent PBMC, where it inhibits inflammasome and caspase-1 activation. First clinical evidence from multiple trauma patients is in line with the results obtained *in vitro* but does not prove causality. As IL-1β and IL-6 are the main inducers of hepatic CRP synthesis during systemic inflammation and IL-1β is an important stimulus for IL-6 expression, we suggest that CRP is a negative feedback regulator of the ATP-dependent production of mature IL-1β by human monocytes (**Figure 7**).

CRP is commonly regarded as an opsonizing agent that binds to PC present on the surfaces of some pro- and eukaryotic pathogens as well as on dying cells (14, 15). Phagocytosis of these opsonized particles is mediated *via* complement fixation and binding of CRP to different Fc-receptors expressed by phagocytic cells (14, 15). The suggested role of CRP in host defense against infections is, however, in sharp contrast to a large body

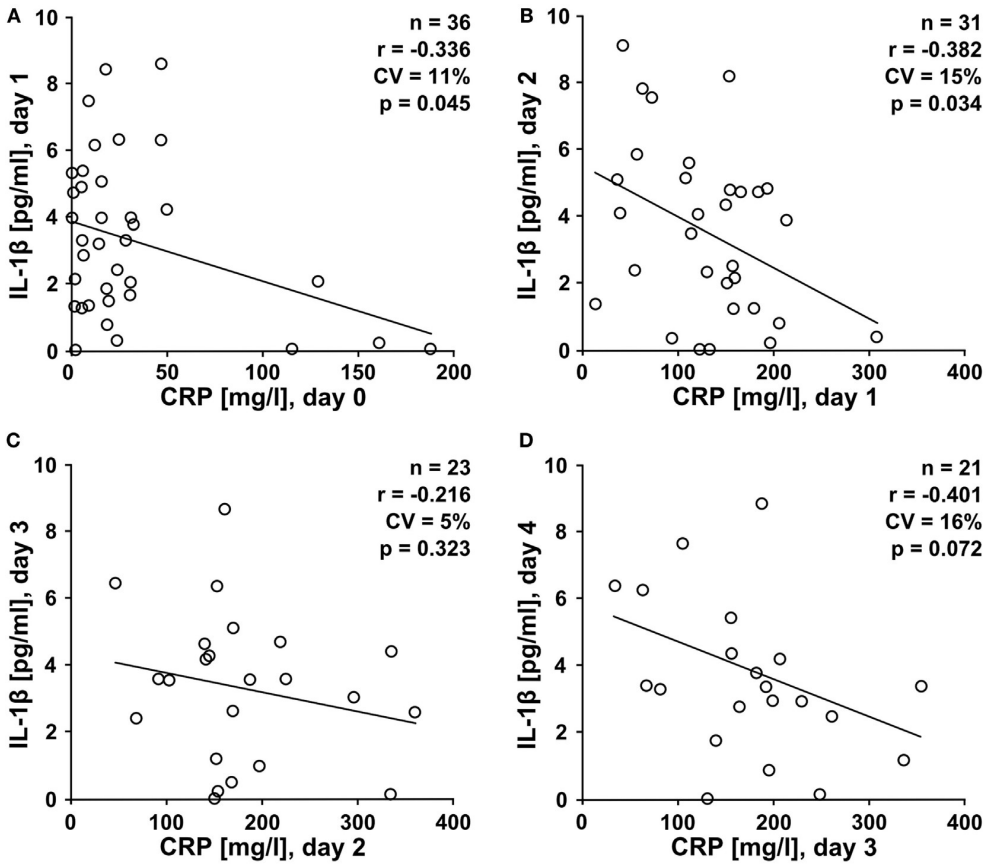

FIGURE 6 | High levels of C-reactive protein (CRP) negatively correlate with low interleukin-1β (IL-1β) levels in trauma patients. A prospective clinical study was performed on patients suffering from multiple traumata. Plasma levels of IL-1β were measured at daily intervals from the day of admission (day 0) until day 4 after trauma and a correlation analysis was performed with CRP values obtained one day earlier. **(A)** IL-1β at day 1 versus CRP at day 0 (n = 36), **(B)** IL-1β at day 2 versus CRP at day 1 (n = 31), **(C)** IL-1β at day 3 versus CRP at day 2 (n = 23), and **(D)** IL-1β at day 4 versus CRP at day 3 (n = 21). Linear regression analysis, r = correlation coefficient, CV = coefficient of variation.

of literature showing that PC-containing cell surface molecules of bacteria as well as PC-modified proteins secreted by helminths exert anti-inflammatory functions leading to immune evasion and chronic colonization of the host (47, 48). We demonstrated before that PC-modified bovine serum albumin and PC-modified lipooligosaccharides from *Haemophilus influenzae* control the release of IL-1β *via* mechanisms resembling those of CRP (30). This suggests that pathogens with PC-modified surfaces hijacked the here described mechanism, which underscores the biological and medical relevance of CRP.

We identify nAChRs composed of subunits α7, α9, and α10 as receptors for eCRP. Our data corroborate the almost 30 years old finding, that CRP binds Ca^{2+}-dependently to human monocytes with an EC_{50} of about 2.3 mg/l (49). Presumably, these authors measured the PC-dependent binding of CRP to monocytic nAChRs. We showed that PC-free CRP is inactive, and its activity can be reconstituted by adding low concentrations of PC that are in the range of those present in the serum of healthy persons (50). Thus, the interaction of eCRP with the ligand-binding site of nAChRs is probably mediated by CRP-bound PC. It has been shown before that endogenous human CRP can be laden with PC and other molecules with a PC group (14, 15, 29). In the same line, we demonstrated recently that high concentrations of free PC also inhibit the ATP-mediated release of IL-1β *via* nAChR (30–33), suggesting that CRP potentiates the effect of free PC. This is of importance *in vivo*, since typical human blood plasma concentrations of free PC are about 2 μM (50), while the IC_{50} of free PC is in the range of 10 μM, and 100 μM are needed for a full inhibition of the ATP-induced release of monocytic IL-1β (30).

Ligand-binding sites of conventional pentameric nAChRs that function as ligand-gated ion channels are formed by two neighboring nAChR subunits each and close upon binding of their cognate agonists (51). Interestingly, binding of a single ligand to only one of the five sites is sufficient for maximal ionotropic response of α7 nAChRs and additional binding sites enhance agonist sensitivity (52). The nAChR binding sites are dimensioned for classical nicotinic agonists but are certainly too small to enclose a macromolecule such as eCRP, suggesting

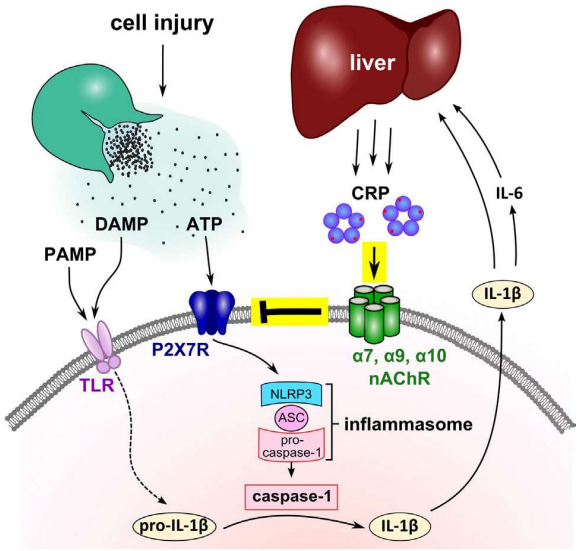

FIGURE 7 | Suggested mechanism of the mutual control of C-reactive protein (CRP) and interleukin-1β (IL-1β) during trauma-associated sterile inflammation. Injury causes the release of cytoplasmic danger-associated molecular patterns (DAMP) and ATP, and frequently microbiota, a source of pathogen-associated molecular patterns (PAMP), get access to the damaged tissue. DAMP and PAMP can bind to pattern recognition receptors such as toll-like receptors (TLR) at monocytic cells and induce the synthesis of pro-IL-1β, the inactive cytoplasmic precursor of IL-1β. Extracellular ATP activates the P2X7R and leads to inflammasome assembly and caspase-1 activation. Activated caspase-1 cleaves pro-IL-1β and enables the release of mature bioactive IL-1β that in turn, induces IL-6. IL-1β and IL-6 activate the hepatic synthesis of CRP and blood levels of CRP quickly rise to 1,000-fold. We demonstrated that purified human endogenous CRP inhibits the response of P2X7R to ATP *via* nAChR subunits α7, α9, and α10. The function of CRP critically depends on its association with small molecules such as phosphocholine (red dots) that seem to mediate the interaction with nicotinic receptors. We suggest that the CRP-mediated control of ATP-induced IL-1β release is a negative feedback loop that controls excessive systemic inflammation in response to injury. Although the nAChR is illustrated as a pentamer, its molecular structure remains to be elucidated.

that the structures of monocytic nAChRs are different. The activation of nAChR by a large molecule like eCRP is surprising but not unprecedented, as we demonstrated before that PC covalently bound to bovine serum albumin, PC-modified lipooligosaccharides and dipalmitoyl phosphatidylcholine function as potent agonists at unconventional monocytic nAChRs (30, 32). The IC_{50} values of PC-modified bovine serum albumin and lipooligosaccharides are also in the nanomolar range and hence, considerably lower than free PC, choline, or acetylcholine (30). If the structure of monocytic nAChRs differs from classical pentamers, it might be speculated that one eCRP pentamer interacts with several monocytic nAChRs. Once CRP binds to a nAChR, other receptors might be quickly recruited and activated. This hypothesis might explain the observed low IC_{50} values and the steep dose-response curve. This hypothesis may also apply to PC-modified albumin and to PC-lipooligosaccharides, because the PC-albumin investigated contained nine PC groups per BSA (30) and lipooligosaccharides tend to form micelles due to their amphiphilic structure. In contrast to the above-mentioned molecules with covalent PC modifications, it is also possible that eCRP delivers free PC to nAChRs because the affinities of PC to both molecules are in the same range (53). However, the considerably lower IC_{50} value of CRP compared to free PC (30) speaks against his theory. It may, however, explain, why eCRP acts as a silent agonist at canonical nAChRs heterologously expressed by *Xenopus* oocytes. Nevertheless, the structure of such unconventional nAChRs that exert metabotropic functions remains to be elucidated and it is even unclear if the leukocytic nAChR subunits form pentameric receptors at all.

Increasing evidence suggests that leukocytes in general respond to nicotinic stimuli *via* nAChRs with metabotropic responses, and no ligand-gated ion channel functions have been reported so far (44, 45, 54). A prominent example is the cholinergic regulation of the transcription and translation of pro-inflammatory cytokines by macrophages that is mediated *via* nAChR subunit α7 (54, 55). Here, we demonstrate that the P2X7R function of monocytic cells and the ATP-dependent release of IL-1β are fully inhibited by eCRP *via* stimulation of nAChRs containing subunits α7, α9, and α10. Nicotine, choline, and free PC exert similar effects albeit at much higher molar concentrations (30, 31, 33). The molecular signaling mechanism down-stream of monocytic nAChRs is currently under investigation.

Of note, eCRP neither induces ionotropic functions at monocytic nAChRs containing subunits α7, α9, and α10, nor at conventional nAChRs that were heterologously expressed by *Xenopus* oocytes. Similar results were shown before for free PC (31). Seemingly, eCRP is a novel agonist of nAChRs that exclusively induces metabotropic receptor functions and does not activate canonical ionotropic nAChR functions of excitable cells. Furthermore, we show that eCRP down-modulates the choline-induced ionotropic activity of heterologously expressed α9α10 nAChRs and provide evidence that CRP might be a silent agonist of nAChRs. As CRP and the nAChR subunits α7, α9, and α10 were highly conserved during evolution (14, 15, 51, 56), and mononuclear phagocytes are already present in primitive multicellular organisms (57), we speculate that the control of the P2X7R by nAChRs might be, in an evolutionary sense, older than neurotransmission.

We showed for the first time that in patients suffering from multiple traumata, IL-1β blood levels negatively correlated with preceding CRP levels. These results are in line with the hypothesis that the CRP-mediated control of IL-1β release is active *in vivo*, albeit they do not prove causality. According to Hill's criteria for causation (58), "a small association does not mean that there is not a causal effect, though the larger the association, the more likely that it is causal." Hill lists further criteria for causation including temporality, biological gradients, plausibility, and experimental evidence. Although, our data by and large meet with these criteria, more experimental and larger clinical multicenter studies are warranted, before we can dare to claim causality. The absence of a negative correlation of CRP levels with IL-18 and HMGB1 may be due to the ubiquitous expression of these inflammasome-dependent mediators, in contrast to IL-1β which is mainly produced by monocytes/macrophages (59).

Our data suggest that elevated CRP levels attenuate inflammatory diseases that are caused by ATP-induced inflammasome activation. It remains to be investigated if the here described mechanism also contributes to the protection against experimental inflammation in animals overexpressing human CRP (25–28). Interestingly, CRP was recently shown to impair dendritic cell development, maturation and function (60). This anti-inflammatory mechanism involves the inhibitory Fcγ receptor IIB (60). The potential clinical implications of these and our findings deserve further investigation, including a careful consideration of the known pro-inflammatory functions of CRP (14, 15, 22).

Several therapeutics targeting IL-1 or its receptor, among them the IL-1 receptor antagonist anakinra, the decoy receptor rilonacept and the neutralizing monoclonal anti-IL-1β antibody canakinumab, were tested in large trials but never reached the clinical arena for the treatment of SIRS (4). A major disadvantage of these approaches might be the general inhibition of IL-1β that should result in an impaired host defense against infections. By contrast, we showed that eCRP specifically inhibits the P2X7R-mediated response to extracellular ATP that is a danger signal mainly associated with mechanical cell damage (61). Viral, bacterial, and fungal pathogens activate numerous additional ATP-independent pathways of inflammasome activation and IL-1β maturation (1). Hence, we speculate that CRP predominantly inhibits trauma-associated release of IL-1β without preventing the IL-1β response to infection.

In conclusion, CRP efficiently inhibits ATP-dependent inflammasome activation and IL-1β release from human monocytic blood cells *in vitro*. This effect of CRP seems to depend on bound PC and activation of non-canonical nAChRs that efficiently inhibit the ion channel functions of monocytic ATP receptors. In the same line, we provide the first clinical evidence that elevated CRP levels might reduce systemic IL-1β release in patients suffering from multiple traumata.

ETHICS STATEMENT

The local ethics committee at the University of Giessen approved all studies on primary human cells (approval No. 81/13). The study protocol for clinical sample collection (trial registration: DRKS00010991) was approved by the ethics committee of the medical faculty Giessen, Germany (No. 164/14) and performed in accordance with the Helsinki Declaration. All patients completed written informed consent prior to study entry.

AUTHOR CONTRIBUTIONS

KR, SS, ATZ, MK, JD, SH, SW, and AJH performed experiments and interpreted results; IA, MP, SR, AH, and CK recruited healthy donors and patients and interpreted results; IRK performed statistical analyses and interpreted results; WK, MS, K-DS, WP, JMM, and CK were involved in study design, interpretation of the results, and in writing; in addition, JMM provided seminal reagents; VG designed the study, interpreted data, and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01604/full#supplementary-material.

REFERENCES

1. Broz P, Dixit VM. Inflammasomes: mechanism of assembly, regulation and signalling. *Nat Rev Immunol* (2016) 16(7):407–20. doi:10.1038/nri.2016.58
2. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* (1992) 101(6):1644–55. doi:10.1378/chest.101.6.1644
3. Xiao W, Mindrinos MN, Seok J, Cuschieri J, Cuenca AG, Gao H, et al. A genomic storm in critically injured humans. *J Exp Med* (2011) 208(13): 2581–90. doi:10.1084/jem.20111354
4. Dinarello CA, Simon A, van der Meer JW. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat Rev Drug Discov* (2012) 11(8):633–52. doi:10.1038/nrd3800
5. Manson J, Thiemermann C, Brohi K. Trauma alarmins as activators of damage-induced inflammation. *Br J Surg* (2012) 99(Suppl 1):12–20. doi:10.1002/bjs.7717
6. Westphal M, Kampmeier T. The race against the "septic shark". *Crit Care* (2015) 19(Suppl 3):S11. doi:10.1186/cc14729
7. Gross O, Thomas CJ, Guarda G, Tschopp J. The inflammasome: an integrated view. *Immunol Rev* (2011) 243(1):136–51. doi:10.1111/j.1600-065X.2011.01046.x
8. Rathinam VA, Vanaja SK, Fitzgerald KA. Regulation of inflammasome signaling. *Nat Immunol* (2012) 13(4):333–42. doi:10.1038/ni.2237
9. Rathinam VA, Fitzgerald KA. Inflammasome complexes: emerging mechanisms and effector functions. *Cell* (2016) 165(4):792–800. doi:10.1016/j.cell.2016.03.046
10. Grahames CB, Michel AD, Chessell IP, Humphrey PP. Pharmacological characterization of ATP- and LPS-induced IL-1beta release in human monocytes. *Br J Pharmacol* (1999) 127(8):1915–21. doi:10.1038/sj.bjp.0702732
11. Cekic C, Linden J. Purinergic regulation of the immune system. *Nat Rev Immunol* (2016) 16(3):177–92. doi:10.1038/nri.2016.4
12. Novick D, Kim S, Kaplanski G, Dinarello CA. Interleukin-18, more than a Th1 cytokine. *Semin Immunol* (2013) 25(6):439–48. doi:10.1016/j.smim.2013.10.014

13. Yang H, Antoine DJ, Andersson U, Tracey KJ. The many faces of HMGB1: molecular structure-functional activity in inflammation, apoptosis, and chemotaxis. *J Leukoc Biol* (2013) 93(6):865–73. doi:10.1189/jlb.1212662
14. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest* (2003) 111(12):1805–12. doi:10.1172/JCI200318921
15. Mantovani A, Garlanda C, Doni A, Bottazzi B. Pentraxins in innate immunity: from C-reactive protein to the long pentraxin PTX3. *J Clin Immunol* (2008) 28(1):1–13. doi:10.1007/s10875-007-9126-7
16. Badimon L, Peña E, Arderiu G, Padró T, Slevin M, Vilahur G, et al. C-reactive protein in atherothrombosis and angiogenesis. *Front Immunol* (2018) 9:430. doi:10.3389/fimmu.2018.00430
17. Del Giudice M, Gangestad SW. Rethinking IL-6 and CRP: why they are more than inflammatory biomarkers, and why it matters. *Brain Behav Immun* (2018) 70:61–75. doi:10.1016/j.bbi.2018.02.013
18. O'Toole MS, Bovbjerg DH, Renna ME, Lekander M, Mennin DS, Zachariae R. Effects of psychological interventions on systemic levels of inflammatory biomarkers in humans: a systematic review and meta-analysis. *Brain Behav Immun* (2018). doi:10.1016/j.bbi.2018.04.005
19. Ballou SP, Lozanski G. Induction of inflammatory cytokine release from cultured human monocytes by C-reactive protein. *Cytokine* (1992) 4(5):361–8. doi:10.1016/1043-4666(92)90079-7
20. Galve-de Rochemonteix B, Wiktorowicz K, Kushner I, Dayer JM. C-reactive protein increases production of IL-1 alpha, IL-1 beta, and TNF-alpha, and expression of mRNA by human alveolar macrophages. *J Leukoc Biol* (1993) 53(4):439–45. doi:10.1002/jlb.53.4.439
21. Ridker PM. From C-reactive protein to interleukin-6 to interleukin-1: moving upstream to identify novel targets for atheroprotection. *Circ Res* (2016) 118(1):145–56. doi:10.1161/CIRCRESAHA.115.306656
22. Braig D, Nero TL, Koch H-G, Kaiser B, Wang X, Thiele JR, et al. Transitional changes in the CRP structure lead to the exposure of proinflammatory binding sites. *Nat Commun* (2017) 8:14188. doi:10.1038/ncomms14188
23. Thiele JR, Zeller J, Kiefer J, Braig D, Kreuzaler S, Lenz Y, et al. A conformational change in C-reactive protein enhances leukocyte recruitment and reactive oxygen species generation in ischemia/reperfusion injury. *Front Immunol* (2018) 9:95. doi:10.3389/fimmu.2018.00675
24. Tilg H, Vannier E, Vachino G, Dinarello CA, Mier JW. Antiinflammatory properties of hepatic acute phase proteins: preferential induction of interleukin 1 (IL-1) receptor antagonist over IL-1 beta synthesis by human peripheral blood mononuclear cells. *J Exp Med* (1993) 178(5):1629–36. doi:10.1084/jem.178.5.1629
25. Mold C, Rodriguez W, Rodic-Polic B, Du Clos TW. C-reactive protein mediates protection from lipopolysaccharide through interactions with Fc gamma R. *J Immunol* (2002) 169(12):7019–25. doi:10.4049/jimmunol.169.12.7019
26. Heuertz RM, Xia D, Samols D, Webster RO. Inhibition of C5a des Arg-induced neutrophil alveolitis in transgenic mice expressing C-reactive protein. *Am J Physiol* (1994) 266(6 Pt 1):L649–54.
27. Jiang S, Xia D, Samols D. Expression of rabbit C-reactive protein in transgenic mice inhibits development of antigen-induced arthritis. *Scand J Rheumatol* (2006) 35(5):351–5. doi:10.1080/03009740600757963
28. Torzewski J, Fan J, Schunkert H, Szalai A, Torzewski M. C-reactive protein and arteriosclerosis. *Mediators Inflamm* (2014) 2014:646817. doi:10.1155/2014/683598
29. Thompson D, Pepys MB, Wood SP. The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure* (1999) 7(2):169–77. doi:10.1016/S0969-2126(99)80023-9
30. Hecker A, Küllmar M, Wilker S, Richter K, Zakrzewicz A, Atanasova S, et al. Phosphocholine-modified macromolecules and canonical nicotinic agonists inhibit ATP-induced IL-1β release. *J Immunol* (2015) 195(5):2325–34. doi:10.4049/jimmunol.1400974
31. Richter K, Mathes V, Fronius M, Althaus M, Hecker A, Krasteva-Christ G, et al. Phosphocholine – an agonist of metabotropic but not of ionotropic functions of α9-containing nicotinic acetylcholine receptors. *Sci Rep* (2016) 6:28660. doi:10.1038/srep28660
32. Backhaus S, Zakrzewicz A, Richter K, Damm J, Wilker S, Fuchs-Moll G, et al. Surfactant inhibits ATP-induced release of interleukin-1β via nicotinic acetylcholine receptors. *J Lipid Res* (2017) 58(6):1055–66. doi:10.1194/jlr.M071506
33. Zakrzewicz A, Richter K, Agné A, Wilker S, Siebers K, Fink B, et al. Canonical and novel non-canonical cholinergic agonists inhibit ATP-induced release of monocytic interleukin-1β via different combinations of nicotinic acetylcholine receptor subunits α7, α9 and α10. *Front Cell Neurosci* (2017) 11:491. doi:10.3389/fncel.2017.00189
34. Whiteaker P, Christensen S, Yoshikami D, Dowell C, Watkins M, Gulyas J, et al. Discovery, synthesis, and structure activity of a highly selective alpha7 nicotinic acetylcholine receptor antagonist. *Biochemistry* (2007) 46(22):6628–38. doi:10.1021/bi7004202
35. Innocent N, Livingstone PD, Hone A, Kimura A, Young T, Whiteaker P, et al. Alpha-conotoxin Arenatus IBV11L,V16D corrected is a potent and selective antagonist at rat and human native alpha7 nicotinic acetylcholine receptors. *J Pharmacol Exp Ther* (2008) 327(2):529–37. doi:10.1124/jpet.108.142943
36. Romero HK, Christensen SB, Di Cesare Mannelli L, Gajewiak J, Ramachandra R, Elmslie KS, et al. Inhibition of α9α10 nicotinic acetylcholine receptors prevents chemotherapy-induced neuropathic pain. *Proc Natl Acad Sci U S A* (2017) 114(10):E1825–32. doi:10.1073/pnas.1621433114
37. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (1970) 227(5259):680–5. doi:10.1038/227680a0
38. Taylor CW. Regulation of IP3 receptors by cyclic AMP. *Cell Calcium* (2017) 63:48–52. doi:10.1016/j.ceca.2016.10.005
39. Baker SP, O'Neill B, Haddon W, Long WB. The injury severity score: a method for describing patients with multiple injuries and evaluating emergency care. *J Trauma* (1974) 14(3):187–96. doi:10.1097/00005373-197403000-00001
40. North RA. Molecular physiology of P2X receptors. *Physiol Rev* (2002) 82(4):1013–67. doi:10.1152/physrev.00015.2002
41. Kushner I, Antonelli MJ. What should we regard as an "elevated" C-reactive protein level? *Ann Intern Med* (2015) 163(4):326. doi:10.7326/L15-5126
42. Papke RL, Bencherif M, Lippiello P. An evaluation of neuronal nicotinic acetylcholine receptor activation by quaternary nitrogen compounds indicates that choline is selective for the alpha 7 subtype. *Neurosci Lett* (1996) 213(3):201–4. doi:10.1016/0304-3940(96)12889-5
43. Baker ER, Zwart R, Sher E, Millar NS. Pharmacological properties of alpha 9 alpha 10 nicotinic acetylcholine receptors revealed by heterologous expression of subunit chimeras. *Mol Pharmacol* (2004) 65(2):453–60. doi:10.1124/mol.65.2.453
44. Peng H, Ferris RL, Matthews T, Hiel H, Lopez-Albaitero A, Lustig LR. Characterization of the human nicotinic acetylcholine receptor subunit alpha (alpha) 9 (CHRNA9) and alpha (alpha) 10 (CHRNA10) in lymphocytes. *Life Sci* (2004) 76(3):263–80. doi:10.1016/j.lfs.2004.05.031
45. Papke RL. Merging old and new perspectives on nicotinic acetylcholine receptors. *Biochem Pharmacol* (2014) 89(1):1–11. doi:10.1016/j.bcp.2014.01.029
46. Erttmann SF, Härtlova A, Sloniecka M, Raffi FA, Hosseinzadeh A, Edgren T, et al. Loss of the DNA damage repair kinase ATM impairs inflammasome-dependent anti-bacterial innate immunity. *Immunity* (2016) 45(1):106–18. doi:10.1016/j.immuni.2016.06.018
47. Grabitzki J, Lochnit G. Immunomodulation by phosphocholine – biosynthesis, structures and immunological implications of parasitic PC-epitopes. *Mol Immunol* (2009) 47(2–3):149–63. doi:10.1016/j.molimm.2009.09.035
48. Clark SE, Weiser JN. Microbial modulation of host immunity with the small molecule phosphorylcholine. *Infect Immun* (2013) 81(2):392–401. doi:10.1128/IAI.01168-12
49. Ballou SP, Buniel J, Macintyre SS. Specific binding of human C-reactive protein to human monocytes in vitro. *J Immunol* (1989) 142(8):2708–13.
50. Ilcol YO, Ozbek R, Hamurtekin E, Ulus IH. Choline status in newborns, infants, children, breast-feeding women, breast-fed infants and human breast milk. *J Nutr Biochem* (2005) 16(8):489–99. doi:10.1016/j.jnutbio.2005.01.011
51. Boffi JC, Marcovich I, Gill-Thind JK, Corradi J, Collins T, Lipovsek MM, et al. Differential contribution of subunit interfaces to α9α10 nicotinic acetylcholine receptor function. *Mol Pharmacol* (2017) 91(3):250–62. doi:10.1124/mol.116.107482
52. Andersen N, Corradi J, Sine SM, Bouzat C. Stoichiometry for activation of neuronal α7 nicotinic receptors. *Proc Natl Acad Sci U S A* (2013) 110(51):20819–24. doi:10.1073/pnas.1315775110
53. Christopeit T, Gossas T, Danielson UH. Characterization of Ca^{2+} and phosphocholine interactions with C-reactive protein using a surface plasmon resonance biosensor. *Anal Biochem* (2009) 391(1):39–44. doi:10.1016/j.ab.2009.04.037
54. Olofsson PS, Rosas-Ballina M, Levine YA, Tracey KJ. Rethinking inflammation: neural circuits in the regulation of immunity. *Immunol Rev* (2012) 248(1):188–204. doi:10.1111/j.1600-065X.2012.01138.x

55. Kawashima K, Fujii T, Moriwaki Y, Misawa H, Horiguchi K. Reconciling neuronally and nonneuronally derived acetylcholine in the regulation of immune function. *Ann N Y Acad Sci* (2012) 1261:7–17. doi:10.1111/j.1749-6632.2012.06516.x
56. Lipovsek M, Im GJ, Franchini LF, Pisciottano F, Katz E, Fuchs PA, et al. Phylogenetic differences in calcium permeability of the auditory hair cell cholinergic nicotinic receptor. *Proc Natl Acad Sci U S A* (2012) 109(11):4308–13. doi:10.1073/pnas.1115488109
57. Buchmann K. Evolution of innate immunity: clues from invertebrates via fish to mammals. *Front Immunol* (2014) 5:459. doi:10.3389/fimmu.2014.00459
58. Hill AB. The environment and disease: association or causation? *Proc R Soc Med* (1965) 58:295–300.
59. Vladimer GI, Marty-Roix R, Ghosh S, Weng D, Lien E. Inflammasomes and host defenses against bacterial infections. *Curr Opin Microbiol* (2013) 16(1):23–31. doi:10.1016/j.mib.2012.11.008
60. Jimenez RV, Wright TT, Jones NR, Wu J, Gibson AW, Szalai AJ. C-reactive protein impairs dendritic cell development, maturation, and function: implications for peripheral tolerance. *Front Immunol* (2018) 9:372. doi:10.3389/fimmu.2018.00372
61. Cauwels A, Rogge E, Vandendriessche B, Shiva S, Brouckaert P. Extracellular ATP drives systemic inflammation, tissue damage and mortality. *Cell Death Dis* (2014) 5:e1102. doi:10.1038/cddis.2014.70

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Amyloid Beta Peptide ($A\beta_{1-42}$) Reverses the Cholinergic Control of Monocytic IL-1β Release

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Abstract: Amyloid-β peptide ($A\beta_{1-42}$), the cleavage product of the evolutionary highly conserved amyloid precursor protein, presumably plays a pathogenic role in Alzheimer's disease. $A\beta_{1-42}$ can induce the secretion of the pro-inflammatory cytokine intereukin-1β (IL-1β) in immune cells within and out of the nervous system. Known interaction partners of $A\beta_{1-42}$ are α7 nicotinic acetylcholine receptors (nAChRs). The physiological functions of $A\beta_{1-42}$ are, however, not fully understood. Recently, we identified a cholinergic mechanism that controls monocytic release of IL-1β by canonical and non-canonical agonists of nAChRs containing subunits α7, α9, and/or α10. Here, we tested the hypothesis that $A\beta_{1-42}$ modulates this inhibitory cholinergic mechanism. Lipopolysaccharide-primed monocytic U937 cells and human mononuclear leukocytes were stimulated with the P2X7 receptor agonist 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate triethylammonium salt (BzATP) in the presence or absence of nAChR agonists and $A\beta_{1-42}$. IL-1β concentrations were measured in the supernatant. $A\beta_{1-42}$ dose-dependently (IC_{50} = 2.54 μM) reversed the inhibitory effect of canonical and non-canonical nicotinic agonists on BzATP-mediated IL-1β-release by monocytic cells, whereas reverse $A\beta_{42-1}$ was ineffective. In conclusion, we discovered a novel pro-inflammatory $A\beta_{1-42}$ function that enables monocytic IL-1β release in the presence of nAChR agonists. These findings provide evidence for a novel physiological function of $A\beta_{1-42}$ in the context of sterile systemic inflammation.

Keywords: amyloid beta peptide; interleukin-1β; nicotinic acetylcholine receptors; monocytes; systemic inflammation; purinergic signaling; P2X7 receptor; adenosine triphosphate

1. Introduction

In the context of trauma including major surgical trauma, the pro-inflammatory cytokine interleukin-1 β (IL-1β) is released and importantly contributes to sterile hyperinflammation that may result in barrier dysfunction, translocation of bacteria from the gastro-intestinal tract to the circulation, and, eventually, in sepsis. At the same time, however, IL-1β is needed for an effective prevention and resolution of septic complications [1–4]. Hence, context-specific mechanisms controlling the production and release of IL-1β are of utmost clinical interest.

Synthesis, maturation, and secretion of IL-1β by monocytic cells are carefully regulated. Pathogen- or danger-associated molecular patterns (PAMPs and DAMPs) are first signals that stimulate pattern recognition receptors expressed, among others, by monocytic cells [3]. An example for a PAMP is lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria. LPS is a prototypical first signal, that stimulates Toll-like receptor (TLR)-4 and induces the synthesis of the inactive precursor protein pro-IL-1β [4]. Extracellular ATP originating from damaged and/or lytic cells is a typical second DAMP promoting the maturation of pro-IL-1β [2,4]. After binding to the P2X7 receptor (P2X7R), ATP induces the assembly of the NLRP3 (NACHT, LRR, and PYD domains containing protein 3) inflammasome that activates the protease caspase-1. Caspase-1 cleaves pro-IL-1β and finally, mature IL-1β is secreted [2–4]. Mechanisms that specifically control the ATP-induced secretion of IL-1β might prevent post-operative hyperinflammation, without fully inhibiting host defense against pathogens that involves several ATP-independent mechanisms of IL-1β release.

Recently, we discovered a novel endogenous cholinergic mechanism that efficiently controls the ATP-mediated NLRP3 inflammasome activation and, hence, release of mature IL-1β by human monocytic cells [5]. We found that activation of nicotinic acetylcholine receptors (nAChRs) containing the evolutionary highly conserved subunits α7, α9, and α10 by classical agonists like acetylcholine (ACh), choline, or nicotine, dose-dependently inhibits the ATP-induced IL-1β release by human monocytic cells [6–8]. In this context, phosphocholine (PC) was identified as an agonist of nAChRs [6–8]. Interestingly, PC is a ligand of the pentraxin C-reactive protein (CRP), an acute phase protein present in human blood that is a widely used clinical marker for inflammation [9,10]. We found that endogenous CRP-PC complexes are potent nAChR agonists that also inhibit the ATP-dependent inflammasome assembly and IL-1β release by human monocytic cells, whereas PC-free CRP is ineffective [11]. Moreover, we discovered that dipalmitoyl phosphatidylcholine (DPPC) and metabolites of phosphatidylcholines such as lysophosphatidylcholine or glycerophosphocholine (GPC) also function as nAChR agonists [8,12].

Ample evidence suggests that amyloid precursor protein (APP) and its cleavage products interfere with the cholinergic system. The α7 nAChR subunits are direct interaction partners for Aβ peptides [13–19]. Therefore, we decided to investigate the interaction between APP, nAChRs, and the IL-1β system. APP is an evolutionary highly conserved cell surface protein that is expressed by tissues of all germ layers such as the nervous system, skin, adipose tissue, skeletal muscle, heart, kidney, spleen, gastrointestinal tract, thymus, lung, and liver [20–24]. APP and its cleavage products, including the Aβ peptides, are mainly known for their putative roles in Alzheimer's disease (AD), the most common dementia and primary neurodegenerative disorder in elderly persons [23,25–28]. The essential physiological functions of APP that can be predicted because of its evolutionary conservation, however, are incompletely understood.

APP can be cleaved via the non-amyloidogenic pathway that involves the participation of α- and γ-secretases and results in the release of a secreted APP from (sAPP) [21]. In contrast, the amyloidogenic way, that seems to promote the pathogenesis of AD, involves double cleavage by β-secretase (β-site

APP-cleaving enzyme1, BACE1) and γ-secretase (a complex containing presenilin-1 or -2) resulting in different types of Aβ, among them $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ [21,22]. $A\beta_{1\text{-}42}$ is prone to form fibrils and contributes to plaque formations in the brain of AD patients [21–23,29–31]. In addition, there are several non-canonical pathways through which APP can be processed as reviewed by Müller and colleagues [32].

Several physiological binding partners of Aβ peptides were identified like apolipoprotein E (ApoE), the receptor for advanced glycosylation end products (RAGE) and serpin–enzyme complex receptor (SEC-R) [33,34]. Moreover, APP can function as a cell surface receptor-like protein that interacts with more than 200 extracellular and intracellular binding partners [32]. In the skin, APP and Aβ peptides seem to regulate epidermal cell differentiation and modulate proliferation and cell migration [20,35]. Intestinal APP and Aβ peptides were suggested to regulate enteric neurons, macrophages, and epithelial cells and even seem to influence absorption and barrier function [20,36]. In addition, multiple non-neuronal immunological effects on leukocytes have been described. Both fibrillary and monomeric $A\beta_{1\text{-}42}$ can trigger inflammasome activation in mononuclear leukocytes and induce the secretion of the pro-inflammatory cytokines IL-1β and IL-18 [29,37–40]. In addition, inflammasome-independent cytokines IL-6, tumor necrosis factor-α (TNF-α), and monocyte chemotactic protein-1 (MCP-1) are released [29,37,39–42].

In this study, we test the hypothesis that $A\beta_{1\text{-}42}$ reverses the inhibitory effect of nicotinic agonists on ATP-mediated release of IL-1β by human monocytic cells. We provide evidence that $A\beta_{1\text{-}42}$ functions as an antagonist at monocytic nAChRs containing subunit α7, α9, and α10, in human monocytic U937 cells and primary peripheral mononuclear blood leukocytes (PBMCs). Moreover, we show first clinical data on patients, who underwent major surgery, that support the hypothesis, that this mechanism is also active in vivo.

2. Materials and Methods

2.1. Reagents

Acetylcholine chloride, nicotine hydrogen tartrate salt, phosphocholine chloride calcium salt tetrahydrate (PC), L-α-glycerophosphorylcholine (GPC), 1,2-dipalmitoyl-SN-glycero-3-phosphocholine (DPPC), C-reactive protein (CRP) isolated from human pleural fluid (Merck Millipore AG732), $A\beta_{1\text{-}42}$ (Sigma-Aldrich A9810) as well as reverse $A\beta_{42\text{-}1}$ (Sigma-Aldrich SCP0048), and lipopolysaccharide (LPS) from *Escherichia coli* (Sigma-Aldrich L2654) were purchased from Merck (Darmstadt, Germany). $A\beta_{1\text{-}42}$ and $A\beta_{42\text{-}1}$ were dissolved in an ultrasonic bath and stock solutions were immediately frozen at −20 °C. Later, stock solutions were thawed in an ultrasonic bath for at least 5 min before use. 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate trieethylammonium salt (BzATP) was provided by Jena Bioscience (Jena, Germany). [V11L, V16D]ArIB and RgIA4 were produced and characterized as described previously [5,7,43–45]. Concentrations of all compounds used in this study were optimized in previous experiments on human monocytic U937 cells [6–8,11,12].

2.2. U937 Cells

Human monocytic U937 cells from a histiocytic lymphoma cell line were obtained from the German Collection of Microorganisms and Cell Culture (Braunschweig, Germany). Cells were cultured at 37 °C, 5% CO_2 in RPMI 1640 medium (Gibco, Life Technologies, Darmstadt, Germany) with 10% fetal calf serum (FCS, Biochrome, Berlin, Germany) and 2 mM L-glutamine (Gibco). After seeding in a 24-well plate (10^6 cells/mL), U937 cells were primed for 5 h with 1 µg/mL LPS. Thereafter, cells were stimulated with the P2X7R agonist BzATP (100 µM) for 30 min in the presence or absence of $A\beta_{1\text{-}42}$, $A\beta_{42\text{-}1}$, or agonists of nAChRs. Cell-free cell culture supernatants were collected and stored at −20 °C.

2.3. Human Peripheral Blood Mononuclear Cells (PBMCs)

The study was approved by the ethics committee of the medical faculty Giessen, Germany (No. 90/18) and performed in accordance with the Helsinki Declaration. Each volunteer gave written informed consent. Peripheral blood mononuclear cells (PBMCs) were freshly isolated from blood obtained from self-reported healthy, non-smoking adult volunteers. Blood was drawn into sterile syringes containing 1 mM EDTA (bioWORLD, Dublin, OH, USA) per ml blood and PBMCs were separated on Leucosep gradients (Greiner Bio-One, Frickenhausen, Germany). Before gradient centrifugation, LPS (5 ng/mL) was added to blood samples. Thereafter, PBMCs were cultured in 24-well plates at a density of 1×10^6 cells/mL in Monocyte Attachment Medium (PromoCell, Heidelberg, Germany) for 3 h. Non-adherent cells were removed, and cell culture medium was replaced by fresh RPMI 1640 medium (Sigma-Aldrich R8758). Stimulation with BzATP in the presence or absence of $A\beta_{1\text{-}42}$, agonists or antagonists of nAChRs was done as described for U937 cells.

2.4. Human Plasma Samples of Patients Undergoing Major Surgery

The study was approved by the ethics committee of the medical faculty Giessen, Germany (No. 159/17) and performed in accordance with the Helsinki Declaration. Written informed consent was given by each patient or patient's legal representative. Male and female patients aged 48–83 years (median age = 67) undergoing major surgery of the pancreas (n = 8; female = 4, male = 4) or esophagectomy (n = 4; all male) were recruited at the University Hospital of Giessen, Germany. All patients underwent elective surgery for oncological tumor resection of the esophagus (n = 8) or the pancreas (n = 4). Patients with preexisting increased inflammatory parameters and septic patients were excluded from the study. The medication prescribed prior to the surgery was checked for potential interactions with IL-1β, as far as known from the literature. All patients underwent anesthesia according to a standardized regimen. The first venous blood sample was collected shortly before the operation as well as 0 to 2 h, 24 to 48 h, 72 to 96 h, and 7 to 9 days after the operation. Venous blood was collected into EDTA-buffered collection tubes (Sarstedt, Nürnberg, Germany) and subsequently subjected to a Ficoll-Hypaque (Sigma-Aldrich, Darmstadt, Germany) gradient. After centrifugation, the upper blood plasma layer was harvested and stored at −20 °C.

2.5. Cell Viability and Cytokine Measurements

To test for cell viability at the end of the cell culture experiments on U937 cells and PBMCs, non-radioactive cytotoxicity assay (Promega, Madison, WI, USA) was used to measure lactate dehydrogenase (LDH) activity in cell free supernatants as indicated by the supplier. LDH values are given as percentage of the total LDH content of lysed control cells.

The concentrations of IL-1β were measured in the cell free supernatants and human plasma samples by using the Human IL-1 beta/IL-1F2 DuoSet® enzyme-linked immunosorbent assay (ELISA) from R&D Systems (Minneapolis, MN, USA; sensitivity: less than 1 pg/mL human IL-1β) according to the manufacturer. $A\beta_{1\text{-}42}$ concentrations in human plasma samples were measured by the human amyloid beta (aa1-42) Quantikine ELISA Kit from R&D Systems (Minneapolis, MN, USA; sensitivity: 4.73 pg/mL human $A\beta_{1\text{-}42}$) as indicated by the manufacturer.

2.6. Human P2X7R Expressing HEK293 Cell Line

To generate a stable human P2X7R expressing cell line, a previously cloned cDNA [46] encoding the full-length human P2X7R (hP2X7R) subunit was directionally subcloned via HindIII/ApaI restriction sites into the inducible expression vector pcDNA5/FRT/TO (Invitrogen, Thermo Fisher Scientific, Dreieich, Germany). Except for a H155Y polymorphism, the deduced 595 residues hP2X7R sequence corresponds to UniProt accession ID Q99572.4. The resulting hP2X7R-pcDNA5/FRT/TO plasmid was co-transfected with the Flp recombinase vector, pOG44, into Flp-InTM T-REx™-293 host cells (Invitrogen) using Lipofectamine LTX reagent (Invitrogen). Stably transfected cells (P2X7R-HEK cells)

were selected for hygromycin B resistance in DMEM high glucose medium (Gibco) supplemented with 10% FCS (Biochrome), 2 mM L-glutamine (Gibco), 100 µg/mL hygromycin B (InvivoGen, Toulouse, France), 15 µg/mL blasticidin (InvivoGen), 1 µg/mL % penicillin-streptomycin (Gibco). The Flp-In T-REx system creates isogenic clones, making monoclonal selection unnecessary.

2.7. Whole-Cell Patch-Clamp Recordings

For electrophysiological experiments, P2X7R-HEK cells were seeded in cell culture dishes (Nunc, Roskilde, Denmark). P2X7R expression was induced by the addition of 1 µg/mL tetracycline (Sigma-Aldrich) and incubation at 37 °C, 5% CO for 24–48 h. Thereafter, the culture medium was replaced by a bath solution containing 5.4 mM KCl, 120 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid), and 25 mM glucose (all purchased from Merck; pH 7.4). Whole-cell patch clamp recordings were performed as described previously [6,11]. In brief, patch pipettes were pulled from borosilicate glass capillaries (outer diameter 1.6 mm, Hilgenberg, Malsfeld, Germany) to a resistance of 2 to 4 MΩ using an automated puller (Zeitz, Augsburg, Germany). Pipettes were filled with pipette solution containing 120 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 11 mM ethylene glycol tetra acetic acid and 20 mM glucose (all purchased from Merck; pH 7.3). The membrane potential of P2X7R-HEK cells was voltage-clamped to −60 mV and transmembrane currents were amplified with an EPC 9 amplifier (HEKA, Lambrecht, Germany) and acquired via an ITC-16 interface with the Pulse software (HEKA). A pressure-driven microperfusion system was used to apply BzATP (100 µM) and $A\beta_{1\text{-}42}$ (5 µg/mL). All experiments were performed at room temperature.

2.8. Measurements of Intracellular Ca^{2+}

To measure intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), P2X7R-HEK cells were seeded in glass bottom culture dishes (CELLview™, Greiner Bio-One, Kremsmünster, Austria) and P2X7R expression was induced by the addition of 1 µg/mL tetracycline (Sigma-Aldrich T7660) and culturing at 37 °C, 5% CO_2, for 24–48 h. Thereafter, the culture medium was replaced by the same bath solution as described for the whole-cell patch-clamp recordings. Measurements were performed as described previously [11]. In brief, P2X7R-HEK cells were loaded with 3.3 µM Fura-2/AM (Thermo Fisher Scientific) for 25 min at 37 °C. Fura-2/AM was excited at 340 and 380 nm wavelengths and the fluorescence emission was measured at 510 nm. Three independent batches of P2X7R-HEK cells were used in these experiments and a total number of 411 cells were tracked individually. The fluorescence intensity ratio of 340:380 nm was recorded. All experiments were performed at room temperature.

2.9. Statistical Analyses and Data Processing

Results were analyzed using SPSS® (Version 23, IBM®, Armonk, NY, USA). The IC_{50} value of $A\beta_{1\text{-}42}$ in human U937 cells was determined in GraphPad Prism® (Version 6, GaphPad Software) by fitting log-transformed concentration values and the original effect data. Multiple groups were first analyzed by the non-parametric Kruskal–Wallis test. In case of $p \leq 0.05$, the non-parametric Mann–Whitney U test was performed to compare between individual groups and again, a $p \leq 0.05$ was considered as evidence for statistical significance. Paired data were analyzed first by the Friedman test followed by the Wilcoxon signed-rank test. Data were visualized using Inkscape version 0.48.5 r10040 (Free and Open Source Software licensed under the GPL).

3. Results

3.1. Amyloid Beta Peptide ($A\beta_{1\text{-}42}$) Attenuates the Inhibitory Effect of Acetylcholine (ACh) and Nicotine (Nic) on BzATP-Induced Relase of IL-1β by Human Monocytic U937 Cells

Human monocytic U937 cells were primed with 1 µg/mL LPS for 5 h to induce pro-IL-1β synthesis. As expected, LPS-primed U937 cells did not spontaneously release IL-1β, whereas additional

application of BzATP (100 µM, 30 min) induced a release of about 50 pg/mL IL-1β in the cell culture supernatant (Figure 1a). ACh and Nic are known canonical agonists of monocytic nAChRs containing subunits α7, α9, and α10 [5]. In accordance with previous studies [6,8], the BzATP-induced release of IL-1β was inhibited in the presence of ACh (7.5 µM; $p = 0.002$; Figure 1a). Interestingly, the inhibitory effect of ACh was dose-dependently reversed by addition of $A\beta_{1\text{-}42}$ with an IC_{50} of 2.54 µM; the effect was statistically significant at $A\beta_{1\text{-}42}$ concentrations of 5 and 10 µM ($p = 0.01$; Figure 1a). In contrast, reverse $A\beta_{42\text{-}1}$ had no impact on the inhibitory effect of ACh. (Figure 1a). Neither $A\beta_{1\text{-}42}$ nor $A\beta_{42\text{-}1}$ induced IL-1β release from LPS-primed cells (Figure 1b). Moreover, similar results were found for the nAChR agonist Nic (10 µM; Figure 1c). The inhibitory effect of Nic (10 µM) on BzATP-induced IL-1β release ($p = 0.029$) was reversed in the presence of 10 µM $A\beta_{1\text{-}42}$ ($p = 0.029$). Cell death was evaluated by measuring LDH and revealed that cell viability was largely unimpaired in these experiments (Table S1).

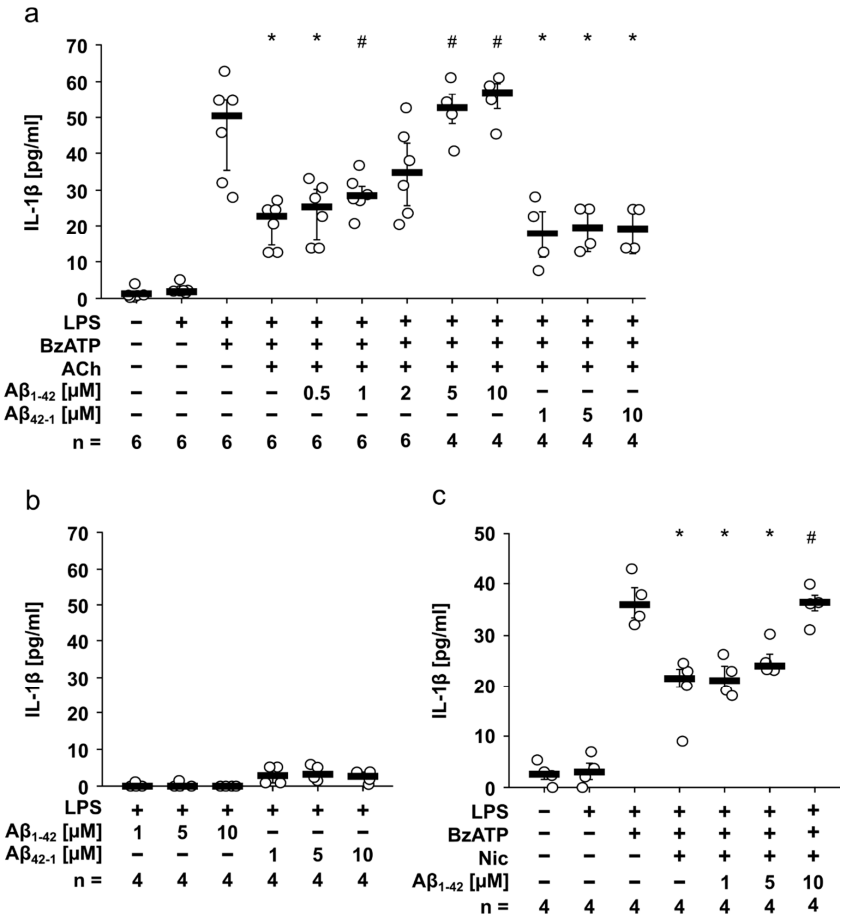

Figure 1. Amyloid beta peptide ($A\beta_{1\text{-}42}$) reverses the inhibitory effect of acetylcholine (ACh) and nicotine (Nic) on BzATP-induced interleukin-1β (IL-1β) release by human monocytic U937 cells. U937 cells were primed with lipopolysaccharide (LPS) from *Escherichia coli* for 5 h. Thereafter, the P2X7 receptor agonist 2(3)-O-(4-benzoylbenzoyl)adenosine-5-triphosphate (BzATP; 100 µM) was added for another 30 min and IL-1β was measured by ELISA in cell culture supernatants. (**a**) The nicotinic agonist ACh (7.5 µM) inhibited the BzATP-induced release of IL-1β. This inhibitory effect was dose dependently blunted by $A\beta_{1\text{-}42}$ with an IC_{50} = 2.54 µM. Reverse $A\beta_{42\text{-}1}$ (1–10 µM) had no impact. (**b**) In control experiments, neither $A\beta_{1\text{-}42}$ nor $A\beta_{42\text{-}1}$ induced changes in IL-1β release by LPS-primed U937 cells. (**c**) The inhibitory effect of Nic (10 µM) was blunted in presence of 10 µM $A\beta_{1\text{-}42}$. All data are shown as individual data points, bar represents median, whiskers encompass the 25th to 75th percentile. Statistical analyses were performed using the Kruskal–Wallis followed by Mann–Whitney U test. * $p \leq 0.05$, different from LPS-primed cells stimulated with BzATP alone. # $p \leq 0.05$, different from LPS-primed cells stimulated with BzATP and the corresponding nicotinic agonist without $A\beta_{1\text{-}42}$.

3.2. $A\beta_{1-42}$ Attenuates the Inhibitory Effect of Non-Canonical Nicotinic Agonists on BzATP-Induced IL-1β Release by Human Monocytic U937 Cells

Recently, we discovered that PC, CRP, GPC, and DPPC function as non-canonical nAChR agonists that inhibit the BzATP-induced release of IL-1β by human monocytic cells via nAChRs containing subunits α7, α9, and/or α10 [6–8,12]. Due to the findings that the inhibitory effect of ACh and Nic on BzATP-induced release of IL-1β was attenuated by $A\beta_{1-42}$, we questioned, if this is also true for non-canonical agonists of nAChRs. Similar to ACh (10 μM; $p = 0.016$), the BzATP-induced IL-1β release was inhibited by PC (100 μM; $p = 0.016$), CRP (5 μg/mL; $p = 0.016$), GPC (10 μM; $p = 0.008$) as well as by DPPC (100 μM; $p = 0.008$; Figure 2). Indeed, the inhibitory effect of all non-canonical agonists was blunted in presence on 5 μM $A\beta_{1-42}$ (PC, CRP: $p = 0.029$; GPC, DPPC: $p = 0.008$; Figure 2). Cell death, estimated by measurement of LDH release, remained below 10% in all experiments (Table S1).

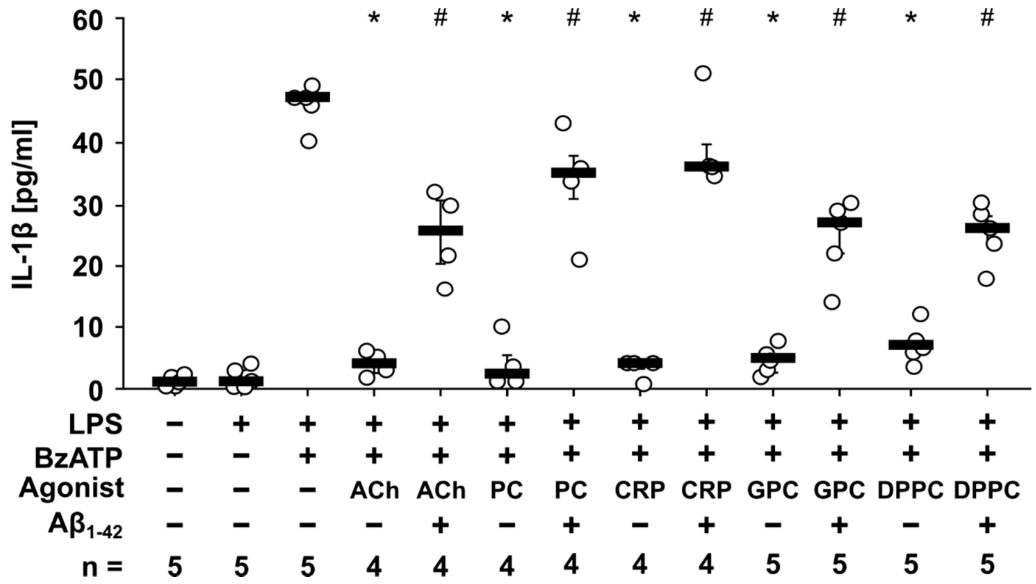

Figure 2. Amyloid beta peptide ($A\beta_{1-42}$) blunts the inhibitory effect of non-canonical nicotinic agonists on BzATP-induced interleukin-1β (IL-1β) release by human monocytic U937 cells. U937 cells were primed with lipopolysaccharide (LPS) from *Escherichia coli* for 5 h. Thereafter, the P2X7 receptor agonist BzATP (100 μM) was added for another 30 min in the presence or absence of $A\beta_{1-42}$ (5 μM) and different nicotinic agonists: acetylcholine (ACh; 10 μM), phosphocholine (PC; 100 μM), C-reactive protein (CRP; 5 μg/mL), glycerophosphorylcholine (GPC; 10 μM) and dipalmitoylphosphatidylcholine (DPPC; 100 μM). BzATP-induced release of IL-1β was measured by ELISA in cell culture supernatants. All data are shown as individual data points, bar represents median, whiskers encompass the 25th to 75th percentile. Statistical analyses were performed using the Kruskal–Wallis followed by Mann–Whitney U test. * $p \leq 0.05$, different from LPS-primed cells stimulated with BzATP alone. # $p \leq 0.05$, different from LPS-primed cells stimulated with BzATP and the corresponding nicotinic agonist without $A\beta_{1-42}$.

3.3. $A\beta_{1-42}$ Attenuates the Inhibitory Effect of PC on BzATP-Induced IL-1β Release by Human PBMCs

Next, we investigated, if the antagonizing effect of $A\beta_{1-42}$ on nAChR agonists is active in primary cells. For this purpose, human PBMCs were freshly isolated and pulsed with 5 ng/mL LPS during the process of PBMC isolation. As expected, the spontaneous secretion of IL-1β by these cells was low (Figure 3a). In response to stimulation with BzATP (100 μM; 30 min) the concentration of IL-1β was increased. This BzATP-induced release of IL-1β was inhibited by PC (200 μM; $p = 0.018$; Figure 3a). In accordance to the findings on U937 cells, 5 μM $A\beta_{1-42}$ reversed the inhibitory effect of PC ($p = 0.018$; Figure 3a). To investigate if PC signals via nAChRs, the conopetide [V11L, V16D]ArIB, a specific antagonist of nAChR containing α7 subunits, and conopeptide RgIA4, specific for nAChRs containing α9 and α10 subunits, were used. [V11L, V16D]ArIB (500 nM; $p = 0.028$) and RgIA4 (50 nM;

p = 0.018) were applied 10 min prior to PC application and both reversed the inhibitory effect of PC on BzATP-induced release of IL-1β (Figure 3a). In control experiments it was shown that $A\beta_{1\text{-}42}$ as well as [V11L, V16D]ArIB and RgIA4 alone had no inhibitory effect on BzATP- or LPS-induced IL-1β concentrations (Figure 3b). Absolute values of IL-1β (pg/mL) obtained in these experiments are given in Table S2. Cell death, estimated by measurement of LDH release, remained below 8.5% in all experiments (Table S3).

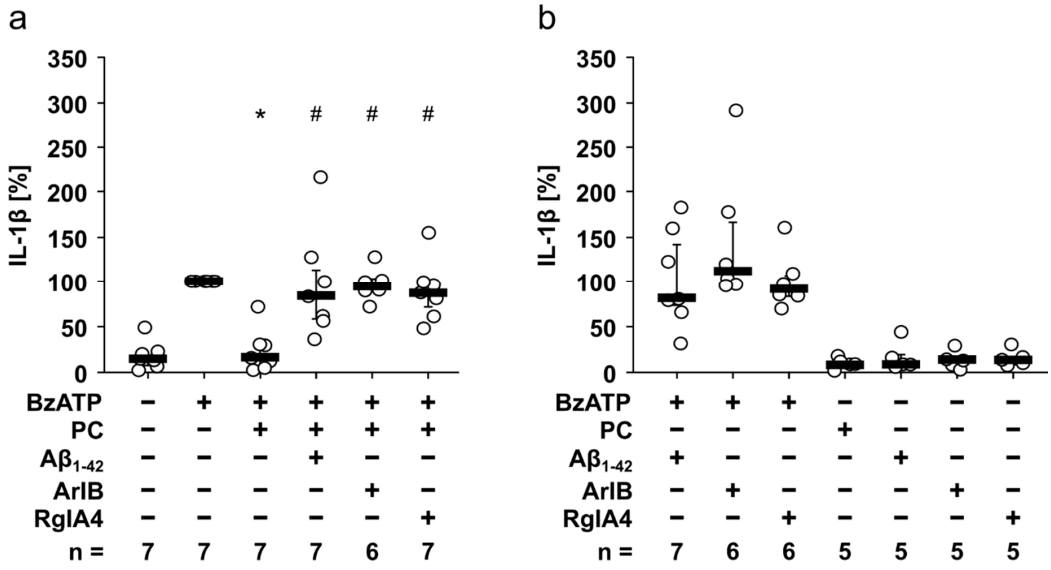

Figure 3. Amyloid beta peptide ($A\beta_{1\text{-}42}$) attenuates the inhibitory effect of phosphocholine (PC) on the BzATP-induced interleukin-1β (IL-1β) release by human peripheral blood mononuclear cells (PBMCs). PBMCs freshly isolated from healthy human volunteers were pulsed with lipopolysaccharide (LPS; 5 ng/mL) during the process of PBMC isolation and cultured for 3 h. The cells were stimulated with the P2X7 receptor agonist BzATP (100 μM) in the presence or absence of phosphocholine (PC; 200 μM), $A\beta_{1\text{-}42}$ (5 μM), [V11L, V16D]ArIB (ArIB; 500 nM) and/or RgIA4 (50 nM) for 30 min. BzATP-induced release of IL-1β was measured by ELISA in cell culture supernatants. The IL-1β concentration in experiments, in which PBMCs were stimulated with BzATP alone, was set to 100% and all other values were calculated accordingly. (**a**) The inhibitory effect of PC was reversed in the presence of $A\beta_{1\text{-}42}$, RgIA4 as well as ArIB. (**b**) In control experiments, $A\beta_{1\text{-}42}$, RgIA4, and ArIB had no impact on BzATP- and LPS-induced IL-1β release. All data are shown as individual data points, bar represents median, whiskers encompass the 25th to 75th percentile. Statistical analyses were performed using the Friedman test followed by the Wilcoxon signed-rank test. * $p \leq 0.05$, different from LPS-primed cells stimulated with BzATP alone. # $p \leq 0.05$, different from LPS-primed cells stimulated with BzATP and PC.

3.4. *$A\beta_{1\text{-}42}$ Has No Impact on the Ion Channel Activity of the Human P2X7R*

Aβ and the ATP-sensitive ionotropic P2X7R are suggested to play an important role in the pathogenesis of Alzheimer's disease, a neurodegenerative disorder characterized by a sustained inflammatory response [28]. To investigate if $A\beta_{1\text{-}42}$ directly changes the ionotropic function of the human P2X7R, we first performed electrophysiological whole-cell patch-clamp measurements on HEK-P2X7R cells (Figure 4). As expected, application of BzATP (100 μM) resulted in repetitive current stimulations due to P2X7R activation (p = 0.52; Figure 4a,c). In the next set of experiments, BzATP was first applied alone, which provoked current stimulation (Figure 4b). After washout, the cells were preincubated with $A\beta_{1\text{-}42}$ (5 μM) for 50 s, followed by an additional application of BzATP (Figure 4b). $A\beta_{1\text{-}42}$ did neither induce changes in ion currents nor impact BzATP-induced current stimulations (p = 0.92; Figure 4b,c).

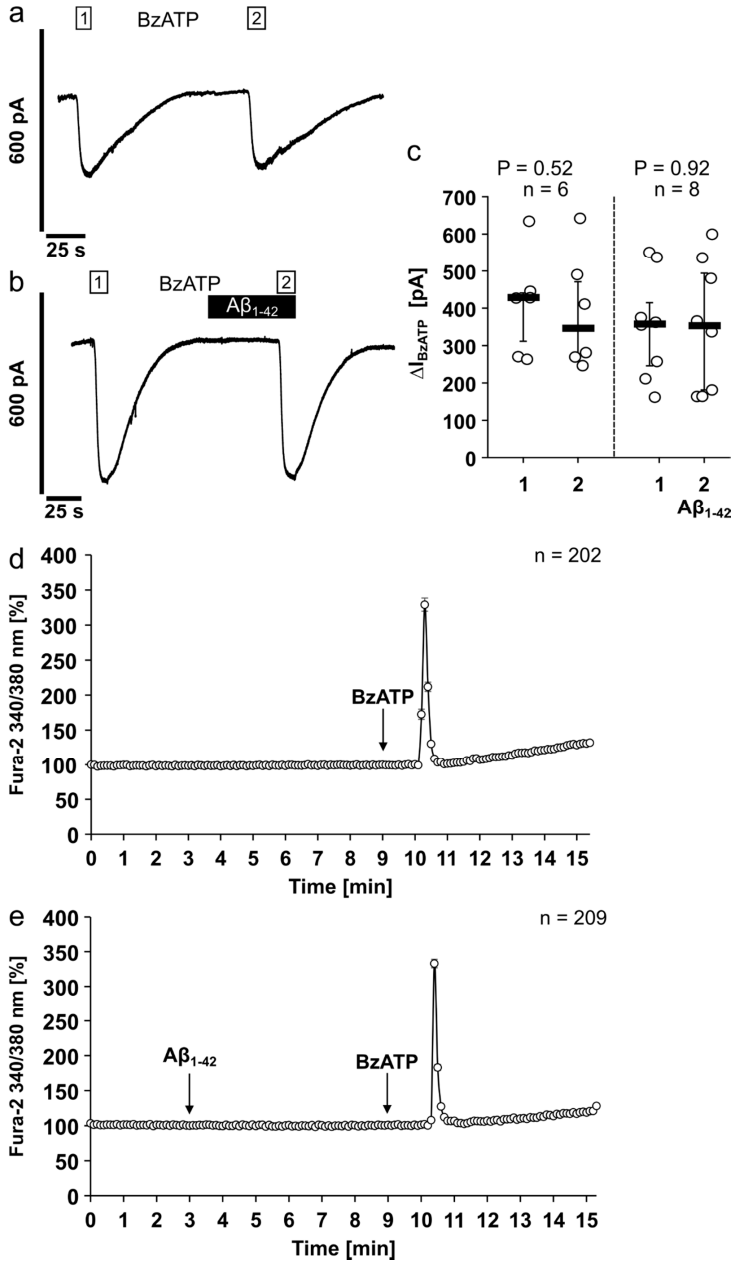

Figure 4. Amyloid beta peptide ($A\beta_{1\text{-}42}$) has no impact on the ion channel activity of the human P2X7 receptor. (**a**–**c**) Whole-cell patch-clamp measurements were performed on HEK293 cells overexpressing human P2X7 receptor (HEK-P2X7R cells). (**a**,**b**) Depicted are representative current traces. (**a**,**c**) In control experiments, consecutive application of the P2X7 receptor agonist BzATP (100 μM, white bar) induced repetitive current stimulations (BzATP1 and 2). (**b**,**c**) After washout of the first BzATP stimulus, $A\beta_{1\text{-}42}$ (5 μM, black bar) was applied and did not induce changes in ion currents. In the presence of $A\beta_{1\text{-}42}$, BzATP induced repetitive current stimulations. (**c**) All BzATP-induced current changes (ΔI_{BzATP}) are shown as individual data points, bar represents median, whiskers encompass the 25th to 75th percentile. Statistical analyses were performed using the Friedman test followed by the Wilcoxon signed-rank test. (**d**,**e**) Calcium imaging experiments were performed on HEK-P2X7R cells. Intracellular calcium concentrations ($[Ca^{2+}]_i$) of HEK-P2X7R cells were recorded as Fura-2/AM (Fura-2) fluorescence intensity ratio of 340:380 nm excitation. For graphical representation and statistical analyses, signal intensities at time point 3 min were set to 100%, and all other values were calculated in accordance and are presented as mean ± SEM. (**a**) In control experiments, application of the P2X7 receptor agonist BzATP (100 μM) induced a rise in $[Ca^{2+}]_i$. (**b**) $A\beta_{1\text{-}42}$ (5 μM) did not alter the $[Ca^{2+}]_i$. In the presence of $A\beta_{1\text{-}42}$ additional

application of BzATP induced a rise in $[Ca^{2+}]_i$. The BzATP-induced $[Ca^{2+}]_i$ changes ($\Delta[Ca^{2+}]_i$) in the absence ((**a**); $\Delta[Ca^{2+}]_i$ = 239 ± 9%) and presence ((**b**); $\Delta[Ca^{2+}]_i$ = 233 ± 6%) of $A\beta_{1\text{-}42}$ were not significantly different (p = 0.92; Mann–Whitney U test).

It is known that activation of the P2X7R results in changes of $[Ca^{2+}]_i$ levels [47]. Therefore, we performed calcium imaging measurements on HEK-P2X7R cells to test for P2X7R ion channel function in the presence or absence of $A\beta_{1\text{-}42}$ (Figure 4d,e). As expected, application of BzATP (100 µM) resulted in an increase of $[Ca^{2+}]_i$. Application of 5 µM $A\beta_{1\text{-}42}$ did not cause alterations in $[Ca^{2+}]_i$ whereas additional application of BzATP induced a rise in $[Ca^{2+}]_i$. The BzATP-induced peak $[Ca^{2+}]_i$ changes in the absence (d) or presence (e) of $A\beta_{1\text{-}42}$ were not significantly different (p = 0.92; Figure 4d,e).

3.5. $A\beta_{1\text{-}42}$ and IL-1β Concentrations in Human Plasma of Patients Undergoing Major Surgery

Finally, we investigated perioperative IL-1β and $A\beta_{1\text{-}42}$ levels in the blood plasma of 12 patients, who underwent major abdominal surgery. Blood was drawn shortly before the operation (n = 12), 0 to 2 h (n = 10), 24 to 48 h (n = 12), 72 to 96 h (n = 12), and 7 to 9 days (n = 8) after the operation, and the data of all samples were investigated collectively. In most patient samples IL-1β remained below the threshold of detection, and a median of 19.70 pg/mL $A\beta_{1\text{-}42}$ was detected (Figure 5). However, in plasma samples with detectable IL-1β levels, significantly ($p \leq 0.001$) higher $A\beta_{1\text{-}42}$ levels were measured (median 56.60 pg/mL) compared to those samples, in which IL-1β remained below the threshold of detection (Figure 5).

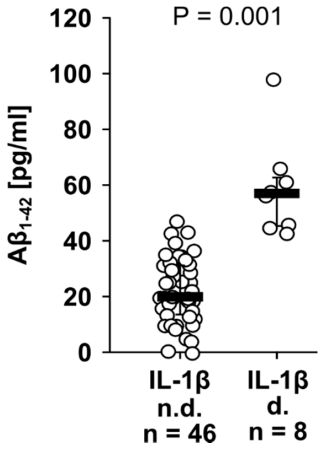

Figure 5. Interleukin-1β (IL-1β) is predominantly detected in the blood of surgical patients together with increased amyloid beta ($A\beta_{1\text{-}42}$) levels. Blood was drawn from patients, who underwent major surgery, shortly before the operation (n = 12), 0 to 2 h (n = 10), 24 to 48 h (n = 12), 72 to 96 h (n = 12), and 7 to 9 days (n = 8) after the operation. $A\beta_{1\text{-}42}$ and IL-1β were measured by ELISA in patient blood plasma. $A\beta_{1\text{-}42}$ values of patients, in whom IL-1β levels were below the threshold of detection (n.d.), were compared to patients with detectable IL-1β (d). Data obtained for all time points were investigated collectively and are shown as individual data points, bar represents median, whiskers encompass the 25th to 75th percentile. Statistical analysis was performed using the Mann–Whitney U test.

4. Discussion

APP and its Aβ peptides as well as nAChRs are evolutionary highly conserved and evolved long before the emergence of a nervous system [48–51], suggesting that their original functions need to be sought in more primitive and ubiquitous systems, such as innate immunity. Most research has investigated the pathophysiological effects of the interaction of Aβ with α7 nAChRs with a special focus on the etiology of AD [52]. In this study, we provide evidence that $A\beta_{1\text{-}42}$ functions as an

antagonist of monocytic nAChRs containing subunits α7, α9, and/or α10, in human monocytic U937 cells and primary human PBMCs (Figure 6). Thus, we have discovered a novel pro-inflammatory function of $A\beta_{1\text{-}42}$ that enables monocytic IL-1β release in the presence of nAChR agonists. In addition, we provide first clinical hints that this mechanism might be functional in vivo.

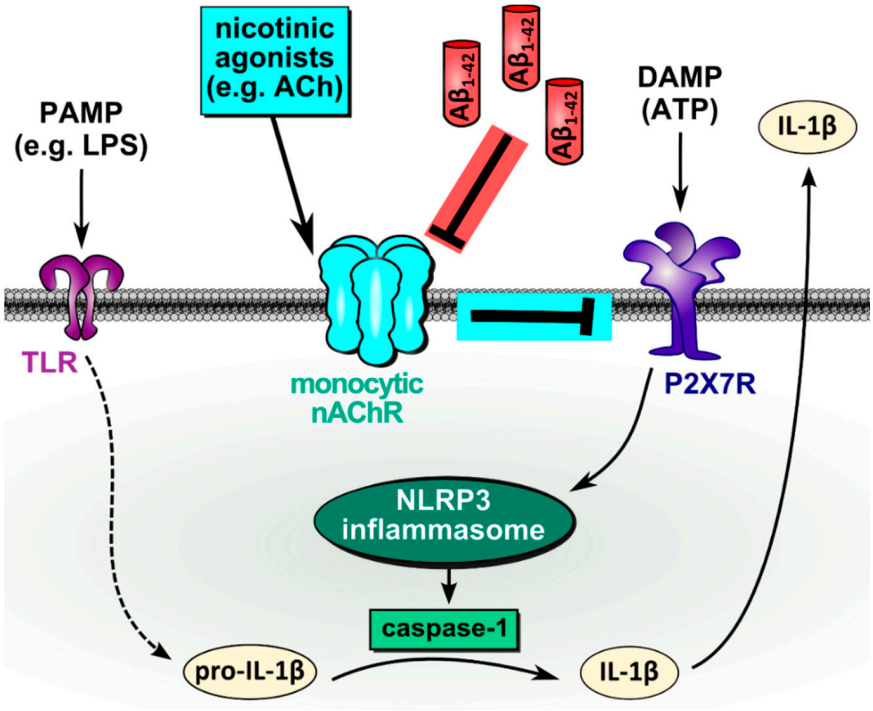

Figure 6. Schematic summary of the proposed mechanism. In LPS-primed monocytic cells, extracellular ATP, originating from dead or injured cells, binds to the ATP-sensitive P2X7R, induces NLRP3 inflammasome assembly, activation of caspase-1, cleavage of pro-IL-1β and release of bioactive IL-1β. This damage-associated release of IL-1β is inhibited by the activation of monocytic nAChRs containing subunits α9, α7, and/or α10. As shown previously, activation of nAChRs inhibits the ion channel function of P2X7R and, thus, ATP-induced IL-1β release [6,7]. Our data suggest that amyloid beta ($A\beta_{1\text{-}42}$) antagonizes this inhibitory effect and enables monocytic release of IL-1β in the presence of nicotinic agonists. ACh, acetylcholine; ATP, adenosine triphosphate; DAMP, danger-associated molecular pattern; IL-1β, interleukin-1β; LPS, lipopolysaccharide; nAChR, nicotinic acetylcholine receptor; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; P2X7R, P2X7 receptor; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptors.

Our data suggest that $A\beta_{1\text{-}42}$ can functionally interact with monocytic nAChRs. $A\beta_{1\text{-}42}$ reverses the inhibitory effect of nicotinic agonists on ATP-mediated release of IL-1β in monocytic U937 cells. In previous studies, we found that beside classical nAChR agonists like ACh, choline, and nicotine, non-canonical agonist like PC, endogenous CRP, GPC, and DPPC can activate monocytic nAChRs containing subunits α9, α7, and/or α10 to induce this anti-inflammatory effect [6–8,11,12]. Here, we show that $A\beta_{1\text{-}42}$ dose-dependently antagonizes the inhibitory effect of classical as well as of non-canonical nicotinic agonists on the BzATP-mediated IL-1β release by human monocytic U937 cells.

Because of the well-known limitations of cell lines, we included primary PBMCs in this study that were freshly isolated from the blood of healthy volunteers. In these experiments, we focused on PC, the lead compound of the non-canonical agonists of monocytic nAChRs [5,7,8] and directly compared the effects of conopeptides [V11L, V16D]ArIB and RgIA4 to the effects of $A\beta_{1\text{-}42}$. We confirmed that, also in human PBMCs, PC signals via subunits α7, α9, and/or α10 to inhibit the BzATP-induced release of IL-1β [6,7]. The effect of $A\beta_{1\text{-}42}$, again, strikingly resembles those of the conopeptides [V11L, V16D]ArIB (α7-specific) and RgIA4 (α9/α10-specific).

In our experimental setting, we found that $A\beta_{1-42}$ antagonizes the inhibitory effects of nicotinic agonists with an IC_{50} of 2.54 μM, corresponding to about 10 μg/mL. Whether the $A\beta_{1-42}$ concentrations used in the present study are physiological for monocytic cells is difficult to decide. Roher and colleagues compared plasma Aβ levels in AD patients and in age-matched control subjects at four time points over a period of 12 months [53]. $A\beta_{1-40}$ and $A\beta_{1-42}$ plasma levels fluctuated widely among the individuals in both AD subjects and controls with overall mean values of 384 pg/mL and 132 pg/mL, respectively [53]. In the light of these data, an IC_{50} of about 10 μg/mL seems to be extremely high. However, APP is expressed by vascular endothelial cells [50]. Moreover, activated platelets and leukocytes can secrete APP and its Aβ peptides, thus contributing to 90% of peripheral serum concentrations [50,54,55]. As monocytes closely interact with endothelial cells and platelets [56,57] and may secrete $A\beta_{1-42}$ themselves, $A\beta_{1-42}$ might function as a paracrine and/or autocrine factor and local concentrations possibly reach micromolar concentrations.

How does $A\beta_{1-42}$ reverse the inhibitory effects of nicotinic agonists on the ATP-induced release of IL-1β? To tackle the question, whether $A\beta_{1-42}$ directly modulates ATP receptor functions, we made use of HEK293 cells overexpressing the human P2X7R. Upon stimulation with BzATP, we observed the expected changes in ion currents and cytoplasmic Ca^{2+} concentrations, which remained unchanged in the presence of $A\beta_{1-42}$. Therefore, in the present study $A\beta_{1-42}$ seems not to modulate P2X7R ion channel activity. Moreover, this result favors the hypothesis that $A\beta_{1-42}$ interacts with monocytic nAChRs. However, some studies pointed out an important role of the P2X7R in Aβ signaling [28,58]. These controversial results might be due to the different experimental protocols and cells used here (monocytic cells) and in the previous studies (microglia).

Ample evidence suggests that $A\beta_{1-42}$ directly interacts with conventional nAChRs [59–62]. Conventional nAChRs of the neuronal type are ligand-gated ion channels composed of α and β subunits that form heteromeric or homomeric pentamers with a conductance for positively charged ions like Na^+, K^+, and Ca^{2+} [63,64]. Homomeric α7 nAChRs and heteromeric α4β2 are the two most abundant types of nAChRs in the human central nervous system [65]. Wang and colleagues were the first to identify an interaction of the α7 nAChRs and $A\beta_{1-42}$ in the brain [59,60]. Later, it was shown that α4β2, α4α5β2, and α2αβ nAChRs can also interact with APP and its Aβ peptides [61,62]. In contrast to our data, however, the affinity of $A\beta_{1-42}$ to α7 nAChRs in the brain is in the low picomolar range [51,59].

A direct modulation of the ion channel functions of α7 nAChR by $A\beta_{1-42}$ was first shown by Petti and colleagues in brain slices, where $A\beta_{1-42}$ inhibited postsynaptic nAChR-mediated currents [15,66]. Seemingly, the $A\beta_{1-42}$ amino acid residues 1–28 are responsible for this interaction [60,66]. The literature about the consequences of Aβ peptide binding to nAChRs is, however, contradictory [52]. Both, agonistic and antagonistic effects of Aβ peptides on the α7 nAChR have been described [15,16,51,52,67–71]. Moreover, we and others demonstrated that leukocytic nAChRs do not function as classical ion channels, but rather exert metabotropic functions [5–7,72–78]. Interestingly, ion channel activity of nAChRs was detected in human monocyte-derived macrophages [79] and macrophages derived from monocytic THP-1 cells [80] in the presence of the positive allosteric modulator PNU-120596. However, as the structure and function of monocytic nAChRs still remain to be elucidated, we can only speculate on their interaction with $A\beta_{1-42}$. Due to the lack of specific antibodies and the low expression of nAChRs by leukocytes, it is extremely difficult to provide evidence for their direct interaction with $A\beta_{1-42}$.

Numerous studies have shown pro-inflammatory functions of Aβ. Treatment of PBMCs with $A\beta_{1-42}$ leads to the secretion of pro-inflammatory cytokines like IL-1β, IL-6, IL-18, monocyte chemotactic protein-1 (MCP-1), and TNF-α [37,39,40,42,81,82]. Fibrillar $A\beta_{1-42}$ is supposed to directly interact with TLR2 and TLR4 to trigger TNF-α secretion in a monocytic cell line [83]. TLR activation is known to induce the expression of IL-1β and of components of the inflammasome. In our experiments, however, $A\beta_{1-42}$ was applied together with BzATP for only 30 min, which is probably not enough time for an efficient induction of gene expression and protein synthesis. Other studies found that fibrillar and monomeric $A\beta_{1-42}$ can activate the NLRP3 inflammasome, thereby inducing the release of mature

IL-1β [29,38,84,85]. This probably depends on the internalization of $A\beta_{1-42}$ fibrils via the scavenger receptor CD36, followed by lysosomal rupture, a known signal of NLRP3 assembly [29,86,87]. In the present study, we used soluble $A\beta_{1-42}$ and did not see a significant release of IL-1β in the absence of BzATP, and the BzATP-induced release of IL-1β was not changed in presence of $A\beta_{1-42}$. Therefore, it is unlikely that $A\beta_{1-42}$ boosted the expression of pro-IL-1β or its cleavage machinery or directly activated the NLRP3 inflammasome in our experimental setting.

To obtain a first indication whether IL-1β and $A\beta_{1-42}$ blood levels are linked in vivo, blood plasma samples from patients, who underwent major surgery, were investigated. We did not perform a correlation analysis because IL-1β was undetectable in a large proportion of the samples. Systemic IL-1β levels are known to be low, even during systemic inflammation, due to its short half-life in the circulation [88,89]. Instead, we compared $A\beta_{1-42}$ levels in samples with undetectable versus detectable IL-1β levels. In plasma samples with detectable IL-1β, $A\beta_{1-42}$ levels were indeed higher compared to samples, in which IL-1β was undetectable. This is in line with our hypothesis that $A\beta_{1-42}$ fosters the release of IL-1β. However, these data should be interpreted with utmost caution and further careful clinical research is warranted, because we only investigated a small cohort of patients, pooled data from blood collections at different perioperative time points and do not prove causality at all. Furthermore, patient $A\beta_{1-42}$ levels measured in our study were about half as low compared to those reported for healthy blood donors [53]. We assume that this is due to technical reasons, as we separated plasma from blood cells on a density gradient that certainly resulted in dilution. Even the highest $A\beta_{1-42}$ concentration measured in patient blood plasma, which is in the range of 100 pg/mL, is far below the $A\beta_{1-42}$ concentrations needed to inhibit ATP-induced IL-1β release in monocytic U937 cells and human PBMCs (IC_{50} = 10 μg/mL). Hence, $A\beta_{1-42}$ is probably not active systemically, but might reach sufficient concentrations in the micro-milieu of traumatized tissues, as inflammation is known to upregulate APP and its cleavage machinery resulting in increased Aβ levels [90,91].

5. Conclusions

We conclude from our study that $A\beta_{1-42}$ enables the ATP-induced release of monocytic IL-1β despite the presence of potent nicotinic agonists. Although we do not know if $A\beta_{1-42}$ directly interacts with leukocytic nAChRs, $A\beta_{1-42}$ most probably functions as a nicotinic antagonist. As enabling an ATP-induced IL-1β release can prevent infection and fatal sepsis, we believe that we discovered one of the vital functions of APP and its peptides that contributed to their striking conservation during evolution.

Our findings are certainly of clinical interest, especially in the context of surgery-induced inflammation. High $A\beta_{1-42}$ concentrations in patient blood might predict inflammatory complications in response to major surgery and our findings might pave the way towards new therapeutic avenues. However, more studies including experimental in vivo studies and extended carefully controlled clinical studies are warranted to estimate the clinical relevance of our results.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/9/2887/s1, Table S1: Lactate dehydrogenase (LDH) concentrations in cell culture supernatants of human monocytic U937 cells; Table S2: Absolute values of interleukin-1β (IL-1β [pg/mL]) in cell culture supernatants of human peripheral blood mononuclear cells (PBMCs); Table S3: Lactate dehydrogenase (LDH) concentrations in cell culture supernatants of human peripheral blood mononuclear cells (PBMCs).

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References

1. Dinarello, C.A.; Simon, A.; van der Meer, J.W.M. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat. Rev. Drug Discov.* **2012**, *11*, 633–652. [CrossRef]
2. Broz, P.; Dixit, V.M. Inflammasomes: Mechanism of assembly, regulation and signalling. *Nat. Rev. Immunol.* **2016**, *16*, 407–420. [CrossRef]
3. Bortolotti, P.; Faure, E.; Kipnis, E. Inflammasomes in tissue damages and immune disorders after trauma. *Front. Immunol.* **2018**, *9*, 1900. [CrossRef]
4. Dinarello, C.A. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol. Rev.* **2018**, *281*, 8–27. [CrossRef]
5. Grau, V.; Richter, K.; Hone, A.J.; McIntosh, J.M. Conopeptides V11L.; V16DArIB and RgIA4: Powerful tools for the identification of novel nicotinic acetylcholine receptors in monocytes. *Front. Pharmacol.* **2018**, *9*, 1499. [CrossRef]
6. Hecker, A.; Küllmar, M.; Wilker, S.; Richter, K.; Zakrzewicz, A.; Atanasova, S.; Mathes, V.; Timm, T.; Lerner, S.; Klein, J.; et al. Phosphocholine-modified macromolecules and canonical nicotinic agonists inhibit ATP-induced IL-1β release. *J. Immunol.* **2015**, *195*, 2325–2334. [CrossRef] [PubMed]
7. Richter, K.; Mathes, V.; Fronius, M.; Althaus, M.; Hecker, A.; Krasteva-Christ, G.; Padberg, W.; Hone, A.J.; McIntosh, J.M.; Zakrzewicz, A.; et al. Phosphocholine—An agonist of metabotropic but not of ionotropic functions of α9-containing nicotinic acetylcholine receptors. *Sci. Rep.* **2016**, *6*, 28660. [CrossRef] [PubMed]
8. Zakrzewicz, A.; Richter, K.; Agné, A.; Wilker, S.; Siebers, K.; Fink, B.; Krasteva-Christ, G.; Althaus, M.; Padberg, W.; Hone, A.J.; et al. Canonical and novel non-canonical cholinergic agonists inhibit ATP-induced release of monocytic interleukin-1β via different combinations of nicotinic acetylcholine receptor subunits α7, α9 and α10. *Front. Cell. Neurosci.* **2017**, *11*, 491. [CrossRef] [PubMed]
9. Pepys, M.B.; Hirschfield, G.M.; Tennent, G.A.; Gallimore, J.R.; Kahan, M.C.; Bellotti, V.; Hawkins, P.N.; Myers, R.M.; Smith, M.D.; Polara, A.; et al. Targeting C-reactive protein for the treatment of cardiovascular disease. *Nature* **2006**, *440*, 1217–1221. [CrossRef]
10. Mantovani, A.; Garlanda, C.; Doni, A.; Bottazzi, B. Pentraxins in innate immunity: From C-reactive protein to the long pentraxin PTX3. *J. Clin. Immunol.* **2008**, *28*, 1–13. [CrossRef]
11. Richter, K.; Sagawe, S.; Hecker, A.; Küllmar, M.; Askevold, I.; Damm, J.; Heldmann, S.; Pöhlmann, M.; Ruhrmann, S.; Sander, M.; et al. C-reactive protein stimulates nicotinic acetylcholine receptors to control ATP-mediated monocytic inflammasome activation. *Front. Immunol.* **2018**, *9*, 1604. [CrossRef] [PubMed]
12. Backhaus, S.; Zakrzewicz, A.; Richter, K.; Damm, J.; Wilker, S.; Fuchs-Moll, G.; Küllmar, M.; Hecker, A.; Manzini, I.; Ruppert, C.; et al. Surfactant inhibits ATP-induced release of interleukin-1β via nicotinic acetylcholine receptors. *J. Lipid Res.* **2017**, *58*, 1055–1066. [CrossRef]
13. Nagele, R.G.; D'Andrea, M.R.; Anderson, W.J.; Wang, H.-Y. Intracellular accumulation of β-amyloid1–42 in neurons is facilitated by the α7 nicotinic acetylcholine receptor in Alzheimer's disease. *Neuroscience* **2002**, *110*, 199–211. [CrossRef]
14. Lee, D.H.S.; Wang, H.-Y. Differential physiologic responses of alpha7 nicotinic acetylcholine receptors to beta-amyloid1-40 and beta-amyloid1-42. *J. Neurobiol.* **2003**, *55*, 25–30. [CrossRef] [PubMed]
15. Parri, R.H.; Dineley, T.K. Nicotinic acetylcholine receptor interaction with beta-amyloid: Molecular, cellular, and physiological consequences. *Curr. Alzheimer Res.* **2010**, *7*, 27–39. [CrossRef]
16. Sadigh-Eteghad, S.; Talebi, M.; Farhoudi, M.; Golzari, S.E.J.; Sabermarouf, B.; Mahmoudi, J. Beta-amyloid exhibits antagonistic effects on alpha 7 nicotinic acetylcholine receptors in orchestrated manner. *J. Med. Hypotheses Ideas* **2014**, *8*, 49–52. [CrossRef]

17. Barykin, E.P.; Garifulina, A.I.; Kruykova, E.V.; Spirova, E.N.; Anashkina, A.A.; Adzhubei, A.A.; Shelukhina, I.V.; Kasheverov, I.E.; Mitkevich, V.A.; Kozin, S.A.; et al. Isomerization of Asp7 in beta-amyloid enhances inhibition of the α7 nicotinic receptor and promotes neurotoxicity. *Cells* **2019**, *8*, 771. [CrossRef]
18. Lasala, M.; Fabiani, C.; Corradi, J.; Antollini, S.; Bouzat, C. Molecular modulation of human α7 nicotinic receptor by amyloid-β peptides. *Front. Cell. Neurosci.* **2019**, *13*, 37. [CrossRef]
19. Li, M.D. (Ed.) *Nicotinic Acetylcholine Receptor Technologies*; Springer: New York, NY, USA, 2016; ISBN 978-1-4939-3768-4.
20. Puig, K.L.; Combs, C.K. Expression and function of APP and its metabolites outside the central nervous system. *Exp. Gerontol.* **2013**, *48*, 608–611. [CrossRef]
21. Thinakaran, G.; Koo, E.H. Amyloid precursor protein trafficking, processing, and function. *J. Biol. Chem.* **2008**, *283*, 29615–29619. [CrossRef]
22. Mattson, M.P. Pathways towards and away from Alzheimer's disease. *Nature* **2004**, *430*, 631–639. [CrossRef] [PubMed]
23. Sinha, S.; Lieberburg, I. Cellular mechanisms of beta-amyloid production and secretion. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11049–11053. [CrossRef] [PubMed]
24. Selkoe, D.J. Normal and abnormal biology of the beta-amyloid precursor protein. *Annu. Rev. Neurosci.* **1994**, *17*, 489–517. [CrossRef] [PubMed]
25. Solfrizzi, V.; D'Introno, A.; Colacicco, A.M.; Capurso, C.; Todarello, O.; Pellicani, V.; Capurso, S.A.; Pietrarossa, G.; Santamato, V.; Capurso, A.; et al. Circulating biomarkers of cognitive decline and dementia. *Clin. Chim. Acta* **2006**, *364*, 91–112. [CrossRef]
26. Karran, E.; Mercken, M.; de Strooper, B. The amyloid cascade hypothesis for Alzheimer's disease: An appraisal for the development of therapeutics. *Nat. Rev. Drug Discov.* **2011**, *10*, 698–712. [CrossRef]
27. Kametani, F.; Hasegawa, M. Reconsideration of Amyloid hypothesis and tau hypothesis in Alzheimer's disease. *Front. Neurosci.* **2018**, *12*, 25. [CrossRef]
28. Godoy, P.A.; Ramírez-Molina, O.; Fuentealba, J. Exploring the role of P2X receptors in Alzheimer's disease. *Front. Pharmacol.* **2019**, *10*, 1330. [CrossRef]
29. Dong, Y.; Li, X.; Cheng, J.; Hou, L. Drug development for Alzheimer's disease: Microglia induced neuroinflammation as a target? *Int. J. Mol. Sci.* **2019**, *20*, 558. [CrossRef]
30. Heneka, M.T.; Carson, M.J.; Khoury, J.E.; Landreth, G.E.; Brosseron, F.; Feinstein, D.L.; Jacobs, A.H.; Wyss-Coray, T.; Vitorica, J.; Ransohoff, R.M.; et al. Neuroinflammation in Alzheimer's disease. *Lancet Neurol.* **2015**, *14*, 388–405. [CrossRef]
31. Han, S.-H.; Park, J.-C.; Mook-Jung, I. Amyloid β-interacting partners in Alzheimer's disease: From accomplices to possible therapeutic targets. *Prog. Neurobiol.* **2016**, *137*, 17–38. [CrossRef]
32. Müller, U.C.; Deller, T.; Korte, M. Not just amyloid: Physiological functions of the amyloid precursor protein family. *Nat. Rev. Neurosci.* **2017**, *18*, 281–298. [CrossRef] [PubMed]
33. El Khoury, J.; Hickman, S.E.; Thomas, C.A.; Cao, L.; Silverstein, S.C.; Loike, J.D. Scavenger receptor-mediated adhesion of microglia to beta-amyloid fibrils. *Nature* **1996**, *382*, 716–719. [CrossRef] [PubMed]
34. Du Yan, S.; Zhu, H.; Fu, J.; Yan, S.F.; Roher, A.; Tourtellotte, W.W.; Rajavashisth, T.; Chen, X.; Godman, G.C.; Stern, D.; et al. Amyloid-beta peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: A proinflammatory pathway in Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 5296–5301. [CrossRef] [PubMed]
35. Herzog, V.; Kirfel, G.; Siemes, C.; Schmitz, A. Biological roles of APP in the epidermis. *Eur. J. Cell Biol.* **2004**, *83*, 613–624. [CrossRef]
36. Puig, K.L.; Swigost, A.J.; Zhou, X.; Sens, M.A.; Combs, C.K. Amyloid precursor protein expression modulates intestine immune phenotype. *J. Neuroimmune Pharmacol.* **2012**, *7*, 215–230. [CrossRef]
37. François, A.; Julian, A.; Ragot, S.; Dugast, E.; Blanchard, L.; Brishoual, S.; Terro, F.; Chassaing, D.; Page, G.; Paccalin, M. Inflammatory stress on autophagy in peripheral blood mononuclear cells from patients with Alzheimer's disease during 24 months of follow-up. *PLoS ONE* **2015**, *10*, e0138326. [CrossRef]
38. Gold, M.; El Khoury, J. β-amyloid, microglia, and the inflammasome in Alzheimer's disease. *Semin. Immunopathol.* **2015**, *37*, 607–611. [CrossRef]
39. Rosenberg, P.B. Clinical aspects of inflammation in Alzheimer's disease. *Int. Rev. Psychiatry* **2005**, *17*, 503–514. [CrossRef]

40. Reale, M.; Kamal, M.A.; Velluto, L.; Gambi, D.; Di Nicola, M.; Greig, N.H. Relationship between inflammatory mediators, Aβ levels and ApoE genotype in Alzheimer disease. *Curr. Alzheimer Res.* **2012**, *9*, 447–457. [CrossRef]
41. Fiala, M.; Zhang, L.; Gan, X.; Sherry, B.; Taub, D.; Graves, M.C.; Hama, S.; Way, D.; Weinand, M.; Witte, M.; et al. Amyloid-beta induces chemokine secretion and monocyte migration across a human blood—Brain barrier model. *Mol. Med.* **1998**, *4*, 480–489. [CrossRef]
42. Kaplin, A.; Carroll, K.A.L.; Cheng, J.; Allie, R.; Lyketsos, C.G.; Calabresi, P.; Rosenberg, P.B. IL-6 release by LPS-stimulated peripheral blood mononuclear cells as a potential biomarker in Alzheimer's disease. *Int. Psychogeriatr.* **2009**, *21*, 413–414. [CrossRef] [PubMed]
43. Ramarao, M.K.; Cohen, J.B. Mechanism of nicotinic acetylcholine receptor cluster formation by rapsyn. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 4007–4012. [CrossRef] [PubMed]
44. Innocent, N.; Livingstone, P.D.; Hone, A.; Kimura, A.; Young, T.; Whiteaker, P.; McIntosh, J.M.; Wonnacott, S. Alpha-conotoxin Arenatus IBV11L, V16D corrected is a potent and selective antagonist at rat and human native alpha7 nicotinic acetylcholine receptors. *J. Pharmacol. Exp. Ther.* **2008**, *327*, 529–537. [CrossRef] [PubMed]
45. Romero, H.K.; Christensen, S.B.; Di Cesare Mannelli, L.; Gajewiak, J.; Ramachandra, R.; Elmslie, K.S.; Vetter, D.E.; Ghelardini, C.; Iadonato, S.P.; Mercado, J.L.; et al. Inhibition of α9α10 nicotinic acetylcholine receptors prevents chemotherapy-induced neuropathic pain. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E1825–E1832. [CrossRef]
46. Klapperstück, M.; Büttner, C.; Schmalzing, G.; Markwardt, F. Functional evidence of distinct ATP activation sites at the human P2X(7) receptor. *J. Physiol.* **2001**, *534*, 25–35. [CrossRef]
47. Kopp, R.; Krautloher, A.; Ramírez-Fernández, A.; Nicke, A. P2X7 Interactions and signaling—Making head or tail of it. *Front. Mol. Neurosci.* **2019**, *12*, 183. [CrossRef]
48. Le Novère, N.; Changeux, J.P. Molecular evolution of the nicotinic acetylcholine receptor: An example of multigene family in excitable cells. *J. Mol. Evol.* **1995**, *40*, 155–172. [CrossRef]
49. Franchini, L.F.; Elgoyhen, A.B. Adaptive evolution in mammalian proteins involved in cochlear outer hair cell electromotility. *Mol. Phylogenet. Evol.* **2006**, *41*, 622–635. [CrossRef]
50. d'Uscio, L.V.; He, T.; Katusic, Z.S. Expression and processing of amyloid precursor protein in vascular endothelium. *Physiology* **2017**, *32*, 20–32. [CrossRef]
51. Fabiani, C.; Antollini, S.S. Alzheimer's disease as a membrane disorder: Spatial cross-talk among beta-amyloid peptides, nicotinic acetylcholine receptors and lipid rafts. *Front. Cell. Neurosci.* **2019**, *13*, 309. [CrossRef]
52. Lombardo, S.; Maskos, U. Role of the nicotinic acetylcholine receptor in Alzheimer's disease pathology and treatment. *Neuropharmacology* **2015**, *96*, 255–262. [CrossRef] [PubMed]
53. Roher, A.E.; Esh, C.L.; Kokjohn, T.A.; Castaño, E.M.; van Vickle, G.D.; Kalback, W.M.; Patton, R.L.; Luehrs, D.C.; Daugs, I.D.; Kuo, Y.-M.; et al. Amyloid beta peptides in human plasma and tissues and their significance for Alzheimer's disease. *Alzheimer's Dement.* **2009**, *5*, 18–29. [CrossRef] [PubMed]
54. Li, Q.X.; Fuller, S.J.; Beyreuther, K.; Masters, C.L. The amyloid precursor protein of Alzheimer disease in human brain and blood. *J. Leukoc. Biol.* **1999**, *66*, 567–574. [CrossRef] [PubMed]
55. Marksteiner, J.; Humpel, C. Platelet-derived secreted amyloid-precursor protein-β as a marker for diagnosing Alzheimer's disease. *Curr. Neurovasc. Res.* **2013**, *10*, 297–303. [CrossRef] [PubMed]
56. Mestas, J.; Ley, K. Monocyte-endothelial cell interactions in the development of atherosclerosis. *Trends Cardiovasc. Med.* **2008**, *18*, 228–232. [CrossRef] [PubMed]
57. Lam, F.W.; Vijayan, K.V.; Rumbaut, R.E. Platelets and their interactions with other immune cells. *Compr. Physiol.* **2015**, *5*, 1265–1280. [CrossRef] [PubMed]
58. Sanz, J.M.; Chiozzi, P.; Ferrari, D.; Colaianna, M.; Idzko, M.; Falzoni, S.; Fellin, R.; Trabace, L.; Di Virgilio, F. Activation of microglia by amyloid β requires P2X7 receptor expression. *J. Immunol.* **2009**, *182*, 4378–4385. [CrossRef]
59. Wang, H.Y.; Lee, D.H.; D'Andrea, M.R.; Peterson, P.A.; Shank, R.P.; Reitz, A.B. beta-Amyloid(1-42) binds to alpha7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology. *J. Biol. Chem.* **2000**, *275*, 5626–5632. [CrossRef]
60. Wang, H.Y.; Lee, D.H.; Davis, C.B.; Shank, R.P. Amyloid peptide Abeta(1-42) binds selectively and with picomolar affinity to alpha7 nicotinic acetylcholine receptors. *J. Neurochem.* **2000**, *75*, 1155–1161. [CrossRef]

61. Wu, J.; Kuo, Y.-P.; George, A.A.; Xu, L.; Hu, J.; Lukas, R.J. beta-Amyloid directly inhibits human alpha4beta2-nicotinic acetylcholine receptors heterologously expressed in human SH-EP1 cells. *J. Biol. Chem.* **2004**, *279*, 37842–37851. [CrossRef]
62. Lamb, P.W.; Melton, M.A.; Yakel, J.L. Inhibition of neuronal nicotinic acetylcholine receptor channels expressed in *Xenopus* oocytes by β-amyloid$_{1\text{-}42}$ peptide. *JMN* **2005**, *27*, 13–22. [CrossRef]
63. Karlin, A. Emerging structure of the nicotinic acetylcholine receptors. *Nat. Rev. Neurosci.* **2002**, *3*, 102–114. [CrossRef] [PubMed]
64. Papke, R.L. Merging old and new perspectives on nicotinic acetylcholine receptors. *Biochem. Pharmacol.* **2014**, *89*, 1–11. [CrossRef] [PubMed]
65. Gotti, C.; Zoli, M.; Clementi, F. Brain nicotinic acetylcholine receptors: Native subtypes and their relevance. *Trends Pharmacol. Sci.* **2006**, *27*, 482–491. [CrossRef] [PubMed]
66. Pettit, D.L.; Shao, Z.; Yakel, J.L. beta-Amyloid(1-42) peptide directly modulates nicotinic receptors in the rat hippocampal slice. *J. Neurosci.* **2001**, *21*, RC120. [CrossRef]
67. Dougherty, J.J.; Wu, J.; Nichols, R.A. β-Amyloid regulation of presynaptic nicotinic receptors in rat hippocampus and neocortex. *J. Neurosci.* **2003**, *23*, 6740–6747. [CrossRef]
68. Liu, Q.; Kawai, H.; Berg, D.K. beta-Amyloid peptide blocks the response of alpha 7-containing nicotinic receptors on hippocampal neurons. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 4734–4739. [CrossRef]
69. Dineley, K.T.; Bell, K.A.; Bui, D.; Sweatt, J.D. Beta-Amyloid peptide activates alpha 7 nicotinic acetylcholine receptors expressed in Xenopus oocytes. *J. Biol. Chem.* **2002**, *277*, 25056–25061. [CrossRef]
70. Sondag, C.M.; Combs, C.K. Amyloid precursor protein cross-linking stimulates beta amyloid production and pro-inflammatory cytokine release in monocytic lineage cells. *J. Neurochem.* **2006**, *97*, 449–461. [CrossRef]
71. Wang, H.-Y.; Stucky, A.; Liu, J.; Shen, C.; Trocme-Thibierge, C.; Morain, P. Dissociating beta-amyloid from alpha 7 nicotinic acetylcholine receptor by a novel therapeutic agent, S 24795, normalizes alpha 7 nicotinic acetylcholine and NMDA receptor function in Alzheimer's disease brain. *J. Neurosci.* **2009**, *29*, 10961–10973. [CrossRef]
72. Peng, H.; Ferris, R.L.; Matthews, T.; Hiel, H.; Lopez-Albaitero, A.; Lustig, L.R. Characterization of the human nicotinic acetylcholine receptor subunit alpha (alpha) 9 (CHRNA9) and alpha (alpha) 10 (CHRNA10) in lymphocytes. *Life Sci.* **2004**, *76*, 263–280. [CrossRef] [PubMed]
73. Razani-Boroujerdi, S.; Boyd, R.T.; Dávila-García, M.I.; Nandi, J.S.; Mishra, N.C.; Singh, S.P.; Pena-Philippides, J.C.; Langley, R.; Sopori, M.L. T cells express alpha7-nicotinic acetylcholine receptor subunits that require a functional TCR and leukocyte-specific protein tyrosine kinase for nicotine-induced Ca^{2+} response. *J. Immunol.* **2007**, *179*, 2889–2898. [CrossRef] [PubMed]
74. Hecker, A.; Mikulski, Z.; Lips, K.S.; Pfeil, U.; Zakrzewicz, A.; Wilker, S.; Hartmann, P.; Padberg, W.; Wessler, I.; Kummer, W.; et al. Pivotal advance: Up-regulation of acetylcholine synthesis and paracrine cholinergic signaling in intravascular transplant leukocytes during rejection of rat renal allografts. *J. Leukoc. Biol.* **2009**, *86*, 13–22. [CrossRef] [PubMed]
75. Mikulski, Z.; Hartmann, P.; Jositsch, G.; Zasłona, Z.; Lips, K.S.; Pfeil, U.; Kurzen, H.; Lohmeyer, J.; Clauss, W.G.; Grau, V.; et al. Nicotinic receptors on rat alveolar macrophages dampen ATP-induced increase in cytosolic calcium concentration. *Respir. Res.* **2010**, *11*, 133. [CrossRef]
76. Mishra, N.C.; Rir-sima-ah, J.; Boyd, R.T.; Singh, S.P.; Gundavarapu, S.; Langley, R.J.; Razani-Boroujerdi, S.; Sopori, M.L. Nicotine inhibits Fc epsilon RI-induced cysteinyl leukotrienes and cytokine production without affecting mast cell degranulation through alpha 7/alpha 9/alpha 10-nicotinic receptors. *J. Immunol.* **2010**, *185*, 588–596. [CrossRef] [PubMed]
77. Valbuena, S.; Lerma, J. Non-canonical signaling, the hidden life of ligand-gated ion channels. *Neuron* **2016**, *92*, 316–329. [CrossRef] [PubMed]
78. Godin, J.-R.; Roy, P.; Quadri, M.; Bagdas, D.; Toma, W.; Narendrula-Kotha, R.; Kishta, O.A.; Damaj, M.I.; Horenstein, N.A.; Papke, R.L.; et al. A silent agonist of α7 nicotinic acetylcholine receptors modulates inflammation ex vivo and attenuates EAE. *Brain Behav. Immun.* **2019**. [CrossRef] [PubMed]
79. Báez-Pagán, C.A.; Delgado-Vélez, M.; Lasalde-Dominicci, J.A. Activation of the macrophage α7 nicotinic acetylcholine receptor and control of inflammation. *J. Neuroimmune Pharmacol.* **2015**, *10*, 468–476. [CrossRef]
80. Siniavin, A.E.; Streltsova, M.A.; Kudryavtsev, D.S.; Shelukhina, I.V.; Utkin, Y.N.; Tsetlin, V.I. Activation of α7 nicotinic acetylcholine receptor upregulates HLA-DR and macrophage receptors: Potential role in adaptive immunity and in preventing immunosuppression. *Biomolecules* **2020**, *10*, 507. [CrossRef]

81. Rocha, N.P.; Teixeira, A.L.; Coelho, F.M.; Caramelli, P.; Guimarães, H.C.; Barbosa, I.G.; da Silva, T.A.; Mukhamedyarov, M.A.; Zefirov, A.L.; Rizvanov, A.A.; et al. Peripheral blood mono-nuclear cells derived from Alzheimer's disease patients show elevated baseline levels of secreted cytokines but resist stimulation with β-amyloid peptide. *Mol. Cell. Neurosci.* **2012**, *49*, 77–84. [CrossRef]
82. Reale, M.; Di Nicola, M.; Velluto, L.; D'Angelo, C.; Costantini, E.; Lahiri, D.K.; Kamal, M.A.; Yu, Q.-S.; Greig, N.H. Selective acetyl- and butyrylcholinesterase inhibitors reduce amyloid-β ex vivo activation of peripheral chemo-cytokines from Alzheimer's disease subjects: Exploring the cholinergic anti-inflammatory pathway. *Curr. Alzheimer Res.* **2014**, *11*, 608–622. [CrossRef] [PubMed]
83. Udan, M.L.D.; Ajit, D.; Crouse, N.R.; Nichols, M.R. Toll-like receptors 2 and 4 mediate Abeta(1-42) activation of the innate immune response in a human monocytic cell line. *J. Neurochem.* **2008**, *104*, 524–533. [CrossRef] [PubMed]
84. Ebrahimi, T.; Rust, M.; Kaiser, S.N.; Slowik, A.; Beyer, C.; Koczulla, A.R.; Schulz, J.B.; Habib, P.; Bach, J.P. α1-antitrypsin mitigates NLRP3-inflammasome activation in amyloid β1-42-stimulated murine astrocytes. *J. Neuroinflamm.* **2018**, *15*, 282. [CrossRef] [PubMed]
85. Halle, A.; Hornung, V.; Petzold, G.C.; Stewart, C.R.; Monks, B.G.; Reinheckel, T.; Fitzgerald, K.A.; Latz, E.; Moore, K.J.; Golenbock, D.T. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat. Immunol.* **2008**, *9*, 857–865. [CrossRef]
86. El Khoury, J.B.; Moore, K.J.; Means, T.K.; Leung, J.; Terada, K.; Toft, M.; Freeman, M.W.; Luster, A.D. CD36 mediates the innate host response to beta-amyloid. *J. Exp. Med.* **2003**, *197*, 1657–1666. [CrossRef]
87. Sheedy, F.J.; Grebe, A.; Rayner, K.J.; Kalantari, P.; Ramkhelawon, B.; Carpenter, S.B.; Becker, C.E.; Ediriweera, H.N.; Mullick, A.E.; Golenbock, D.T.; et al. CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. *Nat. Immunol.* **2013**, *14*, 812–820. [CrossRef]
88. Kudo, S.; Mizuno, K.; Hirai, Y.; Shimizu, T. Clearance and tissue distribution of recombinant human interleukin 1 beta in rats. *Cancer Res.* **1990**, *50*, 5751–5755.
89. Lopez-Castejon, G.; Brough, D. Understanding the mechanism of IL-1β secretion. *Cytokine Growth Factor Rev.* **2011**, *22*, 189–195. [CrossRef]
90. Schmidt, J.; Barthel, K.; Wrede, A.; Salajegheh, M.; Bähr, M.; Dalakas, M.C. Interrelation of inflammation and APP in sIBM: IL-1 beta induces accumulation of beta-amyloid in skeletal muscle. *Brain* **2008**, *131*, 1228–1240. [CrossRef]
91. Alasmari, F.; Alshammari, M.A.; Alasmari, A.F.; Alanazi, W.A.; Alhazzani, K. Neuroinflammatory cytokines induce amyloid beta neurotoxicity through modulating amyloid precursor protein levels/metabolism. *BioMed Res. Int.* **2018**, *2018*, 3087475. [CrossRef]

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Activation of endothelial NO synthase and P2X7 receptor modification mediates the cholinergic control of ATP-induced interleukin-1β release by mononuclear phagocytes

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Objective: The pro-inflammatory cytokine interleukin-1β (IL-1β) plays a central role in host defense against infections. High systemic IL-1β levels, however, promote the pathogenesis of inflammatory disorders. Therefore, mechanisms controlling IL-1β release are of substantial clinical interest. Recently, we identified a cholinergic mechanism inhibiting the ATP-mediated IL-1β release by human monocytes *via* nicotinic acetylcholine receptor (nAChR) subunits α7, α9 and/or α10. We also discovered novel nAChR agonists that trigger this inhibitory function in monocytic cells without eliciting ionotropic functions at conventional nAChRs. Here, we investigate the ion flux-independent signaling pathway that links nAChR activation to the inhibition of the ATP-sensitive P2X7 receptor (P2X7R).

Methods: Different human and murine mononuclear phagocytes were primed with lipopolysaccharide and stimulated with the P2X7R agonist BzATP in the presence or absence of nAChR agonists, endothelial NO synthase (eNOS) inhibitors, and NO donors. IL-1β was measured in cell culture supernatants. Patch-clamp and intracellular Ca^{2+} imaging experiments were performed on HEK cells overexpressing human P2X7R or P2X7R with point mutations at cysteine residues in the cytoplasmic C-terminal domain.

Results: The inhibitory effect of nAChR agonists on the BzATP-induced IL-1β release was reversed in the presence of eNOS inhibitors (L-NIO, L-NAME) as well

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as in U937 cells after silencing of eNOS expression. In peripheral blood mononuclear leukocytes from eNOS gene-deficient mice, the inhibitory effect of nAChR agonists was absent, suggesting that nAChRs signal *via* eNOS to inhibit the BzATP-induced IL-1β release. Moreover, NO donors (SNAP, S-nitroso-N-acetyl-DL-penicillamine; SIN-1) inhibited the BzATP-induced IL-1β release by mononuclear phagocytes. The BzATP-induced ionotropic activity of the P2X7R was abolished in the presence of SIN-1 in both, *Xenopus laevis* oocytes and HEK cells over-expressing the human P2X7R. This inhibitory effect of SIN-1 was absent in HEK cells expressing P2X7R, in which C377 was mutated to alanine, indicating the importance of C377 for the regulation of the P2X7R function by protein modification.

Conclusion: We provide first evidence that ion flux-independent, metabotropic signaling of monocytic nAChRs involves eNOS activation and P2X7R modification, resulting in an inhibition of ATP signaling and ATP-mediated IL-1β release. This signaling pathway might be an interesting target for the treatment of inflammatory disorders.

KEYWORDS

inflammation, P2X7 receptor, CHRNA7, CHRNA9, CHRNA10, endothelial NO synthase, monocytes, macrophages

Introduction

Extracellular ATP is sensed by two main classes of purinergic receptors, the G protein-coupled P2Y receptors and the P2X receptors that form trimeric ligand-gated ion channels (1–5). P2X receptors are of outstanding interest as therapeutic targets, as they are expressed by most human or animal cell types and are involved in pain (6) as well as in the pathogenesis of numerous diseases including cancer, cardiovascular disease (7) and virtually all inflammatory diseases (8). The mammalian P2X receptors largely differ in their rates of desensitization and in their affinity towards ATP (5). In both counts, the P2X7 receptor (P2X7R) is an extreme: ATP concentrations in the high micromolar range are needed for P2X7R activation, and there is a complete lack of desensitization (5). In contrast to other P2X receptors, the P2X7R has a characteristic large cytoplasmic C terminal domain, the structure of which has only been recently elucidated by cryoelectron microscopy (9). It consists of three functionally important domains, first a cysteine-rich domain, which prevents P2X7R desensitization, second a cytoplasmatic cap that interacts with the short N-terminus of the P2X7R and contributes to the ion channel, and third a so-called ballast region (9). Further, the cytoplasmic C terminal domain of the P2X7R contains several consensus sequences for the interaction with other proteins and numerous amino acid residues, which are putative sites for covalent protein modification (9–11).

In mononuclear phagocytes extracellular ATP originating from activated cells or spilled cytoplasm of damaged cells is a well-known danger signal. It triggers the ionotropic function of the P2X7R, which lowers the intracellular concentration of K^+ ions (12). A reduced K^+ concentration is a major trigger for the assembly of the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome. The NLRP3 inflammasome is a multiprotein complex, that specifically activates caspase-1 enabling the proteolytic maturation and secretion of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18 (12–15). Inflammasome activation can result in pyroptosis, a form of cell death that further promotes inflammation (15). Both cytokines, IL-1β and IL-18, are center stage in host defense against infections, which pose a major threat for trauma patients. While the protective functions of IL-1β and IL-18 are vital, overshooting cytokine release can induce systemic inflammation causing barrier dysfunction, shock, sepsis, and, eventually, life-threatening multi-organ dysfunction. This is for instance a frequent complication in patients with multiple traumata or those, who underwent major surgery (16). Hence it is conceivable, that several mechanisms evolved that control the IL-1β release, which have been comprehensively reviewed before (12–15).

Most of the mechanisms controlling the release of inflammasome-dependent cytokines also inhibit ATP-independent pathways. Interestingly, we discovered several pathways by which unconventional nicotinic acetylcholine receptors (nAChRs) specifically inhibit the ionotropic function of monocytic P2X7Rs, without impairing ATP-independent pathways of inflammasome activation that are typical for pathogens (17–19). This is of high clinical relevance, because, at least in the acute phase of trauma and major surgery, host defense against infection is desirable, while trauma-induced sterile inflammation should be avoided. We demonstrated that activation of monocytic nAChRs composed of subunits α9, α7 and/or α10 by classical agonists such as acetylcholine or nicotine efficiently inhibit ATP-induced ion

currents at monocytic P2X7Rs by a ion flux-independent mechanism (17, 18). Further non-canonical endogenous agonists of these nAChRs were identified, including phosphocholine (PC), phosphatidylcholines (20) and other compounds with a PC head-group (17–19, 21). In contrast to classical agonists, these non-canonical agonists do not induce ion currents at human nAChRs heterologously expressed in *Xenopus laevis* oocytes but seem to function as silent agonists or weak antagonists (18, 19). Interestingly, the acute-phase reactants C-reactive protein (CRP) (22), α1-antitrypsin (AAT) (23), and secretory leukocyte protease inhibitor (SLPI) (24) also activate monocytic nAChRs to control the function of the P2X7R (25). This suggests that these proteins are part of negative feed-back loops limiting systemic inflammation (25). None of these nAChR agonists induce ion currents at monocytic nAChRs, but metabotropically inhibit the ionotropic function of the P2X7R.

The purpose of this study is to elucidate key steps of the signal transduction mechanism that links activation of nAChRs to the inhibition of the ionotropic function of the P2X7R and, hence, to an inhibition of NLRP3 inflammasome activation and IL-1β release. We provide evidence that agonists of nAChRs activate the endothelial NO synthase (eNOS, *NOS3*), which results in a modification of C377 in the C terminal cytoplasmic domain of the P2X7R and, thus, in an inactivation of its ionotropic function.

Materials and methods

Chemicals and reagents

ACh chloride (Cat# A6625), apyrase (Cat# A6410), adenosine 5′-triphosphate (ATP) disodium salt hydrate (Cat# A2383), bovine serum albumin (BSA, Cat# A9418), choline chloride (Cat# C7017), CRP (Cat# AG723-M), dimethyl sulfoxide (DMSO, Cat# D2650), lipopolysaccharide (LPS, *E. coli* O111:B4, Cat# L2630; *E. coli* O26:B6, Cat# L2654), macrophage colony-stimulating factor (M-CSF, Cat# SRP3110), nicotine hydrogen tartrate salt (Cat# N5260), nigericin sodium salt (Cat# N7143), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, Cat# N5751), phorbol 12-myristate 13-acetate (PMA, Cat# P1585), PC chloride calcium salt tetrahydrate (Cat# P0378), recombinant mouse interferon-γ (INF-γ, Cat# IF005), S-nitroso-N-acetyl-DL-penicillamine (SNAP, Cat# 3398), SIN-1 hydrochloride (Cat# 567028), and tetracycline hydrochloride (Cat# T7660) were purchased from Merck (Darmstadt, Germany). All chemicals for saline Ringer's buffer preparations were purchased from Merck (Darmstadt, Germany). 1400W dihydrochloride (Cat# 1415), L-NIO dihydrochloride (Cat# 0546), and N^{ω}-propyl-L-arginine hydrochloride (N-PLA, Cat# 1200) were purchased from Tocris (Bio-Techne, Wiesbaden-Nordenstadt, Germany). Gibco penicillin-streptomycin solution and L-glutamine solution were purchased from Thermo Fisher Scientific (Dreieich, Germany). BzATP (2'(3')-O-(4-benzoyl-benzoyl)ATP trieethylammonium salt) was purchased from Jena Bioscience (Jena, Germany), 0.5 M ethylenediaminetetraacetic acid (EDTA) solution from bioWORLD (Dublin, OH, United States, Cat# 40520000), Hygromycin B Gold solution (Cat# ant-hg-1) and blasticidin solution (Cat# ant-bl-05) from InvivoGen (Toulouse, France), and recombinant human INF-γ from R&D Systems (Minneapolis, MN, United States; Cat# 285-IF-100). PMA and SNAP were dissolved in DMSO. Nigericin was dissolved in ethanol (EtOH). When appropriate, control experiments were performed with the corresponding concentrations of DMSO/EtOH without drugs.

U937 cells

Monocytic U937 cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). U937 cells were cultured in Gibco™ RPMI 1640 medium (Thermo Fisher Scientific, Cat# 11530586) supplemented with 10% fetal calf serum (FCS; CellConcepts, Umkirch, Germany) under 5% CO_2 atmosphere at 37°C as described before (18, 19). In some experiments the expression of eNOS (*NOS3*) in U937 cells was reduced by using siRNA technology. For this purpose, U937 cells were transfected with ON-TARGETplus human eNOS siRNA SMARTpool (Thermo Fisher Scientific). To test for unspecific effects of siRNA transfection, cells were transfected in parallel with negative control ON-TARGETplus Non-targeting Control Pool (Thermo Fisher Scientific). The transfection was performed in accordance with the manufacturer's protocol and as described previously (18, 19). In brief, U937 cells were transfected with 30 pM siRNA/1 × 10^6 cells using the Amaxa Cell Line Nucleofector Kit C and Nucleofector II Device (both from Lonza Cologne, Cologne, Germany). The transfected cells were then cultured for 48 h under 5% CO_2 atmosphere at 37°C.

To investigate IL-1β release, untreated or transfected cells were resuspended on the day of the experiments in fresh RPMI medium + 10% FCS. 1 × 10^6 cells/ml were transferred per well to 12-well plates (Greiner Bio-One, Frickenhausen, Germany) and stimulated with 1 μg/ml LPS (*E. coli* O26:B6) for 5 h under 5% CO_2 atmosphere at 37°C. Thereafter, BzATP (100 μM) was added for 30 min in the presence or absence of cholinergic agonists, NOS inhibitors or NO donors. At the end of the experiments, cells were spun down (500 g, 8 min, 4°C) and the cell-free supernatants were collected and stored at −20°C for later cytokine and lactate dehydrogenase (LDH) measurements.

THP-1 cells

THP-1 cells were obtained from the German Collection of Microorganisms and Cell Cultures and cultured under 5% CO_2 atmosphere at 37°C using RPMI 1640 medium from Sigma (Merck, Cat# R8758) supplemented with 10% FCS from Capricorn (Ebsdorfergrund, Germany, Cat# FBS-16A). To investigate IL-1β release, monocytic THP-1 cells were resuspended on the day of the experiments in FCS-free RPMI medium, and 0.5 × 10^6 cells/0.5 ml were transferred per well to 48-well plates (Greiner Bio-One). Thereafter, cells were treated with 1 μg/ml LPS (*E. coli* O26:B6) and cultured for 5 h under 5% CO_2 atmosphere at 37°C as described previously (26). After priming, BzATP (100 μM) was added for 40

min in the presence or absence of cholinergic agonists, NOS inhibitors, or NO donors. At the end of the experiments, cells were spun down (500 g, 8 min, 4°C) and the cell-free supernatants were collected and stored at −20°C for later cytokine measurements.

Monocytic THP-1 cells acquire a macrophage-like phenotype by exposure to PMA (27). To obtain THP-1 cell-derived M1-like macrophages, a previously described protocol (26) was used with modifications. Monocytic THP-1 cells were resuspended in RPMI 1640 medium (Sigma) supplemented with 10% FCS (Capricorn), 50 U penicillin/ml and 50 µg streptomycin/ml. 0.3×10^6 cells/ml and per well were seeded in 12-well plates (Greiner) and incubated with 50 nM PMA for 24 h, followed by 24 h incubation in fresh complete medium without PMA. Thereafter, to induce cell differentiation into M1-like macrophages, cells were cultured in fresh complete medium supplemented with 10 ng/ml human IFN-γ and 10 ng/ml LPS (*E. coli* O111:B4) for 48 h. To investigate IL-1β release on day 5, the medium was replaced by fresh RPMI 1640 medium (Sigma) supplemented with 10% FCS. The cells were stimulated with 1 µg/ml LPS (*E. coli* O26:B6) for 5 h and handled as described for monocytic THP-1 cells. The identity of M1-like macrophages on day 5 was evaluated by flow cytometry (see below). For this purpose, M0-like macrophages were differentiated in parallel to M1-like macrophages, using the above-described protocol without adding human IFN-γ and LPS. Cells were washed once with Dulbecco´s phosphate buffered saline without calcium and magnesium (PBS; Merck Cat# D8537) and detached using TrypLE™ Express (Thermo Fisher Scientific, Cat# 12605010) according to the manufacturer's protocol. The cell number was determined, and cells were stored on ice until flow cytometry.

Murine mononuclear leukocytes and bone marrow-derived macrophages

Male and female wild-type mice from The Jackson Laboratory (C57BL/6J), *Nos3* gene-deficient mice (C57BL/6.129/Ola-eNOS™) (28) and corresponding wild-type mice were used for isolation of peripheral blood mononuclear leukocytes (PBMCs). Experimental animals received humane care according to NIH "Guide for the Care and Use of Laboratory Animals", and animal experiments were approved by the local authorities (University Düsseldorf, reference No. O16/04; Regierungspräsidium Giessen, Germany, reference no. 571_M). Mice were euthanized and blood was immediately drawn from the caval vein into heparinized syringes (2 ml syringes filled with 15 µl heparin solution (5.000 I.E/ml) from Ratiopharm, Ulm, Germany (Cat# 03029843). PBMCs were separated using discontinuous Percoll (Ge Healthcare Bio-Sciences AB, Uppsala, Sweden; 1.082 g/ml) density gradient centrifugation as described before (18). 0.1 x 10^6 cells/0.1 ml were seeded in 96-well plates (Greiner) and cultured for 2 h in RPMI 1640 medium (Gibco or Sigma) at 5% CO_2 at 37°C. Thereafter, non-adherent cells were removed, and fresh RPMI medium was added. For investigation of IL-1β release, BzATP (100 µM) or ATP (1 mM) was added for 30 min in the presence or absence cholinergic agonists or NO donors. Subsequently, cell culture supernatants were collected and stored at −20°C for later cytokine measurements.

Male and female C57BL/6J wild-type mice were used for bone marrow cell isolation and bone marrow-derived macrophage (BMDM) differentiation using a previously published protocol with modifications (26). 1×10^6 bone marrow cells/ml complete medium [RPMI 1640 medium (Cytiva, Marlborough, MA, United States) supplemented with 10% FCS (CellConcepts), 50 U penicillin/ml, 50 µg streptomycin/ml and 10 ng/ml M-CSF were seeded in in Costar 12-well plates (Corning, NY, United States) and cultured for 72 h. Thereafter, to induce cell differentiation into M1-like macrophages, 1 ml of fresh complete medium was added supplemented with a final concentration of 10 ng/ml M-CSF, 10 ng/ml IFN-γ and 10 pg/ml LPS (*E. coli* O26:B6). Cells were cultured for another period of 72 h. On day 6 of cultivation, BMDMs were stimulated with 1 µg/ml LPS (*E. coli* O26:B6) for 5 h. Thereafter, ATP (1 mM) or nigericin (50 µM; together with 0.5 U/ml apyrase) was added in the absence or presence of the NO donors SNAP (10 mM) or SIN-1 (1 mM). Cell-free supernatant was harvested 40 min later and stored at −20°C for IL-1β measurements.

Human primary monocytic cells

Blood samples were obtained from healthy (self-reported) female and male non-smoking adult volunteers. The study was performed in accordance with the Helsinki Declaration and approved by the ethics committee of the medical faculty Giessen, Germany (No. 90/18). Blood was drawn into sterile syringes containing 1 mM EDTA per ml blood. Monocytes were enriched from whole blood by negative selection using the RosetteSep™ human Monocyte Enrichment Cocktail and Lymphoprep™ density gradient (both from Stemcell Technologies, Cologne, Germany) according to the manufacturer's protocol. Thereafter, enriched monocytes were stimulated with 5 ng/ml LPS (*E. coli* O26:B6) for 25 min during the first washing step. After centrifugation at 250 g (15 min, room temperature) and a second wash followed by centrifugation at 300 g (10 min, room temperature), the cells were resuspended in RPMI medium (Sigma). The purity of monocytes was evaluated by flow cytometry (see below).

To investigate IL-1β release, 1×10^5 cells/250 µl RPMI medium were seeded in 96-well plates and cultured at 37°C, 5% CO_2 for 3 h in LPS-free medium. Thereafter, the cells were stimulated with BzATP (100 µM) or nigericin (25 µM plus 0.5 U/ml apyrase) in the presence or absence SNAP or SIN-1. After another 30 min of incubation, cell-free cell culture supernatants were harvested and stored at −20°C until measurement of IL-1β concentrations and LDH activity.

Measurement of IL-1β concentration and LDH activity

IL-1β concentrations in cell-free supernatants obtained in experiments on human monocytic U937 cells, THP-1 cells (monocytic, macrophage-like), and human primary monocytic cells were measured using the Human IL-1 beta/IL-1F2 DuoSet enzyme-linked immunosorbent assay (ELISA) from R&D Systems

(Cat# DY201) according to the supplier's instructions. In samples obtained in the mouse PBMC experiments, IL-1β concentrations were measured by using the Mouse Quantikine IL-1β Immunoassay (R&D Systems Cat# MLB00C), whereas samples obtained in the mouse BMDM experiments were measured by using the Mouse IL-1 beta/IL-1F2 DuoSet ELISA (R&D Systems Cat# DY401). To test for cell viability at the end of the cell culture experiments, the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, United States; Cat# G1780) was used to measure LDH activity according to the supplier's instructions. LDH activities determined in cell-free supernatants are given as percentage of the total LDH activity of lysed control cells (Supplementary Table S1).

RNA isolation, cDNA synthesis and real-time RT-PCR

To confirm efficient and selective silencing of human eNOS mRNA expression by siRNA, real-time RT-PCR was performed on siRNA transfected U937 cells and corresponding controls. First, total RNA was isolated from 1 × 10^6 U937 cells using the RNesay Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The obtained RNA (1 µg) was reversely transcribed using M-MLVH Reverse Transcriptase and 1 µg of random hexamer primers (Promega, Mannheim, Germany). Thereafter, real-time RT-PCR was performed (n = 4 per experimental group, each sample was assessed in duplicates) using the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and Platinum SYBER green qPCR Super Mix-UDG (Invitrogen, Karlsruhe, Germany). Primers specific for human eNOS (fwd: 5′-AGC ACT GAG ATC GGC ACG AGG A-3′; rev: 5′-TGC TGC CTT GTC TTT CCA CAG GG-3′), human neuronal NOS (nNOS, *NOS1*; fwd: 5′-CCA CGG CCC ACG GGA TGT TC-3′; rev: 5′-CTC GGA AGT CGT GCT TGC CGT-3′), and the house keeping gene hydroxymethylbilane synthase (HMBS; fwd: 5′-CCC ACG CGA ATC ACT CTC AT-3′; rev: 5′-TGT CTG GTA ACG GCA ATG CG-3′) (17) were synthesized by Eurofins Genomics (Ebersberg, Germany). As a negative control, samples were run under the same conditions, using nuclease-free water instead of cDNA. Changes in the mRNA expression levels of eNOS were estimated by the $2^{\Delta CT}$ method, where ΔCT represents the difference between the CT value of HMBS gene and the CT value of gene of interest (17, 19, 24). The mean of the mRNA expression values from cells transfected with non-targeting siRNA was set to one and the values from cells transfected with siRNA specific for eNOS were calculated accordingly.

Flow cytometry

Flow cytometry analyses were performed to evaluate cell surface marker expression of THP-1 cell-derived macrophages (M0- and M1-like) and the purity of RosetteSep™ enriched human monocytes. THP-1 cell-derived macrophages were characterized by using a panel of antibodies against cluster of differentiation (CD) 14, CD38, CD80 and HLA-DR (see Supplementary Table S2).

Human monocytes can be classified into three main subsets, classical (~85%), intermediate (~5%), and non-classical (~10%) monocytes, which are characterized by their level of CD14 and CD16 expression (29). A previously published gating strategy for whole blood flow cytometry to identify and characterize human monocyte subsets (30) was modified and used to analyze the purity of RosetteSep™ enriched human monocytes. To assess the monocyte subset proportions a panel of antibodies was used (see Supplementary Table S2). Antibodies against T cells (CD3) and B cells (CD19) were used to assess lymphocytic contaminations. The purity of the enriched cells was displayed by using a CD14/CD16/CD45/HLA-DR panel.

Cells were resuspended in flow cytometry buffer (PBS, 2 mM EDTA, 5% FCS). Thereafter, 5 × 10^5 cells were incubated with appropriate antibodies for 30 min on ice. All antibodies (Supplementary Table S2) were used at the manufacturer's recommended dilution or titrated to determine the optimal staining concentrations (between 1:20 and 1:100). Cells were also incubated with the control isotype antibody corresponding to each primary antibody. Thereafter, cells were washed twice in flow cytometry buffer. Then, the cells were fixed with 1% dissolved paraformaldehyde (PFA) before flow cytometry analysis.

Cells were acquired on a BD FACSVerse™ System (BD Biosciences) using the BD FACSuite™ software, recording at least 10,000 events for each sample. Forward scatter (FSC) files were exported and analyzed in FlowJo software version 10.8.1 (FlowJo, LLC). Monocytes and macrophages were gated by FSC–area (FSC-A) and side scatter–area (SSC-A), then single cells by SSC-A and side scatter-width (SSC-W). Surface marker-positive cells were gated based on Fluorescence Minus One (FMO) or isotype controls and the percentage of surface marker positive cells was measured and exported.

Heterologous expression of human P2X7R and two-electrode voltage-clamp measurements

Electrophysiological two-electrode voltage-clamp (TEVC) measurements were performed on *Xenopus laevis* oocytes stages V and VI purchased from Ecocyte Bioscience (Castrop-Rauxel, Germany). Oocytes were injected with cRNA (0.35 ng per oocyte) encoding for the human P2X7R as previously described (31). In brief, the oocytes were placed in a perfusion chamber, perfused at room temperature with a Ca^{2+}-free frog Ringer's solution containing (mM): 100 NaCl, 2.5 KCl, 5 HEPES (4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid) and 0.1 flufenamic acid (pH 7.4), and impaled with two 1 M KCl-filled electrodes. The membrane voltage was clamped to −40 mV and the transmembrane ion currents (I_M) were recorded.

When the I_M was stabilized, BzATP (dissolved in frog Ringer's solution to a working solution of 10 µM) was applied for 2 min (31). After a wash out period, the oocytes were transferred to a 24-well plate and incubated for 2 h at room temperature in either oocyte Ringer's solution or Ringer's solution supplemented with 1 mM SIN-1. Subsequently, oocytes were placed in the perfusion chamber,

voltage clamped to –40 mV and the BzATP-induced changes in I_M were determined for a second time. Measurements were performed on oocytes from at least two different *Xenopus laevis* frogs.

HEK293 cell lines stably expressing wild-type or mutated human P2X7R upon stimulation with tetracycline

The generation of a stable Tet-on HEK293 cell line expressing the full-length wild-type human P2X7R (hP2X7R) under the control of the tetracycline-inducible tetR promotor (wt-hP2X7-HEK) has been described previously (32). In the same wt-hP2X7R pcDNA5/FRT/TO plasmid used before, we mutated C377 or C388 singly to alanine using the QuikChange site-directed mutagenesis protocol (33) with Phusion high-fidelity DNA polymerase and Dpn I restriction endonuclease (both from New England BioLabs, Schwalbach, Germany). After verification of the DNA sequences by commercial nucleotide sequencing (Eurofins), we generated stable C377A (hP2X7R_C377A-HEK) and C388A-hP2X7R-HEK cell lines using the Flp-InTM T-RExTM-system (Invitrogen, Thermo Fisher Scientific).

Intracellular Ca^{2+} imaging

Changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were imaged in wild-type human P2X7R-HEK cells as previously described (32). Cells were seeded in high glucose DMEM (Gibco) supplemented with 10% FCS (Biochrome) and 2 mM L-glutamine (Gibco) in glass bottom culture dishes (CELLview™, Greiner Bio-One, Kremsmünster, Austria) and grown for 24 – 48 h. To induce P2X7R expression, 1 µg/ml tetracycline was added, and cells were cultured for another 24 – 48 h at 37°C, 5% CO_2. To image $[Ca^{2+}]_i$, the culture medium was replaced by a saline bath Ringer's solution containing (in mM): 5.4 KCl, 120 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 25 glucose (pH 7.4). Imaging was performed at room temperature and as described previously (22, 32). Three independent batches of P2X7R-HEK cells were used in these experiments and a total number of 411 cells were tracked individually.

Whole-cell patch-clamp measurements on human wild-type P2X7R and P2X7R mutants

Whole-cell patch-clamp measurements were performed on wt-hP2X7R-HEK, hP2X7R_C377A-HEK and hP2X7R_C388A-HEK cells. The cells were seeded in high glucose DMEM (Gibco) supplemented with 10% FCS (Biochrome) and 2 mM L-glutamine (Gibco) in cell culture dishes (Nunc, Roskilde, Denmark) and cultured at 37°C, 5% CO_2 for 24 – 48 h. P2X7R expression was induced by adding 1 µg/ml tetracycline (Sigma-Aldrich) and a subsequent incubation for 24 – 48 h, as described previously (32). For the measurements, DMEM was replaced by bath solution (for details see $[Ca^{2+}]_i$ measurements) and whole-cell patch clamp recordings were performed as described previously (22, 31, 32). The whole-cell recordings were performed at room temperature. The membrane potential was clamped to –60 mV and the whole-cell transmembrane currents (measurement I) were recorded. BzATP (100 µM) and SIN-1 (1 mM) were dissolved in bath solution and applied by using a pressure-driven microperfusion system (measurement II). At least three independent batches of wt-hP2X7R-HEK, hP2X7R_C377A-HEK and hP2X7R_C388A-HEK cells were used in these experiments.

Structural protein images of the rat P2X7R

Structural images of the rP2X7R protein were generated with the molecular visualization system PyMOL by Schrödinger (2022, Version 2.5) using the cryo-EM PDB files PDB 6u9v and 6u9w for the closed and open structures of rP2X7R, respectively (9).

Statistical analyses and data processing

Results obtained in IL-1β release or electrophysiological experiments were analyzed using SPSS® (Version 23, IBM®, Armonk, NY, USA). Multiple independent data sets were first analyzed by the non-parametric Kruskal–Wallis test. In case of a $p \leq 0.05$, the non-parametric Mann–Whitney U test was performed to compare between individual groups and again, a $p \leq 0.05$ was considered as evidence for statistical significance. Dependent data sets were analyzed first by the Friedman test followed by the Wilcoxon signed-rank test. All numbers (n) of the individual experiments are indicated in the Results section and in the Figures. The n-number given in experiments on primary cells represent data obtained from the cells of individual mice or humans. In experiments with cell lines, the n-number refers to independent experiments performed on different days and with different cell passages. No outliers were excluded from the analyses. An exception of this principle are cells used for electrophysiological measurements that lost voltage-clamp or membrane seal, or otherwise failed to remain viable throughout the measurements, and thus were excluded from the analyses. The free and open-source software Inkscape version 0.48.5 r10040 (licensed under the GPL) was used for visualization of the data.

Results

Inhibition of NOS reverts the inhibitory effects of nAChR agonists on IL-1β release. To test the hypothesis, that NOS are involved in the inhibitory effect of nAChR agonists on the ATP-induced release of IL-1β by monocytic cells, we made use of the NOS inhibitors L-NIO (34) (Figures 1A, B), L-NAME (35) (Figures 1C, D), N-PLA (Supplementary Figures S1A, B) and 1400 W (Supplementary Figures S1C, D). Cells of the human monocytic U937 cell line were primed with LPS (1 µg/ml) for 5 h to induce the expression of pro-IL-1β and of essential compounds of the NLRP3 inflammasome, followed by a second stimulation with the P2X7R

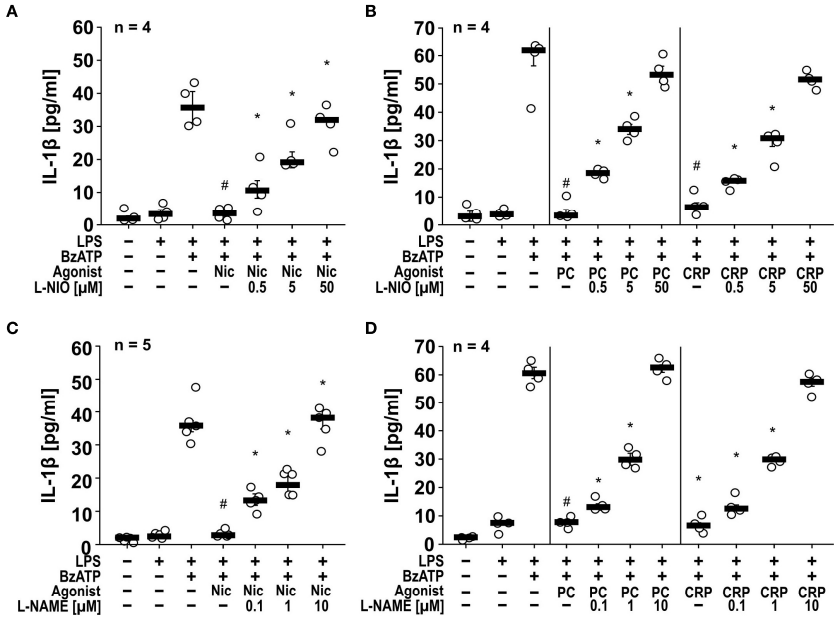

FIGURE 1
The inhibitory potential of nicotine (Nic), phosphocholine (PC) and C-reactive protein (CRP) on the BzATP-induced release of IL-1β by human monocytic U937 cells is prevented by nitric oxide synthase (NOS) inhibitors. U937 cells were primed with LPS (1 µg/ml) for 5 h, and BzATP (2′/3′-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate, tri(triethylammonium) salt; 100 µM) was added for another 30 min to trigger IL-1β release, which was measured by ELISA. The inhibitory potential of the nicotinic acetylcholine receptor (nAChR) agonists Nic (100 µM), PC (100 µM) and CRP (5 µg/ml) on the BzATP-induced release of IL-1β was investigated in absence and presence of the NOS inhibitors **(A, B)** L-NIO (N5-(1-iminoethyl)-L-ornithine dihydrochloride) and **(C, D)** L-NAME (N-omega-nitro-L-arginine methyl ester hydrochloride). The inhibitory potential of nAChR agonists on the BzATP-induced release was concentration-dependently prevented by all NOS inhibitors. **(A, C)** Some IL-1β values (untreated, LPS, LPS+BzATP, LPS +BzATP+Nic) served as controls for multiple NOS inhibitors (Supplementary Figures S1A, C). Data are presented as individual data points, bars represent median, whiskers percentiles 25 and 75. # $p \leq 0.05$ significantly different from samples, in which BzATP was given alone, *$p \leq 0.05$ significantly different from BzATP + nicotine. Kruskal-Wallis followed by Mann-Whitney rank sum test.

agonist BzATP (100 µM) for 30 min to induce inflammasome assembly. These experiments were performed in the presence or absence of nicotine (100 µM), PC (100 µM) or CRP (5 µg/ml). Thereafter, IL-1β was measured in the cell-free culture medium. As described before (17, 18, 22), supernatants of unstimulated cells and cells primed with LPS contained only trace-amounts of IL-1β, while supernatants of cells treated with both LPS and BzATP released 30 – 70 pg/ml IL-1β. As expected (17, 22), the BzATP-induced release of IL-1β was significantly ($p \leq 0.05$) inhibited by nicotine (n = 5), PC (n = 4) and CRP (n = 4) (Figure 1, Supplementary Figure S1). In line with our hypothesis, all investigated NOS inhibitors dose-dependently prevented the effects of the nicotinic agonists (Figure 1, Supplementary Figure S1). In the absence of nicotine, PC or CRP, NOS inhibitors did not affect the BzATP-induced release of IL-1β (n = 4, not shown).

To test if NOS inhibitors also function in another independent monocytic cell line, we repeated some of the experiments with human monocytic THP-1 cells and differentiated macrophage-like THP-1 cells (Figures 2A, B). Like U937 cells, monocytic and macrophage-like THP-1 cells primed with LPS and stimulated with BzATP released IL-1β (monocytic = 109 ± 45 pg/ml, n = 6; macrophage-like = 512 ± 217 pg/ml, n = 6), which was significantly inhibited by nicotine, PC and CRP (Figures 2A, B). In THP-1 cells, the NOS inhibitors L-NIO (50 µM) and L-NAME (10 µM) also significantly reverted the effects of all nAChR agonists tested (Figures 2A, B). Flow cytometrical analyses confirmed that differentiated THP-1 cells expressed cell surface markers typical for M1-like macrophages (Supplementary Figure S2).

The inhibitory effects of nAChR agonists on BzATP-induced IL-1β release depend on eNOS. As NOS function seems to be very quickly activated in response to nAChR agonists, we did not investigate the involvement of iNOS which is constitutively active and regulated on the mRNA level. Therefore, we further investigated if eNOS and nNOS, the functions of which are regulated on the protein level (36, 37), might be involved in the control of BzATP-mediated IL-1β release. In real-time RT-PCR experiments on U937 cells primed with LPS, *NOS3* mRNA was detectable albeit at low levels, while *NOS1* mRNA was in the range of the threshold of detection. Therefore, we hypothesized, that the eNOS is an essential part of the signaling cascade linking nAChR activation to the inhibition of the ionotropic P2X7R function. To test this hypothesis, we followed two independent approaches. First, we transfected U937 cells with control siRNA or siRNA specific for the *NOS3* gene, which significantly (n = 8, $p \leq 0.05$) down-regulated the mRNA expression of *NOS3* (Supplementary Figure S3). With these cells we performed the above-described IL-1β release

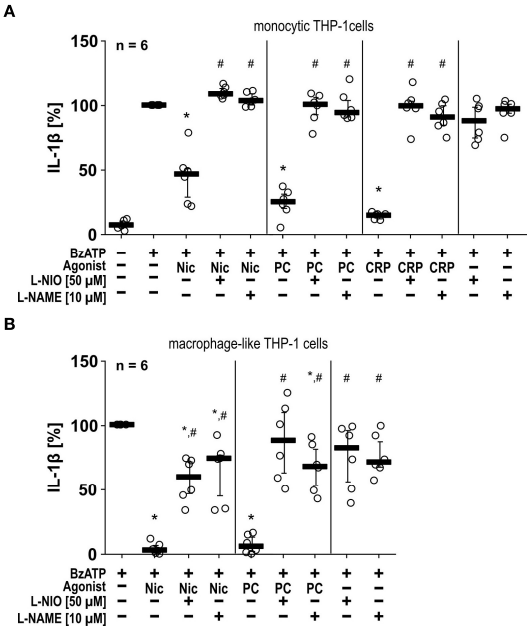

FIGURE 2
The inhibitory potential of nicotine (Nic), phosphocholine (PC) and C-reactive protein (CRP) on the BzATP-induced release of IL-1β by human monocytic and macrophage-like THP-1 cells is reversed by nitric oxide synthase (NOS) inhibitors. Monocytic **(A)** and differentiated macrophage-like **(B)** THP-1 cells were used. Cells were primed with lipopolysaccharide (LPS; 1 µg/ml, 5 h). Thereafter, the P2X7 receptor agonist BzATP ((2′/3′-O-(4-benzoylbenzoyl) adenosine-5′-triphosphate, tri(triethylammonium) salt) was added for another 40 min to trigger IL-1β release, which was measured by ELISA. The inhibitory potential of Nic (100 µM), PC (200 µM) and CRP (10 µg/ml) on the BzATP-induced release of IL-1β was investigated in absence and presence of the NOS inhibitors L-NIO (50 µM; N5-(1-iminoethyl)-L-ornithine dihydrochloride) or L-NAME (10 µM; N-omega-nitro-L-arginine methyl ester hydrochloride). **(B)** In experiments on macrophage-like THP-1 cells, the concentration of IL-1β released in response to BzATP was calculated by subtracting the IL-1β concentrations measured in supernatants of cells treated with LPS alone. In each experiment, the IL-1β concentration obtained after stimulation with BzATP was set to 100% and all other values were calculated accordingly. Data are presented as individual data points, bars represent median, whiskers encompass the 25th to 75th percentile. *p ≤ 0.05, different from LPS-primed cells stimulated with BzATP alone; #p ≤ 0.05 significantly different from samples, in which BzATP plus agonist was given. Friedman test followed by the Wilcoxon signed-rank test.

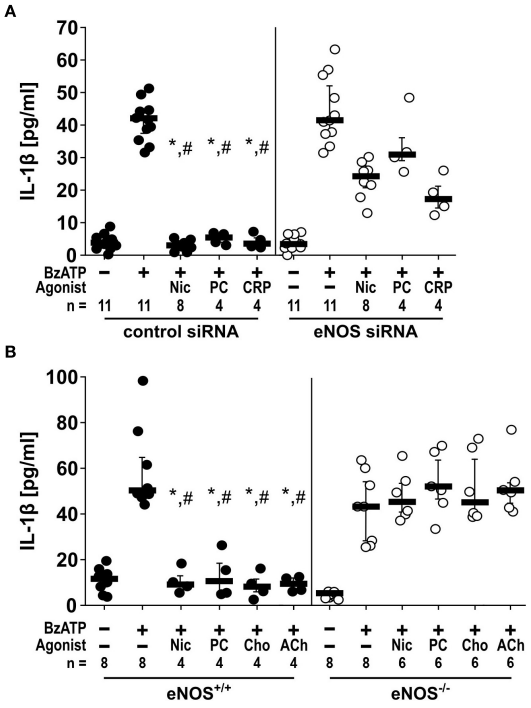

FIGURE 3
The inhibitory potential of cholinergic agonists on BzATP-induced IL-1β release by monocytic cells depends on endothelial nitric oxide synthase (eNOS, *NOS3*) activity. **(A)** U937 cells were transfected with siRNA targeting eNOS or with control siRNA. Forty-eight hours after transfection, cells were primed with lipopolysaccharide (LPS; 1 µg/ml) for 5 h and BzATP ((2′/3′-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate, tri(triethylammonium) salt; 100 µM) was given for additional 30 min in the presence or absence of nicotinic agonists nicotine (Nic; 100 µM), phosphocholine (PC; 100 µM) or C-reactive protein (CRP; 5 µg/ml). While in cells transfected with control siRNA, the BzATP-induced release of IL-1 β was inhibited by nicotinic agonists, the inhibitory activity of all agonists was blunted in eNOS siRNA-transfected cells. Data were analyzed by Kruskal-Wallis test followed by Mann-Whitney rank sum test. **(B)** Freshly isolated mouse peripheral blood mononuclear cells (PBMCs) isolated from wild-type ($eNOS^{+/+}$) mice and mice deficient in eNOS ($eNOS^{-/-}$) were left untreated or stimulated with BzATP for 30 min, in the presence or absence of nicotinic agonists Nic, PC, choline (Cho; 100 µM) or acetylcholine (ACh; 10 µM). BzATP induced the release of IL-1β in PBMCs obtained from all mouse strains. Nicotinic agonists significantly inhibited the IL-1β release by PBMCs from $eNOS^{+/+}$ mice. In contrast, the IL-1β release by PBMCs from $eNOS^{-/-}$ mice was not affected by nicotinic agonists. Friedman test followed by the Wilcoxon signed-rank test. Data are presented as individual data points, bars represent median, whiskers percentiles 25 and 75. ∗p ≤ 0.05 significantly different from samples, in which BzATP was given alone #p ≤ 0.05 significantly different from corresponding samples control siRNA vs. eNOS siRNA and $eNOS^{+/+}$ vs. $eNOS^{-/-}$.

experiments in the presence or absence of nAChR agonists. While transfection of control siRNA did not impair the inhibitory effect of nicotine, PC, and CRP on the BzATP-mediated release of IL-1β, transfection of siRNA specific for *NOS3* blunted the inhibitory effect (Figure 3A).

In a second approach, freshly isolated PBMCs from mice deficient in *NOS3* (C57BL/6.129/Ola-eNOS^{TM}) (28) and corresponding wild-type mice were used and left untreated or stimulated with BzATP for 30 min, in the presence or absence of nicotine, PC, choline (Cho; 100 µM), or acetylcholine (ACh; 10 µM). As expected, BzATP induced the release of IL-1β by PBMCs obtained from both mouse strains. Nicotinic agonists signifilicantly inhibited the BzATP-induced IL-1β release by PBMCs from wild-type mice. In contrast, the IL-1β release by PBMCs from *Nos3*-deficient mice was unaffected by all nicotinic agonists tested (Figure 3B).

NO and peroxynitrite mimic the inhibitory effects of nAChR agonists on IL-1β release. NOS catalyzes the conversion of L-arginine into NO and L-citrulline (34). Highly reactive peroxynitrite

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can be formed from NO in the presence of superoxide anions (38). To test our hypothesis that NO can inhibit the BzATP-induced release of IL-1β by LPS-primed monocytic U937 cells and THP-1 cells, we used the NO/peroxynitrite donors SNAP and SIN-1 (39) and performed IL-1β release assays in U937 cells (Figures 4A-C). In line with our hypothesis SNAP showed a dose-dependent inhibitory effect with first statistically significant effects at a 100 µM concentration (n = 4, $p \leq 0.05$) and a virtually complete inhibition at 10 mM (n = 4, $p \leq 0.05$; Figure 4A). In the same line, SIN-1 (1 mM) fully inhibited the BzATP-induced release of IL-1β, both when given together with BzATP or when applied 30 min earlier (n = 4, $p \leq 0.05$, each; Figure 4B). When the IL-1β secretion was triggered by the pore-forming bacterial toxin nigericin (50 µM), SIN-1 had no effect on the release of IL-1β (Figure 4C). In these experiments, the ATP-degrading enzyme apyrase (0.5 U/ml), was added together with nigericin to ensure, that only ATP-independent mechanisms are investigated.

In the monocytic cell line THP-1 similar results were obtained (Figures 4D, E). SNAP (10 mM) and SIN-1 (1 mM) significantly

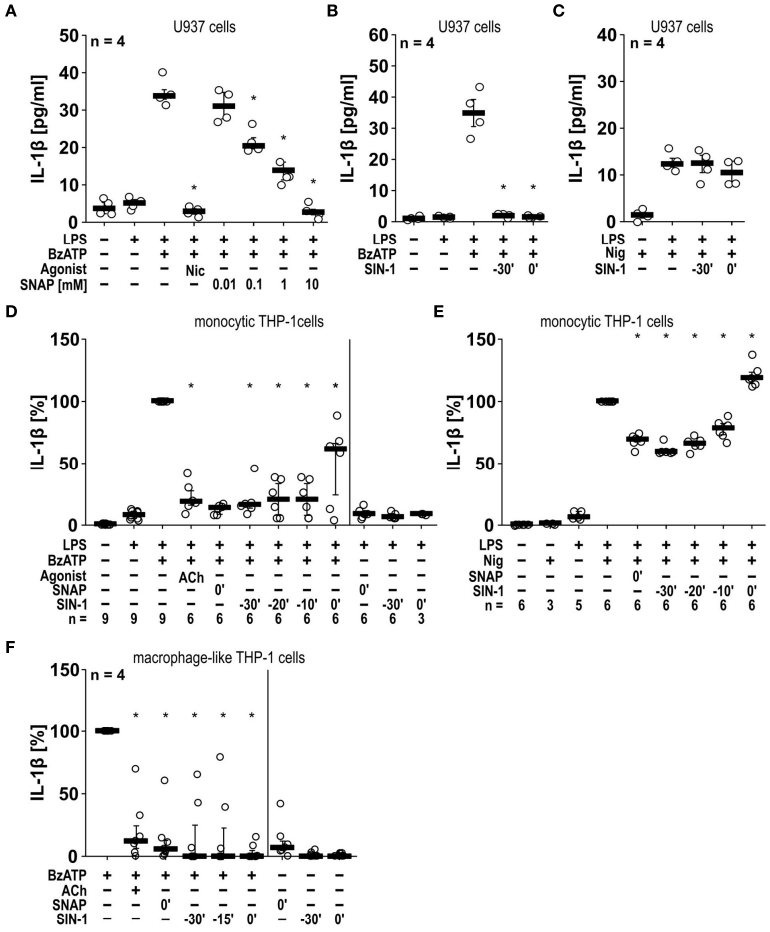

FIGURE 4
The BzATP-induced release of IL-1β by monocytic and macrophage-like cells is inhibited by the NO donors SNAP and SIN-1. **(A, B)** Monocytic U937 cells were primed with lipopolysaccharide (LPS; 1 µg/ml) for 5 h, and BzATP ((2′/3′-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate, tri (triethylammonium) salt; 100 µM) was added for another 30 min to trigger IL-1β release, which was measured by ELISA. The NO donor SNAP (S-nitroso-N-acetyl-DL-penicillamine) concentration-dependently inhibited the BzATP-induced release of IL-1β like nicotine (Nic; 100 µM), which served as a positive control **(A)**. Addition of SIN-1 (1 mM) 30 min before BzATP (t = -30′) or shortly before BzATP (t = 0′) inhibited the BzATP-induced release of IL-1β **(B)**. U937 cells were primed with LPS for 5 h, and nigericin (Nig) was added together with apyrase (0.5 U/ml) for another 30 min to trigger IL-1 β release. The Nig-induced IL-1β release was unimpaired by SIN-1 **(C)**. Kruskal–Wallis followed by Mann–Whitney rank sum test **(A–C)**. **(D, E)** Similar results were found on LPS-primed monocytic THP-1 cells stimulated with BzATP **(D)** or Nig **(E)** for 40 min in the presence and absence of SNAP and SIN-1. Acetylcholine (ACh; 10 µM) was used as a positive control. The IL-1β concentration in supernatants of primed THP-1 cells stimulated with BzATP or Nig alone was set to 100%, and all other values were calculated accordingly. Friedman test followed by the Wilcoxon signed-rank test. **(F)** Macrophage-like THP-1 cells were left untreated or primed with LPS (1 µg/ml, 5 h). Thereafter, BzATP (100 µM) was added for another 40 min to trigger IL-1β release. The amount of IL-1β released in response to BzATP was calculated by subtracting the IL-1β concentrations measured in supernatants of cells treated with LPS alone. In the presence of ACh (10 µM) the BzATP-induced release of IL-1β was blunted. Application of the NO donors SNAP or SIN-1 at different time points, reversed the BzATP-induced release of IL-1β. Friedman test followed by the Wilcoxon signed-rank test. **(A–F)** Data are presented as individual data points, bars represent median, whiskers percentiles 25 and 75. ∗ $p \leq 0.05$ significantly different from samples, in which BzATP or Nig was given alone.

inhibited the BzATP-mediated release of IL-1β by LPS-primed cells (n = 6, p ≤ 0.05, each). However, when SIN-1 was given together with BzATP, IL-1β levels showed a broader distribution, while 30 min preincubation with SIN-1 seemed to result in a more robust inhibitory effect. This prompted us to preincubate the monocytic THP-1 cells with SIN-1 20 and 10 min before application of BzATP, with similar results like those obtained after 30 min of preincubation (Figure 4D). When LPS-primed monocytic THP-1 cells were treated with SNAP or SIN-1 in the absence of BzATP, essentially no IL-1β was released. In contrast to U937 cells, application of SNAP significantly reduced the nigericin-induced release of IL-1β by LPS-primed THP-1 cells by about 40% (n = 6, p ≤ 0.05; Figure 4E). The same holds true for SIN-1, when preincubated for 30, 20 or 10 min before application of nigericin (n = 6, p ≤ 0.05, each; Figure 4E). When SIN-1 was, however, applied together with nigericin, the IL-1β levels in cell culture supernatants were even slightly but significantly increased (n = 6, p ≤ 0.05; Figure 4E). We also included experiments on THP1-derived M1-like macrophages, in which treatment with SNAP and SIN also significantly reduced the BzATP-induced release of IL-1β (n = 7, p ≤ 0.05; Figure 4F).

Next, we tested, if SNAP and SIN-1 exerts the same effects on primary murine cells. Murine PBMCs freshly isolated from wild-type C57BL/6J mice were pulsed with LPS (10 ng/ml) before separation and cultured for 2 h. Non-adherent cells were removed, and remaining cells were stimulated with ATP (1 mM) for another 30 min. The IL-1β levels in cell culture supernatants were significantly (n ≥ 6, p ≤ 0.05, each) lower, when cells were pretreated with SIN-1 for 30 min before applying ATP or, when SIN-1 was given together with ATP (Supplementary Figure S4). In the absence of ATP, only very low amounts of IL-1β were detected, irrespective of the absence or presence of SIN-1 (Supplementary Figure S4). A more extended set of experiments was performed with murine BMDMs (Figures 5A–D). As shown before for U937 cells and THP-1 cells, SNAP efficiently and significantly (n ≥ 6, p ≤ 0.05) inhibited the ATP-induced release of IL-1β, when applied 30 min before or together with ATP but did not induce IL-1β release in the absence of ATP (Figure 5A). The same holds true for SIN-1 (n ≥ 6, p ≤ 0.05; Figure 5B). When SNAP was combined with nigericin and apyrase to investigate ATP-independent mechanisms of inflammasome activation and IL-1β secretion, the IL-1β release by murine BMDMs was also reduced but not fully abolished (n ≥ 6, p ≤ 0.05; Figure 5C). Incubation with SIN-1 for 30 min before application of ATP also reduced IL-1β levels (n ≥ 7, p ≤ 0.05), while SIN-1 application concomitantly with ATP did not (Figure 5D).

Finally, we enriched human monocytes from the blood of healthy volunteers using the RosetteSep™ technique to a purity of 76.07%, 77.33% and 79.51% as analyzed in samples from three volunteers by flow cytometry. Among these monocytes, 88.90 ± 2.20% (median ± standard error of mean) were classical, 4.20 ± 0.47% were intermediate, and 6.19 ± 2.15% were nonclassical monocytes (the gating strategy can be found in Supplementary Figure S5). Similar experiments like those performed on murine BMDMs were also performed with these cells using BzATP and nigericin plus apyrase (Figures 5E–H). Although the variability of the data was higher, essentially the same results were obtained for human monocytes as for murine BMBMs (Figure 5).

Taken together, SNAP and SIN-1 efficiently and consistently inhibit the ATP/BzATP-mediated release of IL-1β by monocytic cell lines, THP-1-dependent macrophages as well as by human and murine primary monocytes and macrophages. ATP-independent IL-1β release can also be reduced, however, this reduction is less prominent.

SIN-1 inhibits the ionotropic function of P2X7Rs. As NO and peroxynitrite cause protein nitration or nitrosylation and thereby modulate their function (39–44), we hypothesized that modification of the P2X7R inhibits its ionotropic function. To address this question, the human P2X7R was heterologously expressed in *Xenopus laevis* oocytes and TEVC measurements were performed. BzATP (10 μM) elicited non-desensitizing inward currents, which were repeatable after 2 h of incubation in ATP-free control solution (Figures 6A, C). The BzATP-induced ion current changes were drastically reduced, when oocytes were incubated with SIN-1 (1 mM) for 2 h in the same solution (n ≥ 7; p ≤ 0.001; Figures 6B, C).

In a similar experiment, changes in intracellular Ca^{2+} levels, which are caused by the BzATP-induced ion currents, were measured. In HEK cells stably expressing the tetracycline-inducible wild-type hP2X7R, Ca^{2+} transients were robustly induced by BzATP (Figure 6D). After preincubation with SIN-1, the BzATP-induced Ca^{2+} signals were virtually absent (Figure 6E) and significantly different from control experiments (Figure 6D; ∗ p ≤ 0.05). To study, if the cells are still able of eliciting Ca^{2+} transients after treatment with SIN-1, forskolin (40 μM) was added, which induced robust signals (Figure 6E). Finally, whole cell patch-clamp experiments were performed on HEK cells expressing the human wild-type *P2RX7* to investigate ion current changes in response to BzATP. As expected (32), BzATP (100 μM) induced repeatable inward currents (Figures 7A, C), which were significantly blunted, when SIN-1 (1 mM) was applied shortly before a second application of BzATP (n ≥ 9, p ≤ 0.05; Figures 7B, C).

Mutation of C377 to alanine abolishes P2X7R sensitivity to SIN-1. The C terminal cytoplasmic domain of the P2X7R constitutes about 40% of the whole P2X7 protein (45) and is highly conserved between rat (rP2X7R) and hP2X7R (80.17% amino acid sequence identity) (Supplementary Figure S6). The C terminal domain contains several domains that potentially interact with other proteins and numerous amino acid residues, which are putative sites for covalent protein modification (9, 10, 45). We tested the two cysteines C377 and C388 as possible targets for P2X7R modification. C377 is the last cysteine of six cysteine residues that, starting at C362 (Figure 8), form the so-called cysteine-rich domain of the P2X7R (9, 47). C377 and C388 are located in the same plane near the cytoplasmic end of the transmembrane domains (Figure 8). C377 is located on the outer surface of the endodomain, C388 in its interior, ~23 Å vertically below the gate defined mainly by the ring of three S342 residues (46), one from each of the three TM2 helices. To test if the cysteine at position

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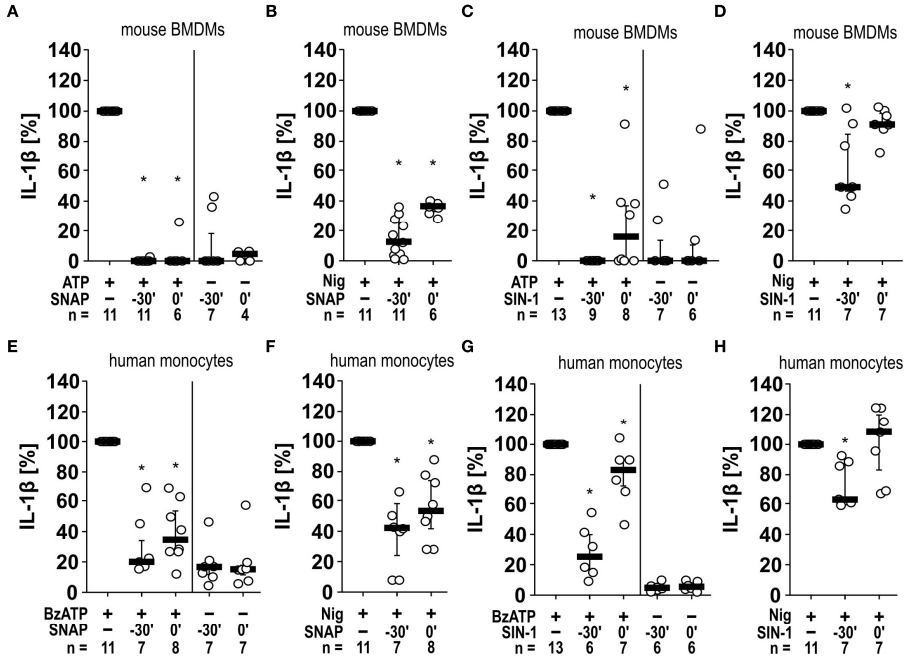

FIGURE 5
Inhibition of IL-1β release by the NO donors SNAP and SIN-1 in murine bone marrow-derived macrophages (BMDMs) and primary human monocytes. **(A–D)** Mouse BMDMs were primed with lipopolysaccharide (LPS; 1 µg/ml, 5 h). **(A, C)** Thereafter, ATP (1 mM) was added for another 40 min to trigger IL-1β release, which was measured by ELISA. Application of the NO donor SNAP (S-nitroso-N-acetyl-DL-penicillamine; 1 mM) 30 min prior to ATP (t = -30´) or shortly before ATP (t = 0´) reversed the ATP-induced release of IL-1β. Similar results were found by using SIN-1 (1 mM). **(B, D)** To test for P2X7 receptor-independent IL-1β release, nigericin (Nig; 50 µM) was added together with apyrase (0.5 U/ml) for 40 min to LPS-primed mouse BMDMs. The Nig-induced IL-1β release was unimpaired by SIN-1 at t = 0´ and slightly reversed in presence of SNAP and SIN-1 at t = -30´. The amount of IL-1β released in response to ATP/Nig was calculated by subtracting the IL-1β concentrations measured in supernatants of cells treated with LPS alone. ∗ $p \leq 0.05$ significantly different from samples, in which ATP or Nig was given alone. **(E–H)** Similar experiments were performed on primary human monocytes enriched from freshly collected human whole blood. Cells were primed with LPS (5 ng/ml, 20 min pulse) during the enrichment process. After 3 h, BzATP ((2′/3′-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate, tri(triethylammonium) salt; 100 µM); **(E, G)** or Nig **(F, H)** was added for another 30 min to trigger IL-1β release. The IL-1β concentration in experiments, in which primed cells were stimulated with BzATP or Nig alone, was set to 100% and all other values were calculated accordingly. ∗ $p \leq 0.05$ significantly different from samples, in which BzATP was given alone. **(A–H)** Data are presented as individual data points, bars represent median, whiskers percentiles 25 and 75. Friedman test followed by the Wilcoxon signed-rank test.

C377 of the P2X7R is involved in signaling, hP2X7R_C377A-HEK cells were used with the C377 mutated to an alanine (C377A), which cannot be modified by nitrosylation. When the above-described kind of patch-clamp experiments were performed with the hP2X7R_C377A-HEK cells, the ion current response towards BzATP remained unchanged, which is in contrast to the wild-type hP2X7R (Figure 7C). Of note, consistent with previous studies on HEK cells expressing different mutant P2X7Rs the mutated P2X7 receptors were still able to channel (48). In the presence of SIN-1, however, the BzATP-induced ion current changes did not differ from control-treated cells (Figure 7C), suggesting, that C377 plays an essential role in the SIN-1-mediated effects. Similarly, the response to BzATP was unchanged in C388A-hP2X7R-HEK cells compared to wild-type P2X7R (Figure 7C). The response to BzATP in the presence of SIN-1, however, was variable with the C388A mutant, ranging from no change in ion currents to a more than twofold increased response (Figure 7C).

Discussion

Activation of nAChRs in mononuclear phagocytes and epithelial cells down-regulates the response of the ATP-sensitive P2X7R, reduces NLRP3 inflammasome assembly and, consequently, the maturation as well as the release of IL-1β (17, 22, 23, 26). This mechanism is activated by several redundant endogenous signals (18, 19, 21–24, 26, 49) and, therefore, seems to be of high biomedical relevance. We demonstrated before, that signaling of AChRs in monocytes is ion flux-independent (17, 18, 22) and no changes in intracellular Ca^{2+} concentrations were detected (22). Flux-independent signaling mechanisms of nAChRs are poorly understood. In this study we investigated the mechanism of how activation of nAChRs translates into an inhibition of the ionotropic function of the P2X7R. It was shown before in neurons and endothelial cells, that activation of nAChR α7 can activate the function of NOS (37, 50–52) and that NO- or

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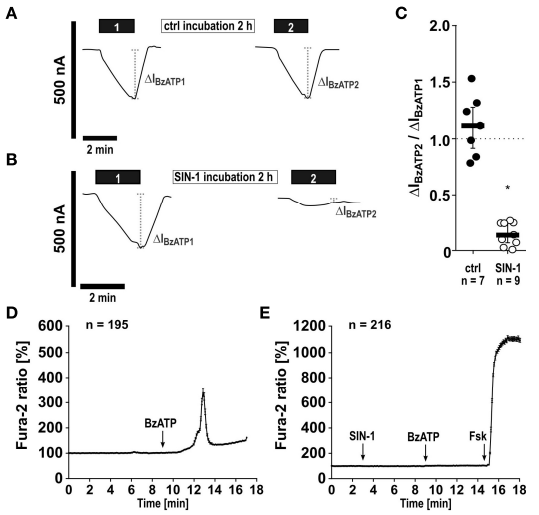

FIGURE 6
The NO and peroxynitrite donor SIN-1 inhibits BzATP-induced ion channel functions of heterologously expressed human P2X7 receptors (hP2X7R). **(A–C)** Two-electrode voltage-clamp measurements were performed on *Xenopus laevis* oocytes expressing the hP2X7R. **(A)** In control experiments (ctrl) application of the P2X7R agonist BzATP ((2′/3′-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate, tri(triethylammonium) salt; 10 μM; 2 min; black bar "1") resulted in a current stimulation (ΔI_{BzATP1}). After a wash out period, the oocytes were incubated for 2 h in oocyte Ringer's solution as a control (ctrl). Thereafter, the BzATP-induced effect was determined for a second time (black bar "2"; ΔI_{BzATP2}). **(B)** After determination of the ΔI_{BzATP1}, oocytes were incubated for 2 h with SIN-1 (1 mM). Subsequently, the ΔI_{BzATP2} was determined. **(C)** The normalized ΔI_{BzATP} values from experiments as shown in panel A and B ($\Delta I_{BzATP2}/\Delta I_{BzATP1}$) were statistically analyzed (* $p \leq 0.05$ significantly different from ctrl). Data are presented as individual data points, bars represent median, whiskers encompass the 25th to 75th percentile. **(D, E)** Calcium imaging experiments were performed on hP2X7 receptor expressing HEK cells. Intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) of HEK-P2X7R cells were recorded as Fura-2/AM (Fura-2) fluorescence intensity ratio of 340:380 nm excitation (mean ± SEM). **(D)** In control experiments (no SIN-1), application of BzATP (100 μM) induced a rise in $[Ca^{2+}]_i$. **(E)** Application of SIN-1 (1 mM) did not cause significant alterations in $[Ca^{2+}]_i$. In the presence of SIN-1 the BzATP-induced rise in $[Ca^{2+}]_i$ was blunted ($p \leq 0.05$ significantly different from BzATP-induced effect in **(D)**. At the end of the experiment, a positive control for cell viability and the Ca^{2+} imaging setup was included: forskolin (Fsk, 40 μM) was applied to induce a cyclic adenosine monophosphate-triggered rise in $[Ca^{2+}]_i$. Friedman test followed by the Wilcoxon signed-rank test.

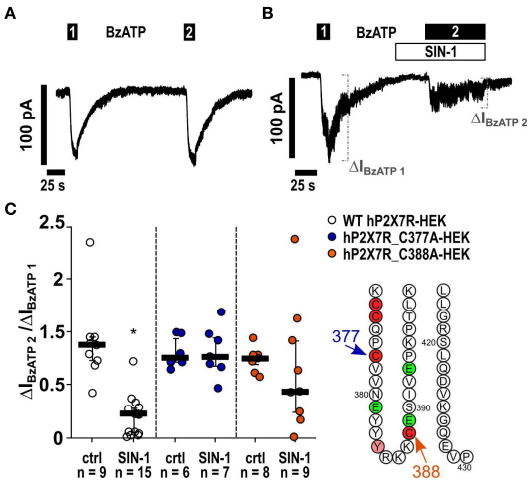

FIGURE 7
The NO and peroxynitrite donor SIN-1 inhibits the BzATP-induced ion channel functions in human P2X7 receptor (hP2X7R) expressing HEK cells *via* the cysteine C377 in the C-terminal intracellular loop. **(A, B)** Depicted are representative current curves of whole-cell patch-clamp measurements. **(A, C)** In control experiments (ctrl) on human wild-type (WT) P2X7R expressing HEK cells (WT hP2X7-HEK), consecutive application of the P2X7R agonist BzATP (100 μM) induced repetitive ion current stimulations (BzATP1 and 2). **(B, C)** In the presence of SIN-1 (1 mM), the BzATP-induced ion current was blunted. **(C)** Similar experiments were performed on HEK cells expressing hP2X7R mutants generated by replacing cysteine 377 or 388 by an alanine (hP2X7_C377A; hP2X7_C388A) in the C-terminal intracellular loop. The BzATP-induced current changes (ΔI_{BzATP1}, ΔI_{BzATP2}) were normalized ($\Delta I_{BzATP2}/\Delta I_{BzATP1}$). **(C)** All BzATP-induced current changes (ΔI_{BzATP}) are shown as individual data points, bars represent median, whiskers encompass the 25th to 75th percentile. *$p \leq 0.05$, Friedman test followed by the Wilcoxon signed-rank test.

peroxynitrite-mediated modifications can regulate protein function (39, 43, 44, 53–57). Moreover, P2X7R was found to co-immunoprecipitate with nNOS in protein extracts of the prefrontal cortex (58). In endothelial cells, stimulation of nAChR α7 with nicotine results in the phosphorylation of eNOS at serine 1177 and induces the production of NO (59). These hints prompted us to hypothesize, that the inhibitory effect of nAChR stimulation on the secretion of IL-1β is mediated *via* NOS and NO- and/or peroxynitrite-mediated modification of the P2X7R (Figure 9).

In line with this hypothesis, we demonstrated that inhibitors of NOS efficiently and dose-dependently reversed the suppressive effect of nicotine, PC and native CRP on the ATP-induced release of IL-1β by LPS-primed U937 cells, suggesting that at least one NOS isoform is involved in signaling. We used the general NOS inhibitors L-NAME (0.1 – 10 μM) (60–62) and L-NIO (0.5 – 50 μM) (63) as well as 1400W (0.01 – 1 μM), which more specifically inhibits iNOS (64) and N-PLA (0.1 – 10 μM), which is regarded as an inhibitor of nNOS (35). In the same line, L-NIO and L-NAME provoked similar effects in THP-1 cells, another monocytic cell line, and in THP-1 cell-derived macrophages. From these inhibitor studies it is, however, difficult to conclude, which NOS isoforms are involved. As iNOS is constitutively active and mainly regulated by its protein expression (36, 37), a swift iNOS mediated inhibition of the P2X7R is not likely. This NOS isoform is expressed by activated pro-inflammatory monocytes and macrophages and plays a decisive role in host defense against infections (36). We decided to further investigate whether eNOS plays an essential role in signaling, because its mRNA was present in LPS-primed monocytic U937 cells, while the mRNA of nNOS was hardly detectable. In line with this assumption, the down-modulation of eNOS expression by siRNA in U937 cells blunted the inhibitory effect of nAChR agonists on the ATP-induced release of IL-1β, while transfection of a control siRNA had no such effect. This was convincingly confirmed by using PBMCs from wild-type and eNOS gene-deficient mice. While in wild-type mice an effective inhibition of the ATP-mediated IL-1β by nAChR agonists was seen, this effect

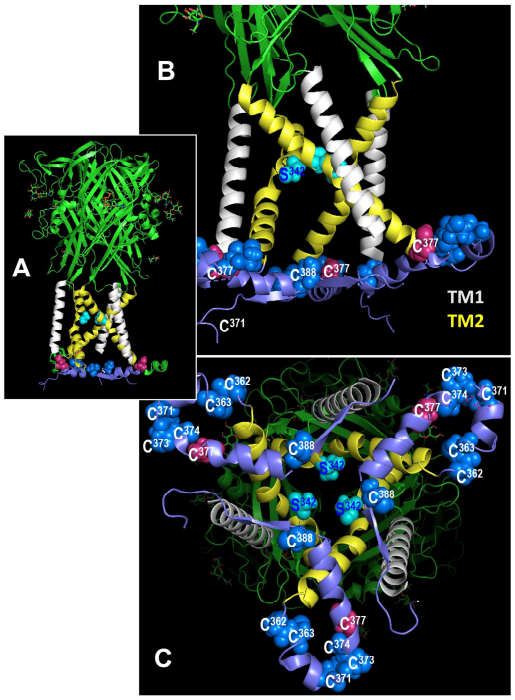

FIGURE 8
Location of cysteines C377 and C388 in the rat P2X7 receptor (rP2X7R) in the open channel conformation. In all figures, the C-terminal residues 400-595 to the end of the 595 residues long subunits are hidden for simplicity. **(A)** Side view of the large extracellular domain harboring the ATP binding sites, the six transmembrane domains (3 x TM1 in white, 3 x TM2, in yellow), and an interfacial helically structured domain lying parallel to the membrane plane. Also shown are the three S342 residues, which constitute the channel gate (46). **(B)** Enlarged side view of the transmembrane domains, the S342 residues (cyan) and the position of two (of three each) residues C377 and C388 in the interfacial region. The other blue spheres indicate additional cysteines of the cysteine-rich domain. **(C)** Perpendicular view from the cytoplasmic side on the numerous cysteine residues of the interfacial region and the transmembrane domains above.

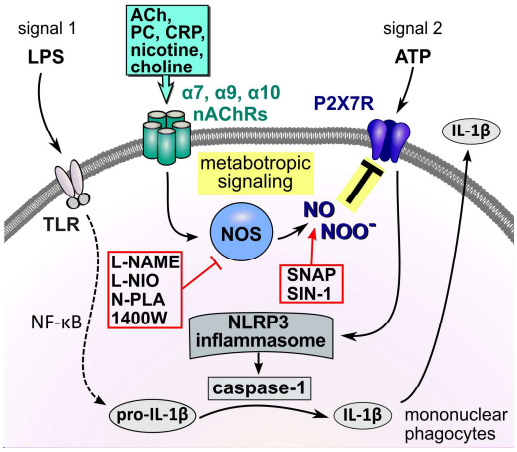

FIGURE 9
Schematic summary of the proposed metabotropic signaling mechanism. In mononuclear phagocytes extracellular ATP originating from activated cells or spilled cytoplasm of damaged cells trigger the ionotropic function of the P2X7R, resulting in NLRP3 inflammasome assembly, activation of caspase-1, cleavage of pro-IL-1β and release of bioactive IL-1β. Activation of nAChRs by classical and unconventional agonists down-regulates the response of the ATP-sensitive P2X7R, impairs NLRP3 inflammasome assembly and, consequently, the maturation as well as the release of IL-1β. We provide evidence that this inhibitory effect of nAChR stimulation on the secretion of IL-1β is mediated *via* endothelial NOS and modification of the P2X7R. ACh, acetylcholine; ATP, adenosine triphosphate; CRP, C-reactive protein; DAMP, danger-associated molecular pattern; IL-1β, interleukin-1β; LPS, lipopolysaccharide; nAChRs, nicotinic acetylcholine receptors; NF-κB, nuclear factor κB; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; NOS, NO synthase; P2X7R, P2X7 receptor; PAMP, pathogen-associated molecular pattern; PC, phosphocholine; SNAP, S-nitroso-N-acetyl-DL-penicillamine; TLR, Toll-like receptors.

was abolished in PBMCs from eNOS gene-deficient mice. Although compensatory up- or down-regulation of other genes has been described for gene-deficient mice in general, the complete absence the cholinergic inhibition of IL-1β release in eNOS gene-deficient cells is remarkable. We conclude, that eNOS plays an essential role in the inhibition of ATP-induced IL-1β release by nAChR stimulation in monocytic cells. The mechanism of how the function of eNOS is activated by stimulation of nAChRs is still unclear and deserves further research. Further, we cannot exclude an additional involvement of nNOS or iNOS in the regulation of P2X7R function.

During the process of activation, eNOS is mainly recruited to membrane domains, where NO is produced locally from its substrate arginine in the presence of the cofactor tetrahydrobiopterin (65–68). NO is a very reactive compound with a biological half-life below one second and thus most likely reacts with targets in its direct vicinity (41). NO and reactive oxygen species, which are often produced by activated mononuclear phagocytes (69), can react to form peroxynitrite, another highly reactive compound (40). Two different pathways of NO signaling have been described. In neurotransmission and vasodilation the soluble guanylyl cyclase is activated, which promotes the formation of the second messenger cyclic guanylyl monophosphate (41). Another pathway, which is most probably involved in the inhibition of the ionotropic function of the P2X7R, involves posttranslational S-nitrosylation at cysteine residues of proteins by NO or nitration by peroxynitrite, which both can modify protein structure and function (40, 41). While nitrosylation is a reversible modification, nitration is rather stable (40, 41).

To confirm the involvement of NO or NO-related compounds in the inhibition of the ATP-induced IL-1β release, we performed a series of experiments using the NO donors SNAP and SIN-1 (39). In all cell types investigated, which included monocytic U937 cells and THP-1 cells, primary human blood monocytes, THP-1-dependent macrophages, and murine BMDMs, SNAP and SIN-1 reduced the BzATP- or ATP-induced secretion of IL-1β. Depending on the cell type investigated, SNAP and SIN-1 fully inhibited or at least significantly blunted the ATP-induced release of IL-1β by LPS-primed monocytes or macrophages. As NO produced by iNOS was

shown before to inhibit the activity of the inflammasome by nitrosylation of NLRP3 (70–72), the question remained if application of a NO- or a peroxynitrite donor inhibits the formation of active inflammasomes or interfered with the function of the P2X7R. Therefore, we included experiments, in which the pore-forming bacterial toxin nigericin was given as an ATP-independent stimulus for inflammasome activation. Apyrase was added in these experiments to exclude possible effects of endogenous ATP. In monocytic U937 cells, SNAP and SIN-1 had no effect on the nigericin-induced secretion of IL-1β, while in monocytic THP-1 cells, murine BMDMs and human monocytes a significant reduction of the secreted IL-1β was seen. In all cases, the effect of NO or NO-related compounds in the nigericin-induced IL-1β secretion was weaker compared to the effects on the ATP-induced secretion. These results suggest that, depending on the cell type, NO and peroxynitrite interferes with the function of the P2X7 receptor, but in addition they can interfere with other mechanisms such as the formation of inflammasomes that are shared with ATP-independent mechanisms of IL-1β maturation and release.

In different experimental settings, we showed that SIN-1 directly affects the ionotropic function of the ATP-sensitive P2X7R. TEVC measurements on *Xenopus laevis* oocytes heterologously expressing the human P2X7R is an accepted method to study its ionotropic function (73, 74). In line with our hypothesis, pre-incubation of the oocytes with SIN-1 significantly blunted the ATP-induced ion currents. In HEK cells over-expressing the human P2X7 receptor, ATP-induced P2X7R function was visualized by imaging of changes in intracellular Ca^{2+} concentrations and by whole cell patch-clamp measurements to monitor changes in transmembrane ion currents. Both, ATP-mediated changes in Ca^{2+} concentrations and ion current changes were indeed significantly blunted in the presence of SIN-1. We conclude from these results, that a NO-dependent modification of the P2X7R impairs its ionotropic function.

HEK cells over-expressing human P2X7R were also used to identify amino acids in the P2X7R C-terminus that might be modified by NO-related compounds. We provide evidence that a NO-dependent modification of the cysteine residue C377 plays an essential role in the inhibition of the ionotropic function of the P2X7R and suggest, that this residue is modified upon stimulation of nAChRs in human mononuclear phagocytes. When C377 is mutated to an alanine and expressed by HEK cells instead of the wildtype receptor, the NO and peroxynitrite donor SIN-1 does not inhibit the ionotropic function of the P2X7R anymore. C377 is the last cysteine out of six cysteine residues that form the cysteine-rich domain of the cytoplasmic C-terminus of the human P2X7R (9, 47). When palmitoylated, the cysteine-rich domains form a membrane anchor, which prevents early receptor desensitization, a typical feature of other ATP receptors such as the P2X3R lacking this cysteine-rich domain (9). Interestingly, cysteine nitrosylation was shown before as a mechanism regulating protein function and intracellular localization by competing with protein fatty acylation (54–57). Acylation at C377 does not seem to be essential for the ionotropic function of the P2X7R, because the mutation of the cysteine at position C377 to an alanine did not prevent P2X7R function. A replacement of C377 by the small and non-polar alanine does not perturb the membrane anchor, which contains five other cysteines that can by palmitoylated that might be functionally sufficient. However, it is conceivable that a polar NO-dependent modification at C377, which is in close contact to the transmembrane region of the P2X7R causes conformational changes that impair ion channel function, while a mutation of C377 to the small lipophilic alanine does not (9).

We further mutated C388 to alanine and expressed the mutant receptor in HEK cells. A cysteine located in a similar amino acid sequence was identified before as a putative nitrosylation site of the NMDA receptor that down-modulates its ionotropic function (10). This cysteine is located close to the region that interacts with the N-terminus in the cytoplasmic cap, which enables ion channel function of the P2X7R (9). The results of our experiments were, however, ambiguous. The ionotropic response of the C388A-P2X7R mutant was unimpaired as was that of the C377A mutant. However, the behavior of the C388A mutant in the presence of SIN-1 and ATP was highly variable ranging from a full inhibition of the ATP-induced ion currents to a more than twofold increase in the ion current changes. At present, we cannot explain these observations, but a mutation at C388 seems to destabilize the P2X7R, when NO donors are present.

Our study has numerous limitations. We did not touch the question, of how eNOS is activated in response to nAChR stimulation. This aspect needs to be investigated in future studies. It is also unclear, if iNOS and nNOS also essentially contribute to signaling, which might be suggested by the fact that N-PLA, a nNOS-specific NOS inhibitor, and 1400W, an iNOS-specific inhibitor, revert the effect of nAChR agonist on the ATP-mediated IL-1β release. As already discussed, we refrained from investigating nNOS and iNOS, but obviously more experiments are needed including experiments on blood cells from nNOS and iNOS gene-deficient mice. Further analyses are needed to directly show protein modification at C377 upon stimulation of nAChRs in mononuclear phagocytes and to directly identify the kind of modification. We also do not answer the question if other cysteines of the P2X7R are also involved in the cholinergic control mechanism. A comprehensive study of nAChR-induced protein modifications of the P2X7R would provide important insights into the functional regulation of the P2X7R.

Conclusions

The conclusions that can be drawn from this study are summarized in Figure 9. We elucidated an essential part of the signaling mechanism that links the stimulation of monocytic nAChRs to the inhibition of the ionotropic function of the P2X7R in LPS-primed human and murine mononuclear phagocytes. Stimulation of nAChRs activates eNOS by a mechanism that still remains to be elucidated. We provide evidence, that an NO- or peroxynitrite-dependent modification of the P2X7R at C377 plays an essential role in the inhibition of its ionotropic function. C377 belongs to the cytoplasmic C-terminal tail of the P2X7R, which contains several cysteine residues that can be palmitoylated to form a membrane anchor. To the best of our

knowledge, this is the first report on a site-specific NOS-mediated inhibition of the ionotropic function of the P2X7R. These results are of high medical interest, because P2X7R modification results in a striking reduction in the ATP-mediated release of monocytic IL-1β. Although more research is needed to elucidate this mechanism in every detail, we provide new insights into the regulation of the P2X7R, which might pave the way towards new therapeutics preventing over-shooting sterile inflammation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by ethics committee of the medical faculty Giessen, Germany (No. 90/18). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by University Düsseldorf, Germany, reference No. O16/04; Regierungspräsidium Giessen, Germany, reference no. 571_M. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

KR participated in the research design, performance of experiments, interpretation of the data, and writing of the manuscript. NA, VKS, SHY, MM and AZ participated in performance of the experiments, interpretation of the data, and editing of the manuscript. SW and SS participated in the performance of experiments. JL, AH, K-DS, MR, AG, WP, IM participated in the research design, interpretation of the data, and editing of the manuscript. GS and VG participated in the research design, interpretation of the data and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1140592/full#supplementary-material

References

1. Abbracchio MP, Burnstock G, Boeynaems J-M, Barnard EA, Boyer JL, Kennedy C, et al. International union of pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* (2006) 58:281–341. doi: 10.1124/pr.58.3.3

2. Di Virgilio F, Jiang L-H, Roger S, Falzoni S, Sarti AC, Vultaggio-Poma V, et al. Structure, function and techniques of investigation of the P2X7 receptor (P2X7R) in mammalian cells. *Methods Enzymol* (2019) 629:115–50. doi: 10.1016/bs.mie.2019.07.043

3. Linden J, Koch-Nolte F, Dahl G. Purine release, metabolism, and signaling in the inflammatory response. *Annu Rev Immunol* (2019) 37:325–47. doi: 10.1146/annurev-immunol-051116-052406

4. Di Virgilio F, Sarti AC, Coutinho-Silva R. Purinergic signaling, DAMPs, and inflammation. *Am J Physiol Cell Physiol* (2020) 318:C832–5. doi: 10.1152/ajpcell.00053.2020

5. Illes P, Müller CE, Jacobson KA, Grutter T, Nicke A, Fountain SJ, et al. Update of P2X receptor properties and their pharmacology: IUPHAR review 30. *Br J Pharmacol* (2021) 178:489–514. doi: 10.1111/bph.15299

6. Inoue K, Tsuda M. Nociceptive signaling mediated by P2X3, P2X4 and P2X7 receptors. *Biochem Pharmacol* (2021) 187:114309. doi: 10.1016/j.bcp.2020.114309

7. Shokoples BG, Paradis P, Schiffrin EL. P2X7 receptors: an untapped target for the management of cardiovascular disease. *Arterioscler Thromb Vasc Biol* (2021) 41:186–99. doi: 10.1161/ATVBAHA.120.315116

8. Mishra A, Behura A, Kumar A, Naik L, Swain A, Das M, et al. P2X7 receptor in multifaceted cellular signalling and its relevance as a potential therapeutic target in different diseases. *Eur J Pharmacol* (2021) 906:174235. doi: 10.1016/j.ejphar.2021.174235

9. McCarthy AE, Yoshioka C, Mansoor SE. Full-length P2X7 structures reveal how palmitoylation prevents channel desensitization. *Cell* (2019) 179:659–670.e13. doi: 10.1016/j.cell.2019.09.017

10. Costa-Junior HM, Sarmento Vieira F, Coutinho-Silva R. C terminus of the P2X7 receptor: treasure hunting. *Purinergic Signal* (2011) 7:7–19. doi: 10.1007/s11302-011-9215-1

11. Becker D, Woltersdorf R, Boldt W, Schmitz S, Braam U, Schmalzing G, et al. The P2X7 carboxyl tail is a regulatory module of P2X7 receptor channel activity. *J Biol Chem* (2008) 283:25725–34. doi: 10.1074/jbc.M803855200

12. Pétrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ* (2007) 14:1583–9. doi: 10.1038/sj.cdd.4402195

13. Gross O, Thomas CJ, Guarda G, Tschopp J. The inflammasome: an integrated view. *Immunol Rev* (2011) 243:136–51. doi: 10.1111/j.1600-065X.2011.01046.x

14. Vladimer GI, Marty-Roix R, Ghosh S, Weng D, Lien E. Inflammasomes and host defenses against bacterial infections. *Curr Opin Microbiol* (2013) 16:23–31. doi: 10.1016/j.mib.2012.11.008

15. Broz P, Dixit VM. Inflammasomes: mechanism of assembly, regulation and signalling. *Nat Rev Immunol* (2016) 16:407–20. doi: 10.1038/nri.2016.58

16. Bortolotti P, Faure E, Kipnis E. Inflammasomes in tissue damages and immune disorders after trauma. *Front Immunol* (2018) 9:1900. doi: 10.3389/fimmu.2018.01900

17. Hecker A, Küllmar M, Wilker S, Richter K, Zakrzewicz A, Atanasova S, et al. Phosphocholine-modified macromolecules and canonical nicotinic agonists inhibit ATP-induced IL-1β release. *J Immunol* (2015) 195:2325–34. doi: 10.4049/jimmunol.1400974

18. Richter K, Mathes V, Fronius M, Althaus M, Hecker A, Krasteva-Christ G, et al. Phosphocholine - an agonist of metabotropic but not of ionotropic functions of α9-containing nicotinic acetylcholine receptors. *Sci Rep* (2016) 6:28660. doi: 10.1038/srep28660

19. Zakrzewicz A, Richter K, Agné A, Wilker S, Siebers K, Fink B, et al. Canonical and novel non-canonical cholinergic agonists inhibit ATP-induced release of monocytic interleukin-1β *via* different combinations of nicotinic acetylcholine receptor subunits α7, α9 and α10. *Front Cell Neurosci* (2017) 11:189. doi: 10.3389/fncel.2017.00189

20. Backhaus S, Zakrzewicz A, Richter K, Damm J, Wilker S, Fuchs-Moll G, et al. Surfactant inhibits ATP-induced release of interleukin-1β *via* nicotinic acetylcholine receptors. *J Lipid Res* (2017) 58:1055–66. doi: 10.1194/jlr.M071506

21. Grau V, Richter K, Hone AJ, McIntosh JM. Conopeptides V11L;V16DArIB and RgIA4: powerful tools for the identification of novel nicotinic acetylcholine receptors in monocytes. *Front Pharmacol* (2018) 9:1499. doi: 10.3389/fphar.2018.01499

22. Richter K, Sagawe S, Hecker A, Küllmar M, Askevold I, Damm J, et al. C-reactive protein stimulates nicotinic acetylcholine receptors to control ATP-mediated monocytic inflammasome activation. *Front Immunol* (2018) 9:1604. doi: 10.3389/fimmu.2018.01604

23. Siebers K, Fink B, Zakrzewicz A, Agné A, Richter K, Konzok S, et al. Alpha-1 antitrypsin inhibits ATP-mediated release of interleukin-1β *via* CD36 and nicotinic acetylcholine receptors. *Front Immunol* (2018) 9:877. doi: 10.3389/fimmu.2018.00877

24. Zakrzewicz A, Richter K, Zakrzewicz D, Siebers K, Damm J, Agné A, et al. SLPI inhibits ATP-mediated maturation of IL-1β in human monocytic leukocytes: a novel function of an old player. *Front Immunol* (2019) 10:664. doi: 10.3389/fimmu.2019.00664

25. Richter K, Amati A-L, Padberg W, Grau V. Negative regulation of ATP-induced inflammasome activation and cytokine secretion by acute-phase proteins: A mini review. *Front Pharmacol* (2022) 13:981276. doi: 10.3389/fphar.2022.981276

26. Richter K, Papke RL, Stokes C, Roy DC, Espinosa ES, Wolf PM, et al. Comparison of the anti-inflammatory properties of two nicotinic acetylcholine receptor ligands, phosphocholine and pCF3-diEPP. *Front Cell Neurosci* (2022) 16:779081. doi: 10.3389/fncel.2022.779081

27. Lund ME, To J, O'Brien BA, Donnelly S. The choice of phorbol 12-myristate 13-acetate differentiation protocol influences the response of THP-1 macrophages to a pro-inflammatory stimulus. *J Immunol Methods* (2016) 430:64–70. doi: 10.1016/j.jim.2016.01.012

28. Gödecke A, Decking UK, Ding Z, Hirchenhain J, Bidmon HJ, Gödecke S, et al. Coronary hemodynamics in endothelial NO synthase knockout mice. *Circ Res* (1998) 82:186–94. doi: 10.1161/01.RES.82.2.186

29. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood* (2010) 116:e74–80. doi: 10.1182/blood-2010-02-258558

30. Marimuthu R, Francis H, Dervish S, Li SC, Medbury H, Williams H. Characterization of human monocyte subsets by whole blood flow cytometry analysis. *J Vis Exp* (2018) (140):57941. doi: 10.3791/57941

31. Marchetti C, Swartzwelter B, Gamboni F, Neff CP, Richter K, Azam T, et al. OLT1177, a β-sulfonyl nitrile compound, safe in humans, inhibits the NLRP3 inflammasome and reverses the metabolic cost of inflammation. *Proc Natl Acad Sci USA* (2018) 115:E1530–9. doi: 10.1073/pnas.1716095115

32. Richter K, Ogiemwonyi-Schaefer R, Wilker S, Chaveiro AI, Agné A, Hecker M, et al. Amyloid beta peptide ($A\beta_{1-42}$) reverses the cholinergic control of monocytic IL-1β release. *J Clin Med* (2020) 9(9):2887. doi: 10.3390/jcm9092887

33. Braman J, Papworth C, Greener A. Site-directed mutagenesis using double-stranded plasmid DNA templates. *Methods Mol Biol* (1996) 57:31–44. doi: 10.1385/0-89603-332-5:31

34. Boucher JL, Moali C, Tenu JP. Nitric oxide biosynthesis, nitric oxide synthase inhibitors and arginase competition for l-arginine utilization. *Cell Mol Life Sci* (1999) 55:1015–28. doi: 10.1007/s000180050352

35. Zhang HQ, Fast W, Marletta MA, Martasek P, Silverman RB. Potent and selective inhibition of neuronal nitric oxide synthase by n omega-propyl-L-arginine. *J Med Chem* (1997) 40:3869–70. doi: 10.1021/jm970550g

36. Andrew P. Enzymatic function of nitric oxide synthases. *Cardiovasc Res* (1999) 43:521–31. doi: 10.1016/S0008-6363(99)00115-7

37. Papadopolou S, Hartmann P, Lips KS, Kummer W, Haberberger RV. Nicotinic receptor mediated stimulation of NO-generation in neurons of rat thoracic dorsal root ganglia. *Neurosci Lett* (2004) 361:32–5. doi: 10.1016/j.neulet.2003.08.064

38. Zielonka J, Sikora A, Joseph J, Kalyanaraman B. Peroxynitrite is the major species formed from different flux ratios of co-generated nitric oxide and superoxide: direct reaction with boronate-based fluorescent probe. *J Biol Chem* (2010) 285:14210–6. doi: 10.1074/jbc.M110.110080

39. Dasgupta S, Gomez J-J, Singh I, Khan M. S-nitrosylation in regulation of inflammation and cell damage. *Curr Drug Targets* (2018) 19:1831–8. doi: 10.2174/1389450119666180213094747

40. Jones LH. Chemistry and biology of biomolecule nitration. *Chem Biol* (2012) 19:1086–92. doi: 10.1016/j.chembiol.2012.07.019

41. Fernando V, Zheng X, Walia Y, Sharma V, Letson J, Furuta S. S-nitrosylation: An emerging paradigm of redox signaling. *Antioxidants (Basel)* (2019) 8(9):404. doi: 10.3390/antiox8090404

42. Lohman AW, Weaver JL, Billaud M, Sandilos JK, Griffiths R, Straub AC, et al. S-nitrosylation inhibits pannexin 1 channel function. *J Biol Chem* (2012) 287:39602–12. doi: 10.1074/jbc.M112.397976

43. Althaus M, Pichl A, Clauss WG, Seeger W, Fronius M, Morty RE. Nitric oxide inhibits highly selective sodium channels and the Na^+/K^+-ATPase in H441 cells. *Am J Respir Cell Mol Biol* (2011) 44:53–65. doi: 10.1165/2009-0335OC

44. Wang P, Wei M, Zhu X, Liu Y, Yoshimura K, Zheng M, et al. Nitric oxide down-regulates voltage-gated na^+ channel in cardiomyocytes possibly through s-nitrosylation-mediated signaling. *Sci Rep* (2021) 11:11273. doi: 10.1038/s41598-021-90840-0

45. Kopp R, Krautloher A, Ramírez-Fernández A, Nicke A. P2X7 interactions and signaling - making head or tail of it. *Front Mol Neurosci* (2019) 12:183. doi: 10.3389/fnmol.2019.00183

46. Pippel A, Stolz M, Woltersdorf R, Kless A, Schmalzing G, Markwardt F. Localization of the gate and selectivity filter of the full-length P2X7 receptor. *Proc Natl Acad Sci USA* (2017) 114:E2156–65. doi: 10.1073/pnas.1610414114

47. Jiang L-H, Caseley EA, Muench SP, Roger S. Structural basis for the functional properties of the P2X7 receptor for extracellular ATP. *Purinergic Signal* (2021) 17:331–44. doi: 10.1007/s11302-021-09790-x

48. Karasawa A, Michalski K, Mikhelzon P, Kawate T. The P2X7 receptor forms a dye-permeable pore independent of its intracellular domain but dependent on membrane lipid composition. *Elife* (2017) 6:e31186. doi: 10.7554/eLife.31186

49. Richter K, Koch C, Perniss A, Wolf PM, Schweda EK, Wichmann S, et al. Phosphocholine-modified lipooligosaccharides of haemophilus influenzae inhibit ATP-induced IL-1β release by pulmonary epithelial cells. *Molecules* (2018) 23(8):1979. doi: 10.3390/molecules23081979

50. el-Mas MM, el-Gowilly SM, Gohar EY, Ghazal A-RM, Abdel-Rahman AA. Estrogen dependence of the renal vasodilatory effect of nicotine in rats: role of α7 nicotinic cholinergic receptor/eNOS signaling. *Life Sci* (2011) 88:187–93. doi: 10.1016/j.lfs.2010.11.009

51. Adams CE, Stevens KE, Kem WR, Freedman R. Inhibition of nitric oxide synthase prevents α7 nicotinic receptor-mediated restoration of inhibitory auditory gating in rat hippocampus. *Brain Res* (2000) 877:235–44. doi: 10.1016/s0006-8993(00)02677-9

52. Haberberger RV, Henrich M, Lips KS, Kummer W. Nicotinic receptor alpha 7-subunits are coupled to the stimulation of nitric oxide synthase in rat dorsal root ganglion neurons. *Histochem Cell Biol* (2003) 120:173–81. doi: 10.1007/s00418-003-0550-3

53. DuVall MD, Zhu S, Fuller CM, Matalon S. Peroxynitrite inhibits amiloride-sensitive na^+ currents in xenopus oocytes expressing alpha beta gamma-rENaC. *Am J Physiol* (1998) 274:C1417–23. doi: 10.1152/ajpcell.1998.274.5.C1417

54. Hess DT, Patterson SI, Smith DS, Skene JH. Neuronal growth cone collapse and inhibition of protein fatty acylation by nitric oxide. *Nature* (1993) 366:562–5. doi: 10.1038/366562a0

55. Baker TL, Booden MA, Buss JE. S-nitrosocysteine increases palmitate turnover on ha-ras in NIH 3T3 cells. *J Biol Chem* (2000) 275:22037–47. doi: 10.1074/jbc.M001813200

56. Ho GP, Selvakumar B, Mukai J, Hester LD, Wang Y, Gogos JA, et al. S-nitrosylation and s-palmitoylation reciprocally regulate synaptic targeting of PSD-95. *Neuron* (2011) 71:131–41. doi: 10.1016/j.neuron.2011.05.033

57. Salaun C, Greaves J, Chamberlain LH. The intracellular dynamic of protein palmitoylation. *J Cell Biol* (2010) 191:1229–38. doi: 10.1083/jcb.201008160

58. Pereira VS, Casarotto PC, Hiroaki-Sato VA, Sartim AG, Guimarães FS, Joca SR. Antidepressant- and anticompulsive-like effects of purinergic receptor blockade: involvement of nitric oxide. *Eur Neuropsychopharmacol* (2013) 23:1769–78. doi: 10.1016/j.euroneuro.2013.01.008

59. Wu JC, Chruscinski A, Jesus Perez VA de, Singh H, Pitsiouni M, Rabinovitch M, et al. Cholinergic modulation of angiogenesis: role of the 7 nicotinic acetylcholine receptor. *J Cell Biochem* (2009) 108:433–46. doi: 10.1002/jcb.22270

60. Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J* (1994) 298(Pt 2):249–58. doi: 10.1042/bj2980249

61. Moore PK, Handy RL. Selective inhibitors of neuronal nitric oxide synthase - is no NOS really good NOS for the nervous system? *Trends Pharmacol Sci* (1997) 18:204–11. doi: 10.1016/s0165-6147(97)01064-x

62. Pfeiffer S, Leopold E, Schmidt K, Brunner F, Mayer B. Inhibition of nitric oxide synthesis by NG-nitro-L-arginine methyl ester (L-NAME): requirement for bioactivation to the free acid, NG-nitro-L-arginine. *Br J Pharmacol* (1996) 118:1433–40. doi: 10.1111/j.1476-5381.1996.tb15557.x

63. Rees DD, Palmer RM, Schulz R, Hodson HF, Moncada S. Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br J Pharmacol* (1990) 101:746–52. doi: 10.1111/j.1476-5381.1990.tb14151.x

64. Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJ, et al. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase *in vitro* and *in vivo*. *J Biol Chem* (1997) 272:4959–63. doi: 10.1074/jbc.272.8.4959

65. Fleming I. Signal transduction of eNOS activation. *Cardiovasc Res* (1999) 43:532–41. doi: 10.1016/s0008-6363(99)00094-2

66. Michel T, Vanhoutte PM. Cellular signaling and NO production. *Pflugers Arch* (2010) 459:807–16. doi: 10.1007/s00424-009-0765-9

67. Kone BC, Kuncewicz T, Zhang W, Yu Z-Y. Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide. *Am J Physiol Renal Physiol* (2003) 285:F178–90. doi: 10.1152/ajprenal.00048.2003

68. Stuehr DJ, Haque MM. Nitric oxide synthase enzymology in the 20 years after the Nobel prize. *Br J Pharmacol* (2019) 176:177–88. doi: 10.1111/bph.14533

69. Canton M, Sánchez-Rodríguez R, Spera I, Venegas FC, Favia M, Viola A, et al. Reactive oxygen species in macrophages: sources and targets. *Front Immunol* (2021) 12:734229. doi: 10.3389/fimmu.2021.734229

70. Hernandez-Cuellar E, Tsuchiya K, Hara H, Fang R, Sakai S, Kawamura I, et al. Cutting edge: nitric oxide inhibits the NLRP3 inflammasome. *J Immunol* (2012) 189:5113–7. doi: 10.4049/jimmunol.1202479

71. Mishra BB, Rathinam VA, Martens GW, Martinot AJ, Kornfeld H, Fitzgerald KA, et al. Nitric oxide controls the immunopathology of tuberculosis by inhibiting NLRP3 inflammasome-dependent processing of IL-1β. *Nat Immunol* (2013) 14:52–60. doi: 10.1038/ni.2474

72. Mao L, Zhou Q, Zhou S, Wilbur RR, Li X. Roles of apolipoprotein e (ApoE) and inducible nitric oxide synthase (iNOS) in inflammation and apoptosis in preeclampsia pathogenesis and progression. *PloS One* (2013) 8:e58168. doi: 10.1371/journal.pone.0058168

73. Pournara D T, Durner A, Kritsi E, Papakostas A, Zoumpoulakis P, Nicke A, et al. Design, synthesis, and *in vitro* evaluation of P2X7 antagonists. *ChemMedChem* (2020) 15:2530–43. doi: 10.1002/cmdc.202000303

74. Schmalzing G, Markwardt F. Established protocols for cRNA expression and voltage-clamp characterization of the P2X7 receptor in *Xenopus laevis* oocytes. *Methods Mol Biol* (2022) 2510:157–92. doi: 10.1007/978-1-0716-2384-8_9

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Comparison of the Anti-inflammatory Properties of Two Nicotinic Acetylcholine Receptor Ligands, Phosphocholine and *p*CF3-diEPP

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Activation of nicotinic acetylcholine receptors (nAChRs) expressed by innate immune cells can attenuate pro-inflammatory responses. Silent nAChR agonists, which down-modulate inflammation but have little or no ionotropic activity, are of outstanding clinical interest for the prevention and therapy of numerous inflammatory diseases. Here, we compare two silent nAChR agonists, phosphocholine, which is known to interact with nAChR subunits α7, α9, and α10, and *p*CF3-N,N-diethyl-*N*′-phenyl-piperazine (*p*CF3-diEPP), a previously identified α7 nAChR silent agonist, regarding their anti-inflammatory properties and their effects on ionotropic nAChR functions. The lipopolysaccharide (LPS)-induced release of interleukin (IL)-6 by primary murine macrophages was inhibited by *p*CF3-diEPP, while phosphocholine was ineffective presumably because of instability. In human whole blood cultures *p*CF3-diEPP inhibited the LPS-induced secretion of IL-6, TNF-α and IL-1β. The ATP-mediated release of IL-1β by LPS-primed human peripheral blood mononuclear leukocytes, monocytic THP-1 cells and THP-1-derived M1-like macrophages was reduced by both phosphocholine and femtomolar concentrations of *p*CF3-diEPP. These effects were sensitive to mecamylamine and to conopeptides RgIA4 and [V11L; V16D]ArIB, suggesting the involvement of nAChR subunits α7, α9 and/or α10. In two-electrode voltage-clamp measurements *p*CF3-diEPP functioned as a partial agonist and a strong desensitizer of classical human α9 and α9α10 nAChRs. Interestingly, *p*CF3-diEPP was more effective as an ionotropic agonist at these nAChRs than at α7 nAChR. In conclusion, phosphocholine and *p*CF3-diEPP are potent agonists at unconventional nAChRs expressed by monocytic and macrophage-like cells. *p*CF3-diEPP inhibits the LPS-induced release of pro-inflammatory cytokines, while

phosphocholine is ineffective. However, both agonists signal *via* nAChR subunits α7, α9 and/or α10 to efficiently down-modulate the ATP-induced release of IL-1β. Compared to phosphocholine, *p*CF3-diEPP is expected to have better pharmacological properties. Thus, low concentrations of *p*CF3-diEPP may be a therapeutic option for the treatment of inflammatory diseases including trauma-induced sterile inflammation.

Keywords: CHRNA7, CHRNA9, CHRNA10, cytokines, inflammation, silent agonist, monocytes, macrophages

INTRODUCTION

Homopentameric nicotinic acetylcholine (ACh) receptors (nAChRs) composed of subunits α7 or α9 as well as α9α10 heteropentamers were originally described as ligand-gated ion channels with a high permeability for Ca^{2+} ions (Katz et al., 2000; Verbitsky et al., 2000; Uteshev et al., 2002; Lipovsek et al., 2014). In the nervous system, α7 nAChRs are widely expressed, whereas nAChR subunits α9 and α10 are confined to the outer hair cells of the inner ear (Elgoyhen et al., 2001; Lustig et al., 2001; Skok, 2002; Gotti et al., 2006). In addition, nAChR subunits α7, α9 and α10 are expressed by immune cells and numerous other non-neuronal cells (Kawashima and Fujii, 2003, 2004; Galvis et al., 2006; Wessler and Kirkpatrick, 2008; Koval et al., 2011; Beckmann and Lips, 2013; Kummer and Krasteva-Christ, 2014; Kawashima et al., 2015; Fujii et al., 2017a,b). While for these non-neuronal cells no convincing ionotropic functions have been published, diverse metabotropic functions were discovered (Peng et al., 2004; De Jonge and Ulloa, 2007; Razani-Boroujerdi et al., 2007; Hecker et al., 2009, 2015; Mikulski et al., 2010; Papke, 2014; Richter et al., 2016, 2018; Valbuena and Lerma, 2016; Zakrzewicz et al., 2017; Grau et al., 2018).

In innate immunity, nAChRs and their agonists are mainly involved in an attenuation of inflammation (Borovikova et al., 2000; Kox et al., 2009; Tracey, 2009; Simard et al., 2013; St-Pierre et al., 2016; Fujii et al., 2017a; Pavlov et al., 2018; Godin et al., 2020). Damage- or pathogen-associated molecular patterns (DAMPs or PAMPs) are sensed by pattern recognition receptors such as Toll-like receptors and typically induce the expression of numerous pro-inflammatory mediators and the release of inflammasome-independent cytokines such as interleukin (IL)-6 or tumor necrosis factor (TNF)-α (Kawai and Akira, 2010). This process is attenuated by activation of nAChRs in monocytes and macrophages (Borovikova et al., 2000; Simard et al., 2013; Yue et al., 2015; St-Pierre et al., 2016; Godin et al., 2020), which is of tremendous clinical interest for the treatment of numerous inflammatory diseases. Treatment of experimental sepsis in mice with specific α7 nAChR agonists or with vagal stimulation, which induces the release of endogenous ACh, attenuated hallmarks of inflammation (Borovikova et al., 2000; Wang et al., 2004; Pavlov et al., 2007; Tracey, 2009).

IL-1β, a potent pro-inflammatory cytokine of innate immunity, is mainly produced by monocytes and macrophages (Broz and Dixit, 2016; Dinarello, 2018). It plays a central role in host defense against infections, but also in the pathogenesis of sepsis as well as in numerous auto-inflammatory and autoimmune diseases (Dinarello, 2018; Mantovani et al., 2019; Galozzi et al., 2021). In the light of these Janus-faced properties of IL-1β, it is reasonable that mechanisms have evolved that tightly control the production and release of IL-1 β. For instance, the biosynthesis of the inactive pro-from of IL-1β (pro-IL-1β) in response to the activation of pattern recognition receptors is also down-regulated by nAChR α7 activation (Borovikova et al., 2000). The release of mature IL-1β typically depends on a second danger signal such as extracellular ATP, an indicator of cytoplasm spilled from damaged cells (Gross et al., 2011; Rathinam et al., 2012; Rathinam and Fitzgerald, 2016; Bortolotti et al., 2018; Dinarello, 2018). When the ATP-sensitive and ionotropic P2X7 receptor is activated, the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome assembles and allows self-activation of caspase-1, which cleaves pro-IL-1β and enables its swift release (Gross et al., 2011; Rathinam et al., 2012; Cekic and Linden, 2016; Rathinam and Fitzgerald, 2016). In addition to ATP, numerous DAMPs and PAMPs can induce the release of IL-1β in a P2X7 receptor-independent way (Broz and Dixit, 2016). We discovered that activation of nAChRs with conventional agonists, such as ACh, choline or nicotine, specifically inhibits the ionotropic function of monocytic ATP receptors and, hence, efficiently attenuates the ATP-induced inflammasome-dependent maturation and release of IL-1β (Hecker et al., 2015; Richter et al., 2016; Zakrzewicz et al., 2017). In this context, phosphocholine (PC) and other molecules with a PC-head group function as nAChR agonists. The inhibitory function induced by PC depends on nAChR subunits α7, α9 and α10 and is independent of ionotropic nAChR functions (Hecker et al., 2015; Richter et al., 2016, 2018). At human α9 nAChR and α9α10 nAChR heterologously expressed by *Xenopus laevis* oocytes, however, PC did not induce ion-currents, but ion-current responses to choline were attenuated at human α9α10 nAChRs in the presence of PC (Richter et al., 2016; Zakrzewicz et al., 2017).

DAMPs, like endogenous Toll-like receptor agonists and ATP, released for instance upon accidental or surgical trauma, induce damage-mediated life-threatening "sterile" inflammation even in the absence of infectious agents (Manson et al., 2012; Bortolotti et al., 2018). Unconventional nAChR agonists are, thus, promising starting points for the development of therapeutics. They ideally control both, the gene expression of pro-inflammatory cytokines (e.g., IL-6, TNF-α, pro-IL-1β) and the ATP-mediated maturation and release of IL-1β. Although there are no experimental data, PC is expected to be unstable and it is unknown, if PC can inhibit the gene expression of cytokines. An alternative approach targeting nAChRs, shown to be effective in animal models of inflammatory pain or autoimmune disease

(Quadri et al., 2017; Godin et al., 2020), relies on the use of a recently described group of synthetic silent agonists of nAChR α7 based on a N,N-diethyl-N′-phenylpiperazine (diEPP) scaffold. These silent agonists need to be tested if they also inhibit the ATP-induced secretion of IL-1β.

Here we characterize the control of cytokine release by mononuclear phagocytes by PC in comparison to *p*CF3-diEPP. As expected, PC turned out to be unstable and ineffective in controlling lipopolysaccharide (LPS)-induced cytokine release. In contrast, we provide evidence, that *p*CF3-diEPP not only reduces cytokine release in response to Toll-like receptor activation with LPS, but also functions as a potent inhibitor of ATP-induced IL-1β release. We further show for the first time, that *p*CF3-diEPP is a partial agonist and a strong desensitizer of classical ionotropic functions at heterologously expressed human α9 and α9α10 nAChRs.

MATERIALS AND METHODS

Chemicals and Reagents

ACh chloride (Cat# A6625), dimethyl sulfoxide (DMSO, Cat# D2650), LPS (*E. coli* 011:B4, Cat# L2630), macrophage colony-stimulating factor (M-CSF, Cat# SRP3110), mecamylamine hydrochloride (Mec, Cat# M9020), phorbol 12-myristate 13-acetate (PMA, Cat# P1585), PC (Cat# P0378), recombinant mouse interferon-γ (INF-γ, Cat# IF005), sodium bicarbonate (Cat# R8758), 3-aminobenzoate methanesulfonate (MS-222, Cat# E10521) and all chemicals for saline Ringer's buffer preparations were purchased from Merck (Darmstadt, Germany) or from Millipore Sigma (St. Louis MO, United States). LPS from *E. coli* O26:B6 was purchased from Merck (Cat# L2654) or eBioscience (Thermo Fisher Scientific, Waltham MA, United States; Cat# L500497693). Gibco penicillin-streptomycin and L-glutamine were purchased from Thermo Fisher Scientific. BzATP [2′(3′)-O-(4-benzoyl-benzoyl)ATP trieethylammonium salt] was purchased from Jena Bioscience (Jena, Germany), EDTA from bioWORLD (Dublin, OH, United States) and recombinant human INF-γ from R&D Systems (Minneapolis, MN, United States).

*p*CF3-diEPP was synthesized as previously published (Quadri et al., 2017) and dissolved in DMSO. When appropriate, control experiments were performed with the corresponding concentrations of DMSO without *p*CF3-diEPP. The conopeptides [V11L, V16D]ArIB (specifically antagonizing α7 nACh (Whiteaker et al., 2008; Hone et al., 2009, 2010) and RgIA4 (antagonizing nAChRs composed of subunits α9 and α10 (Christensen et al., 2017; Romero et al., 2017; Grau et al., 2018) were produced and characterized as described previously (Innocent et al., 2008; Romero et al., 2017).

Mouse Bone Marrow-Derived Macrophages

Male and female mice gene-deficient in *Chrna*7 (α7), *Chrna*9 (α9) or *Chrna*9/*Chrna*10 (α9/α10) as well as corresponding wild-type (WT) mice were used for isolation of bone marrow cells. The α9 (Chrna9tm1Bjmy MGI:5787807) and α10 (Chrna10tm1Bjmy MGI:5787808) knockout lines were generated by Genoway, Inc. (Lyon, France) and backcrossed to congenicity at the Boys Town National Research Hospital, Omaha, NE, United States (BTNRH) using C57Bl/6J mice from The Jackson Laboratory (JAX). The α7 animals were originally received from JAX and a colony maintained at BTNRH. The α7 animals were crossed with the α9 or α9/α10 to produce double and triple knockout lines. The animals used in the studies reported here were derived by crossing the double and triple knockout lines with C57Bl/6J mice to produce single α7 and α9 KOs and double α9/α10 KOs. This was done to produce backgrounds in the strains that were comparable. The use of the animals in the experiments reported here was approved by the BTNRH Institutional Animal Care Committee (protocol #16-04). Mice were bred and housed at BTNRH and shipped to the Laurentian University (Sudbury, Canada). Experimental animals received humane care according to NIH "Guide for the Care and Use of Laboratory Animals." Animal experiments were reviewed and approved by the Laurentian University Animal Care Committee (file number 6013816). Genotypes were analyzed by PCR as described previously (St-Pierre et al., 2016; Morley et al., 2017).

Bone marrow cells were flushed from cleaned femoral and tibial bones using a 10 ml syringe and a 26–27-gauged needle. Debris was removed by passing the suspension through a 40 μm BD Falcon Cell Strainer (Becton, Dickinson (BD), Franklin Lakes, NJ, United States). Red blood cells were lysed by RBC Lysis buffer, as per the supplier's protocol (BioLegend, San Diego, CA, United States). Cells were pelleted at 510 g for 10 min and 1×10^6 cells/ml were resuspended in complete medium [RPMI 1640 medium (Cytiva, Marlborough, MA, United States) supplemented with 10% fetal calf serum (FCS; Life Technologies, Thermo Fisher Scientific, Cat# LS12484028), 50 U penicillin/ml, 50 μg streptomycin/ml and 2 mM L-glutamine] and 10 ng/ml M-CSF. 1×10^6 cells per well were seeded in Costar 12-well plates (Corning, NY, United States) and cultured for 72 h. Thereafter, 1 ml of fresh complete medium was added supplemented with a final concentration of 10 ng/ml M-CSF, 10 ng/ml IFN-γ and 10 pg/ml LPS (*E. coli* O26:B6) to induce their differentiation into M1 macrophages. Cells were cultured for another period of 48 h. Then, the bone marrow-derived macrophages (BMDMs) were stimulated with higher concentrations of LPS (100 ng/ml) as described previously (Godin et al., 2020) in the absence and presence of PC (100 μM) or *p*CF3-diEPP (100 μM). Cell-free supernatant was harvested 6 h later and stored at −20°C for cytokine measurements.

Human Whole Blood

For whole blood experiments, blood samples were obtained from healthy (self-reported) female and male non-smoking adult volunteers. Experiments were reviewed, approved, and performed in accordance with the policies outlined by the Laurentian University Research Ethics Board for Research Involving Human Participants (file number 6012214) and in accordance with the Helsinki Declaration. To prevent blood clotting, blood samples were collected in BD Vacutainer glass blood collection tubes containing sodium heparin (BD, Cat#

B366489) and were individually shaken periodically during and after the blood draw. 200 μl of whole blood was added per well in Costar 24-well flat-bottom plates (Stemcell, Vancouver, Canada, Cat# 38017). Whole blood was then diluted in 700–800 μl of Sigma RPMI 1640 medium (Merck, Cat #R8758). Wells containing whole blood diluted in RPMI were pre-treated with 100 μM *p*CF3-diEPP or 0.1% DMSO for 1 h at 37°C. Immediately following pre-treatment, wells were stimulated with 100 ng/ml LPS (*E. coli* O26:B6) for 24 h (Bruchfeld et al., 2010). Controls wells were treated with an equivalent volume of Sigma RPMI 1640 medium. Following the 24 h of stimulation with LPS, cells were spun down (2,000 g, 5 min, RT). The cell-free supernatants were collected and stored at −20°C for later cytokine measurements.

THP-1 Cells

Monocytic THP-1 cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured in medium (Gibco^{TM} RPMI 1640 medium (Thermo Fisher Scientific, Cat# 11530586) supplemented with 10% FCS (CellConcepts, Umkirch, Germany) and 2 mM L-glutamine). To investigate IL-1β release, monocytic cells were resuspended in FCS-free RPMI medium and transferred to 48-well plates (Greiner Bio-One, Frickenhausen, Germany; 0.5×10^6 cells/0.5 ml and per well). Cells were primed for 5 h with 1 μg/ml LPS (*E. coli* O26:B6) as described previously (Hecker et al., 2015). After priming, the P2X7 receptor agonist BzATP (100 μM) was added for 40 min in presence or absence of different concentrations of cholinergic agonists (PC, *p*CF3-diEPP) and antagonists (mecamylamine, [V11L, V16D]ArIB and RgIA4). After cell treatment, cells were spun down (500 g, 8 min, 4°C), the supernatants were collected and stored at −20°C for later cytokine measurements.

In addition, THP-1 cells were differentiated into M1-like macrophages. Monocytic THP-1 cells were resuspended in complete medium supplemented with 50 U penicillin/ml and 50 μg streptomycin/ml and seeded in 12-well plates (Greiner; 0.3×10^6 cells/ml and per well). Cells were differentiated into macrophages by 24 h incubation with 50 nM PMA followed by 24 h incubation in complete medium supplemented with 50 U penicillin/ml, 50 μg streptomycin/ml. Macrophages were then polarized to M1-like macrophages by incubation in fresh complete medium, supplemented with 50 U penicillin/ml, 50 μg streptomycin/ml, 10 ng/ml recombinant human IFN-γ and 10 ng/ml LPS *(E. coli* 011:B4) for 72 h. To investigate IL-1β release, the medium was replaced by fresh complete medium and the cells were stimulated with LPS (*E. coli* O26:B6) and BzATP as described for monocytic THP-1 cells.

Human Peripheral Blood Mononuclear Cells

Peripheral Blood Mononuclear Cells (PBMCs) were obtained from healthy (self-reported) female and male non-smoking adult volunteers. The study was approved by the ethics committee of the medical faculty Giessen, Germany (No. 90/18) and performed in accordance with the Helsinki Declaration. Blood was drawn into sterile syringes containing EDTA (1 mM per ml blood; bioWORLD, Dublin, OH, United States). LPS (*E. coli* O26:B6; 5 ng/ml) was added to blood samples shortly before PBMC isolation and PBMCs were separated using Leucosep gradients (Greiner) as described previously (Hecker et al., 2015). Thereafter, PBMCs were cultured for 3 h in 24-well plates (Greiner) at a density of 0.5×10^6 cells/0.5 ml in Monocyte Attachment Medium (PromoCell, Heidelberg, Germany). Non-adherent cells were removed, and fresh Sigma RPMI 1640 medium was added. Stimulation with BzATP in the presence or absence of *p*CF3-diEPP was done as described for THP-1 cells. After cell treatment, cells were spun down (500 g, 8 min, 4°C), the supernatants were collected and stored at −20°C.

Cytokine Measurements

In samples obtained in the mouse BMDM experiments, Mouse IL-6 Flex Set (BD, Cat# 558301), Mouse TNF Flex Set (BD, Cat# 558299), Mouse IL-10 Flex Set (BD, Cat# 558300) and Mouse IL-1β Flex Set (BD, Cat# 560232) were used to measure cytokine concentrations with the BD Cytometric Bead Array technique per the supplier's protocol. Human IL-1β Flex kit (BD, Cat# 558279), Human IL-6 Flex kit (BD, Cat# 558276), Human IL-10 Flex kit (BD, Cat# 558274), and Human TNF Flex kit (BD, Cat# 560112) were used to measure cytokine concentrations in the supernatants of human whole blood experiments according to the manufacturer's protocol. The bead fluorescence in the samples was quantified by flow cytometry using a BD FACS Canto II flow cytometer in conjunction with BD FACS Diva Software (v6.1.3). Cytokine concentrations were interpreted using BD FCAP Array Software (v3.0).

IL-1β concentrations in supernatants obtained in experiments on THP-1 cells (monocytic, M1-like) and human PBMCs were measured using the Human IL-1 beta/IL-1F2 DuoSet enzyme-linked immunosorbent assay (ELISA) from R&D Systems. To estimate cell viability at the end of the cell culture experiments on human PBMCs, the non-radioactive cytotoxicity assay (Promega, Madison, WI, United States) was used to measure lactate dehydrogenase (LDH) activity in cell-free supernatants as indicated by the supplier. LDH values are given as percentage of the total LDH content of lysed control cells.

Expression of Human nAChR Subunits in *Xenopus laevis* Oocytes

Plasmid DNA encoding the human *CHRNA7* was obtained from Dr. J. Lindstrom (University of Pennsylvania, Philadelphia, PA, United States). The human resistance-to-cholinesterase 3 (*RIC3*) clone was obtained from Dr. M. Treinin (Hebrew University, Jerusalem, Israel) and co-injected with *CHRNA7* to improve the level and speed of receptor expression without affecting their pharmacological properties (Halevi et al., 2003). Plasmid DNA encoding the human *CHRNA9* and *CHRNA10* as well as the human 43 kDa receptor-associated protein of the synapse (*RAPSN*) with codon optimization for expression in *Xenopus laevis* were obtained from Eurofins Genomics (Ebersberg, Germany), (Richter et al., 2016). After linearization and purification of the plasmid DNAs, RNAs were prepared

using the mMessage mMachine *in vitro* RNA transcription kit (Ambion, Austin, TX, United States).

Frogs were maintained in the Animal Care Service facility of the University of Florida, and all procedures were approved by the University of Florida Institutional Animal Care and Use Committee (approval number 202002669). In brief, the animals were first anesthetized for 15–20 min in 1.5 l frog tank water containing 1 g of MS-222 buffered with sodium bicarbonate. Oocytes were obtained surgically from mature female *Xenopus laevis* (Nasco, Ft. Atkinson, WI, United States) and treated with 1.4 mg/ml type 1 collagenase (Worthington Biochemicals, Freehold, NJ, United States) for 2–4 h at RT in Ca^{2+}-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 15 mM HEPES, and 12 mg/l tetracycline, pH 7.6) to remove the ovarian tissue and the follicular layers. Stage V oocytes were injected with 4–6 ng *CHRNA7* RNA and 2–3 ng *RIC3* RNA (2:1 ratio) in 50 nl water, or with 12 ng *CHRNA9* RNA and 3 ng *RAPSN* RNA, or along with 12 ng *CHRNA10* RNA in 50 nl water. Oocytes were maintained in Barth's solution containing 0.32 mM $Ca(NO_3)_2$ and 0.41 mM $CaCl_2$, and recordings were carried out 2–20 days after injection.

Two-Electrode Voltage-Clamp Electrophysiology

Two-electrode voltage-clamp experiments were conducted using OpusXpress 6000A (Molecular Devices, Union City, CA, United States) (Papke and Stokes, 2010). Both the voltage and current electrodes were filled with 3 M KCl. Oocytes were voltage-clamped at −60 mV at RT. The oocytes were perfused with Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, 1 μM atropine, pH 7.2) at 2 ml/min. To evaluate the effects of experimental compounds, responses were compared to control ACh-evoked responses, defined as the average of two initial applications of 60 μM ACh made before test applications. Drug applications were 12 s in duration followed by 181 s washout periods. In some experiments after obtaining the control responses, the bath perfusion solution was switched to the alternative buffer B, and test solutions delivered during the buffer B perfusion were made up in buffer B solution.

The responses were calculated as both peak-current amplitudes and net charge, as previously described (Papke and Porter Papke, 2002). Data were collected at 50 Hz, filtered at 20 Hz, and analyzed by Clampfit (Molecular Devices) and Excel (Microsoft, Redmond, WA, United States). Data were expressed as means ± SEM from at least four oocytes for each experiment and plotted with Kaleidagraph 4.5.2 (Abelbeck Software, Reading, PA, United States). Each episode of data acquisition was a total of 210 s and included an initial 30 s period used to define the baseline for the drug-evoked responses. After 30 s, drugs were applied, and the following 120 s were defined as the drug response period for analysis. Data reported for α7 were net charged, while peak currents were used for α9 and α9/α10 responses since these receptors do not show the same concentration dependent desensitization that invalidates peak currents as measurements of α7 concentration-dependent responses (Papke and Porter Papke, 2002). Multi-cell averages were calculated for comparisons of complex responses. Averages of the normalized data were calculated for each of the 10,322 points in each of the 206.44 s traces (acquired at 50 Hz), as well as the standard errors for those averages.

Statistical Analyses and Data Processing

Results obtained in the LPS- and BzATP-induced cytokine release experiments were analyzed using SPSS (Version 23, IBM, Armonk, NY, United States). The IC_{50} value of *p*CF3-diEPP in human PBMCs was determined in GraphPad Prism (Version 6, GraphPad Software, San Diego, CA, United States) by fitting log-transformed concentration values and the original effect data. Paired data were analyzed first by the Friedman test followed by the Wilcoxon signed-rank test.

In two-electrode voltage-clamp experiments, the comparisons of results were made using one-way ANOVA or using *t*-tests between the pairs of experimental measurements. In cases, where multiple comparisons were made, a Bonferroni correction for multiple comparisons (Aickin and Gensler, 1996) was applied to correct for possible false positives. A value of $p \leq 0.05$ was used to constitute a minimum level of significance. The statistics were calculated using an Excel template provided in Microsoft Office or ANOVA protocols in Kaleidagraph (4.5.2 Abelbeck Software, Reading, PA). Concentration-response relationships utilized data obtained over a range of concentration at roughly half log units. Average responses were fit to the Hill equation by the Levenberg-Marquardt algorithm in Kaleidagraph.

The number (n) of individual experiments is indicated in the Results section and the Figures. When primary cells were investigated, the n-number represents data obtained from the cells of individual mice or humans. In experiments with cell lines, the n-number refers to independent experiments, which were performed on different days with different cell passages. No outliers were excluded from the analyses. All two-electrode voltage-clamp experiments began with 8 oocytes voltage clamped and treated in parallel. Only cells, which lost voltage-clamp or otherwise failed to remain viable through the measurements, were excluded from the analyses but no viable cells. Data were visualized using Inkscape version 0.48.5 r10040 (Free and Open Source Software licensed under the GPL).

RESULTS

Control of LPS-Induced Cytokine Release by pCF3-diEPP but Not by PC

Agonists of nAChRs have been shown to inhibit the production and release of pro-inflammatory cytokines by mononuclear phagocytes (Kox et al., 2009). Here, we investigated in M1-like murine BMDMs whether the unconventional nAChR agonist PC (Hecker et al., 2015; Richter et al., 2016) and the α7 silent agonist *p*CF3-diEPP (Quadri et al., 2017) change the levels of IL-1β, IL-6, TNF-α and IL-10 in cell culture supernatants in response to treatment with LPS (1 μg/ml for 5 h; **Figure 1**). Throughout, IL-1β levels remained below the level of detection. LPS induced the release of IL-6 in the range of 1 to 1,092 pg/ml, TNF-α in the range of 1,012 to 12,672 pg/ml and IL-10 in the range of 13 to 561 pg/ml.

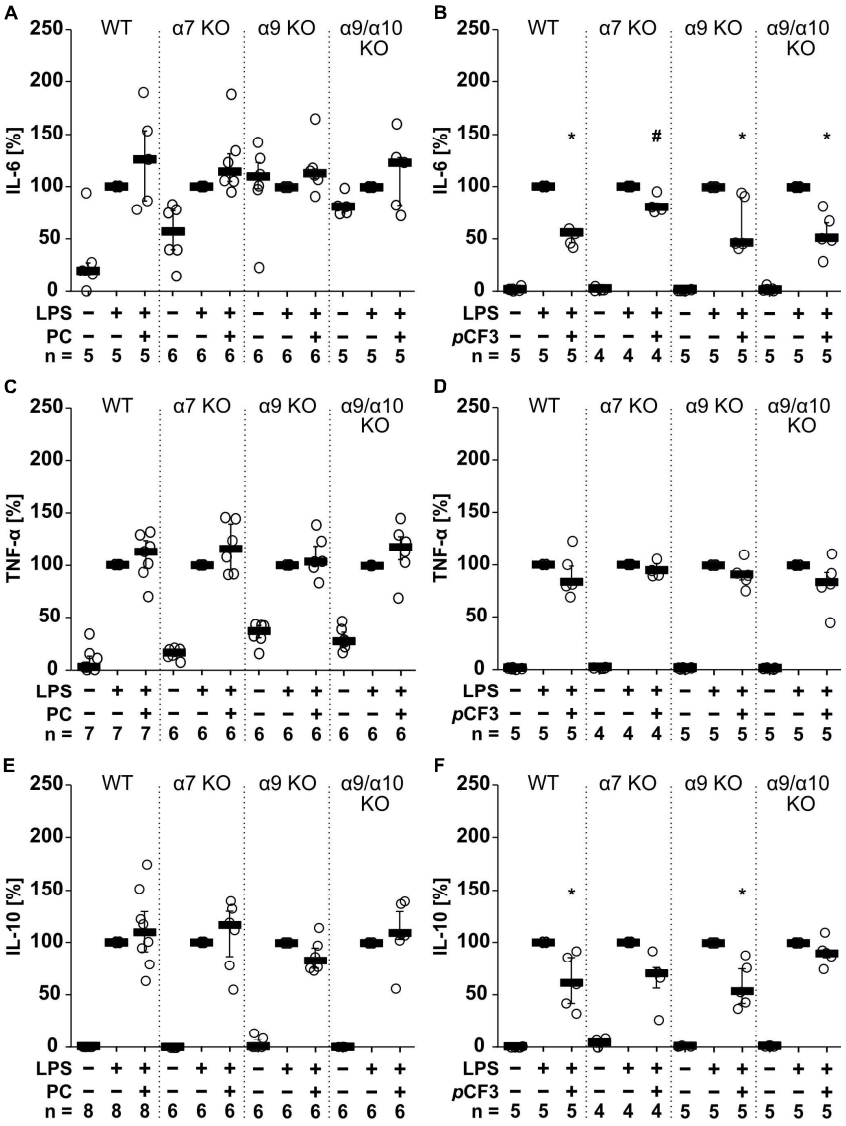

FIGURE 1 | The effect of phosphocholine (PC) and *p*CF3-diEPP (*p*CF3) on lipopolysaccharide (LPS)-mediated cytokine release by mouse bone marrow-derived macrophages (BMDMs). BMDMs were isolated and cultured from wild-type (WT) and mice gene-deficient in *Chrna*7 (α7 KO), *Chrna*9 (α9 KO) or *Chrna*9 and *Chrna*10 (α9/α10 KO). On day 6 of cultivation, BMDMs were primed with lipopolysaccharide (LPS; 1 μg/ml; 5 h) in the absence and presence of PC (100 μM) or *p*CF3 (100 μM). The LPS-induced release of **(A,B)** interleukin-6 (IL-6), **(C,D)** tumor necrosis factor-α (TNF-α) and **(E,F)** IL-10 were measured in cell free cell culture supernatants by BD Cytometric Bead Arrays. Cytokine levels obtained after stimulation with LPS alone, were set to 100%, and all other values were calculated accordingly. Data are presented as individual data points, bars represent median, whiskers encompass the 25th to 75th percentile. *$p \leq 0.05$, different from cells stimulated with LPS alone, Friedman-test followed by the Wilcoxon signed-rank test. #$p \leq 0.05$, significantly different from the same condition in WT, Kruskal–Wallis followed by Mann–Whitney rank sum test.

The LPS-induced release of these cytokines was not affected in the presence or absence of PC (100 μM; **Figures 1A,C,E**). In contrast, *p*CF3-diEPP (100 μM) significantly reduced IL-6 (**Figure 1B**; $p = 0.007$; $n = 5$) and IL-10 concentrations (**Figure 1F**; $p = 0.007$; $n = 5$) but no effect was seen on TNF-α (**Figure 1D**). In BMDMs from mice deficient in nAChR subunits α7, α9 or α10 as well as in mice with a double-deficiency in nAChR subunits α9 and α10, essentially the same results were obtained (**Figure 1**). Only the *p*CF3-diEPP-mediated attenuation of the secretion of IL-6 was not seen in BMDMs from nAChR α7 gene-deficient mice (**Figure 1**). These results are in line with previous reports that *p*CF3-diEPP functions as a silent agonist at nAChR α7 (Quadri

et al., 2017). The fact that PC does not inhibit the LPS-induced production and secretion of cytokines, is most probably due to its expected instability, which is further confirmed in experiments described below (**Supplementary Figure 1**). Therefore, PC was omitted from the experiments on human whole blood cells.

Human whole blood cells secreted IL-6 (in the range of 3 to 22,000 pg/ml), TNF-α (1 to 2,000 pg/ml), IL-1β (in the range of 3 to 5,600 pg/ml) and IL-10 (3 to 800 pg/ml), in response to LPS (100 ng/ml for 24 h; **Figure 2**). The LPS-induced release of IL-6 (p = 0.020), TNF-α (p = 0.003) and IL-1β (p = 0.0002) was significantly inhibited by *p*CF3-diEPP (**Figures 2A–C**), whereas no such effect was seen on IL-10 (p = 0.117; **Figure 2D**). In control experiments, in which LPS was omitted, *p*CF3-diEPP did not change the cytokine levels compared to supernatants of untreated cells (**Figure 2**).

Control of the BzATP-Induced IL-1β Release by Human Monocytic Cells and Macrophages by PC and *p*CF3-diEPP

We showed before that PC functions as an unconventional agonist at monocytic nAChRs containing subunits α7, α9 and α10 and as a potent inhibitor of the BzATP-induced maturation of IL-1β in monocytic U937 cells and human as well as murine peripheral mononuclear blood leukocytes (Hecker et al., 2015; Richter et al., 2016). To test if PC and *p*CF3-diEPP exert similar effects on monocytic THP-1 cells (**Figure 3A**) and THP-1-derived M1 macrophages (**Figure 3B**), cells were primed with LPS (1 μg/ml) for 5 h, followed by stimulation with the P2X7 receptor agonist BzATP (100 μM) for another 40 min in the presence or absence of PC (200 μM) or *p*CF3-diEPP (100 μM). Thereafter, the concentration of IL-1β was measured in cell culture supernatants by ELISA. As expected, untreated cells and cells primed with LPS did not release relevant amounts of IL-1β, whereas stimulation with BzATP resulted in elevated IL-1β levels released by monocytic THP-1 cells in the range of 19 to 122 pg/ml and by THP-1-derived M1-macrophages in the range of 53 to 314 pg/ml. In both monocytic cells and macrophage-like cells, PC and *p*CF3-diEPP significantly inhibited the BzATP-induced release of IL-1β. To examine the subtype(s) of nAChRs that underlie this effect, we used selective peptide antagonists beside the non-selective nAChR antagonist mecamylamine (Innocent et al., 2008). Homomeric α7, and α9-containing nAChRs can be difficult to pharmacologically differentiate. α-Bungarotoxin, for example, blocks both α7 and α9-containing nAChRs with low nM potency (Johnson et al., 1995; Briggs and McKenna, 1996; Elgoyhen et al., 2001). However, certain analogs of conotoxins have been developed that can effectively distinguish between these receptor subtypes (Grau et al., 2018). The inhibitory effect of PC and *p*CF3-diEPP on the BzATP-induced release of IL-1β was completely reverted by mecamylamine and blunted by the nAChRα7-specific conopeptide (Innocent et al., 2008; Whiteaker et al., 2008). [V11L, V16D]ArIB (500 nM) and by the α9α10 nAChR-specific conopeptide RgIA4 (200 nM), suggesting that nAChR subunits α7, α9 and/or α10 are involved in signaling (**Figure 3A**). In control experiments, in which mecamylamine, RgIA4 and [V11L, V16D]ArIB were given together with BzATP, the release of IL-1β was not significantly changed in monocytic THP-1 cells (**Figure 3A**). In THP-1-derived macrophage-like cells, however, a slightly reduced IL-1β concentration was measured in the presence of RgIA4, but not in the presence of [V11L, V16D]ArIB (**Figure 3B**). Further, PC, *p*CF3-diEPP, RgIA4, and [V11L, V16D]ArIB did not induce a relevant release of IL-1β in the absence of BzATP (**Figures 3A,B**).

To investigate the stability of PC and *p*CF3-diEPP in cell culture, PC (200 μM) and *p*CF3-diEPP (100 μM) were added to LPS-primed monocytic THP-1 cells 5, 15, and 30 min before stimulation with BzATP (100 μM). As shown in **Supplementary Figure 1**, the inhibitory effect of PC on the release of IL-1β was

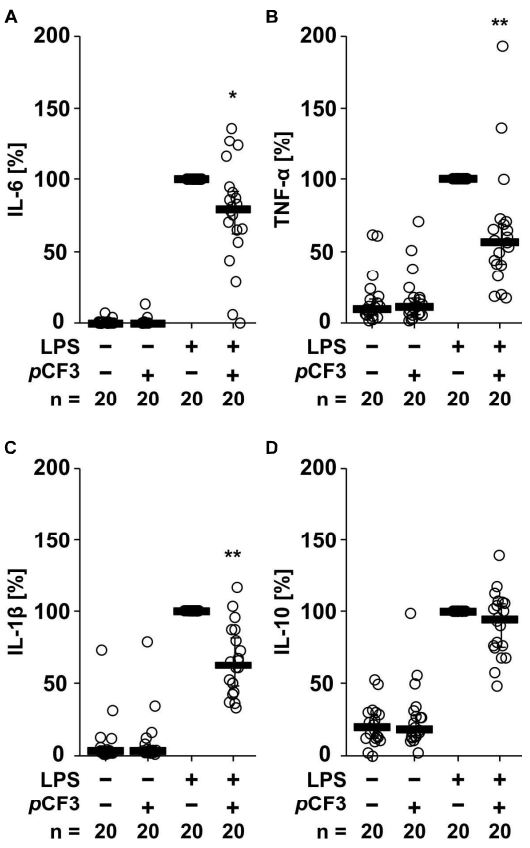

FIGURE 2 | *p*CF3-diEPP (*p*CF3) inhibits the lipopolysaccharide (LPS)-induced release of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and IL-1β by human whole blood cells. Human whole blood was left untreated or pre-treated with 100 μM *p*CF3 for 1 h, followed by a 24 h stimulation with 100 ng/ml LPS. Thereafter, LPS-induced cytokine release was measured in cell-free cell culture supernatants by BD Cytometric Bead Arrays. In the presence of *p*CF3 the LPS-induced release of **(A)** IL-6, **(B)** TNF-α and **(C)** IL-1β was blunted, whereas no effect was seen on **(D)** IL-10. Cytokine levels obtained after stimulation with LPS alone were set to 100%, and all other values were calculated accordingly. Data are presented as individual data points, bars represent median, whiskers encompass the 25th to 75th percentile. *$p \leq 0.05$ and **$p \leq 0.01$ different from cells stimulated with LPS alone. Friedman-test followed by the Wilcoxon signed-rank test.

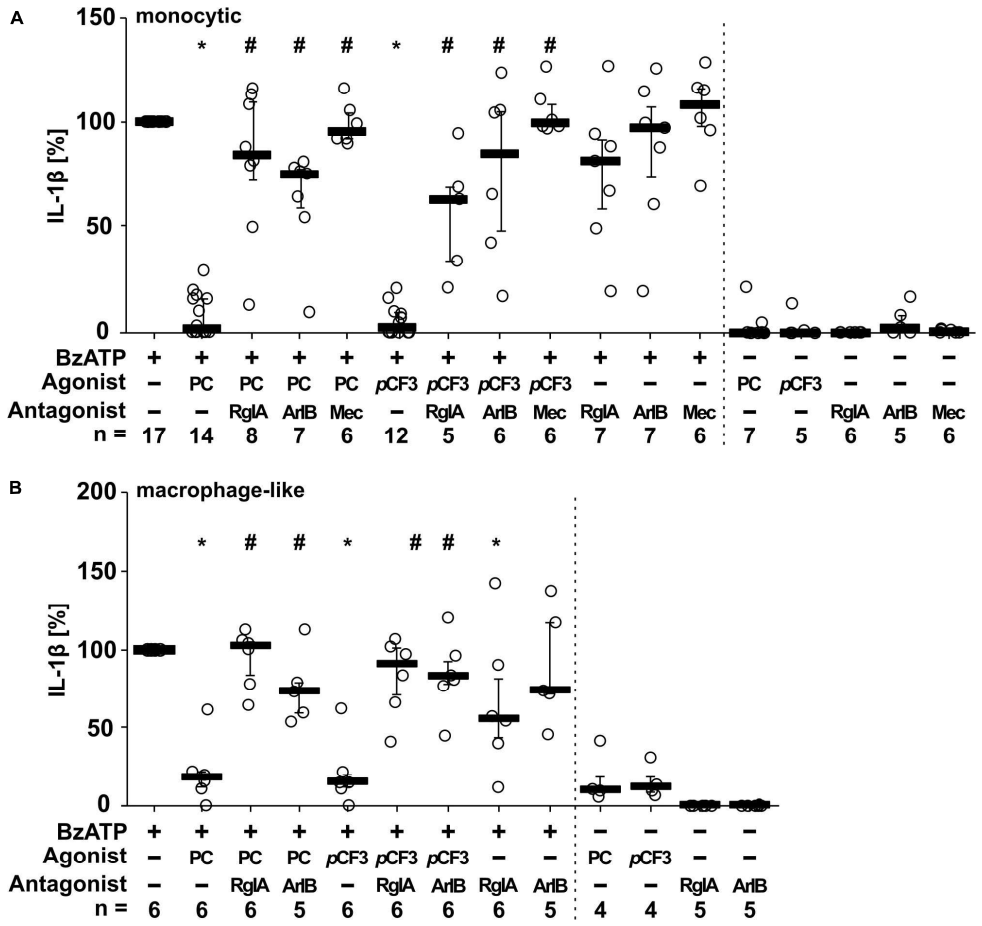

FIGURE 3 | Phosphocholine (PC) and the silent agonist *p*CF3-diEPP (*p*CF3) inhibit the BzATP-mediated release of interleukin-1β (IL-1β) by monocytic and macrophage-like THP-1 cells *via* nAChR subunits α7, α9 and/or α10. Monocytic **(A)** and differentiated macrophage-like **(B)** THP-1 cells were used. Cells were primed with lipopolysaccharide (LPS; 1 μg/ml, 5 h). Thereafter, the P2X7 receptor agonist BzATP [2′/3′-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate, tri(triethylammonium) salt] was added for another 40 min to trigger IL-1β release, which was measured by ELISA. The BzATP (100 μM)-induced release of IL-1β was investigated in the presence and absence of PC (200 μM), the synthetic silent agonists *p*CF3 (100 μM), antagonistic conopeptides RgIA4 (RgIA; 200 nM; specific for nAChRs composed of subunits α9 and α10) or [V11L, V16D]ArIB (500 nM; specific for α7 nAChR subunit) and the non-competitive, non-specific nAChR antagonist mecamylamine (Mec; 100 μM). The concentration of IL-1β released in response to BzATP was calculated by subtracting the IL-1β concentrations measured in supernatants of cells treated with LPS alone. In each experiment, the IL-1β concentration obtained after stimulation with BzATP was set to 100% and all other values were calculated accordingly. Data are presented as individual data points, bars represent median, whiskers encompass the 25th to 75th percentile. $^{*}p \leq 0.05$, different from LPS-primed cells stimulated with BzATP alone; $^{\#}p \leq 0.05$ significantly different from samples, where BzATP plus agonist was given. Friedman-test followed by the Wilcoxon signed-rank test.

lost within 15 min, whereas *p*CF3-diEPP was effective throughout (**Supplementary Figure 1**).

We further tested if *p*CF3-diEPP inhibits the BzATP-induced release of IL-1β by freshly isolated, LPS-primed human PBMCs. These cells released IL-1β in response to BzATP (in the range of 499 to 1,931 pg/ml), which was concentration-dependently inhibited by *p*CF3-diEPP (**Figure 4**). Of note, the calculated half maximal inhibitory concentration (IC_{50}) value was as low as 64 fM (**Figure 4**). When different concentrations of *p*CF3-diEPP were added to LPS-primed PBMCs in control experiments without BzATP, IL-1β secretion remained at low levels (**Supplementary Figure 2A**). Cell viability as measured by LDH-release in LPS-primed and BzATP-stimulated PBMCs was not altered by different concentrations of *p*CF3-diEPP (**Supplementary Figure 2B**).

Interference of PC and *p*CF3-diEPP With ACh-Induced Ion-Currents at Human α7, α9 and α9α10 nAChRs

Previous experiments using 1 mM PC revealed an efficient PC-mediated reduction of choline-gated ion-currents at

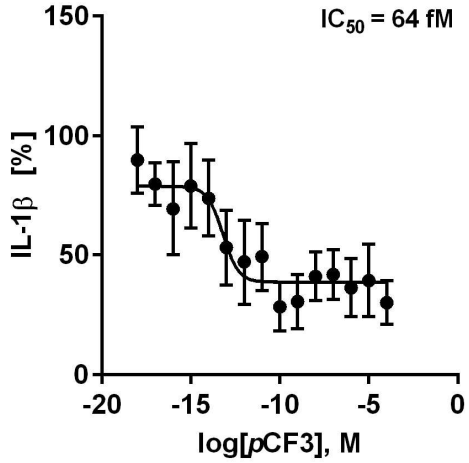

FIGURE 4 | *p*CF3-diEPP (*p*CF3) inhibits the BzATP-mediated release of interleukin-1β (IL-1β) by human peripheral blood mononuclear cells (PBMCs). PBMCs were freshly isolated from blood of healthy human volunteers and primed with lipopolysaccharide (LPS, 5 ng/ml) during the isolation process. After 3 h of culture, non-adherent cells were decanted. *p*CF3 dose-dependently inhibited the BzATP [2'/3'-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate, tri(triethylammonium) salt] (100 μM)-induced release of IL-1β by human PBMCs (IC_{50} = 64 fM). The amount of IL-1β released in response to BzATP was calculated by subtracting the IL-1β concentrations measured in supernatants of cells treated with LPS alone. In each experiment, the IL-1β concentration obtained after stimulation with BzATP was set to 100% and all other values were calculated accordingly. IL-1β (%) is provided on vertical axis and log molar (M) concentrations on horizontal axis. The IC_{50} value obtained by the dose-response curve (n = 5–9 independent experiments, shown as mean ± SEM) was obtained by non-linear regression analysis using Graph Pad Prism 6.0.

heterologously expressed α9α10 nAChRs (Richter et al., 2016). This prompted us to investigate the effects of PC and *p*CF3-diEPP on ACh-induced ion-currents in *Xenopus laevis* oocytes expressing human α7, α9 or α9α10 nAChRs in two-electrode voltage-clamp experiments. When ACh pulses (60 μM, 12 s, 2 ml/min) were applied to nAChR-expressing oocytes, repeatable ion-currents were induced (**Figure 5**). Concomitant application of PC (200 μM) had no effect at α7 nAChRs but provoked a slight reduction of the peak currents measured in oocytes expressing α9 or α9α10 nAChRs (**Figure 5A**). The concentration of 200 μM for PC was selected based on previous experiments (Richter et al., 2020) and on its potency to inhibit ATP-induced release of IL-1β as described above (**Figures 3**, **4**). After wash-out of PC, ACh-induced peak currents increased most prominently in oocytes expressing α9α10 nAChRs, and to a lesser extent in oocytes expressing α9 or α7 nAChRs (**Figure 5A**). Of note, the ACh-induced ion currents in oocytes expressing α7, α9 or α9α10 nAChRs entirely vanished in the presence of 30 μM *p*CF3-diEPP (**Figure 5B**). When a concentration of 1 μM *p*CF3-diEPP was used in the same experimental setting, a minor attenuation of the ACh-induced peak currents was seen in oocytes expressing α7 nAChRs, whereas this concentration still fully inhibited ACh-gated currents at α9 or α9α10 nAChRs (**Figure 5C**). Interestingly,

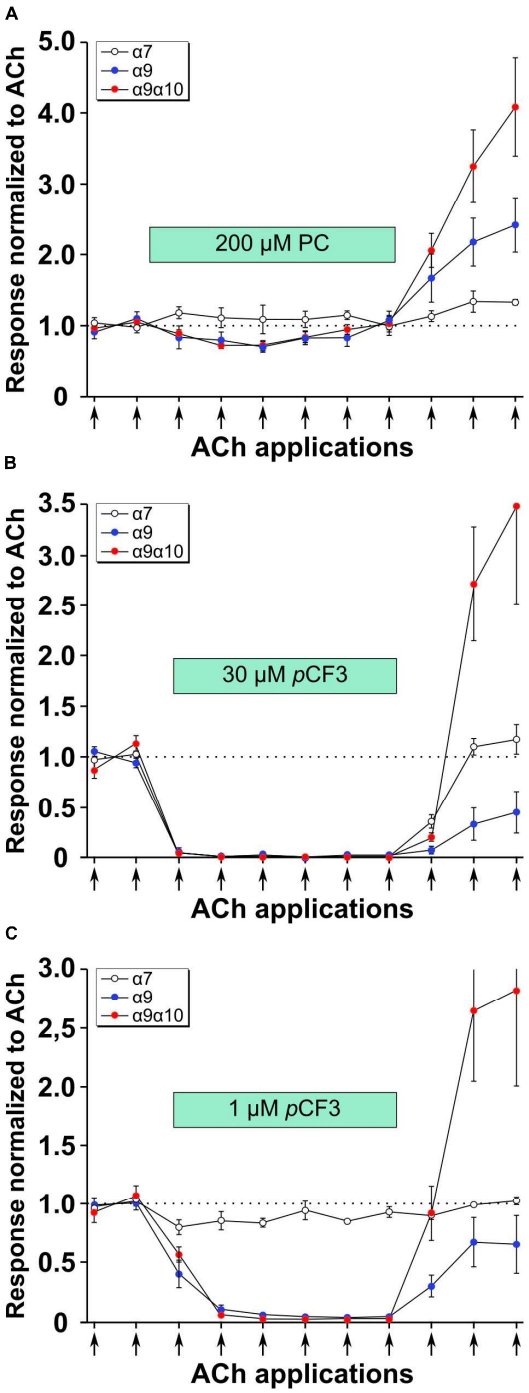

FIGURE 5 | Effects of phosphocholine (PC) and *p*CF3-diEPP (*p*CF3) on the peak current responses evoked by acetylcholine (ACh) on *Xenopus laevis* oocytes expressing human α7, α9 or α9α10 nAChRs. Two-electrode voltage-clamp measurements were performed on *Xenopus oocytes*

(Continued)

FIGURE 5 | heterologously expressing α7, α9 or α9α10 nAChRs. Two initial current responses to 60 μM ACh (12 s pulses indicated by arrows) were obtained in control Ringer's solution. Thereafter, the perfusion was switched to a solution containing **(A)** 200 μM PC, **(B)** 30 μM *p*CF3 or **(C)** 1 μM *p*CF3. The ACh-gated currents were monitored for changes in amplitude for six ACh applications. Thereafter, the perfusion solution was switched back to control and three more ACh-gated currents were monitored. Data are the average responses (± SEM), normalized to the average of the two initial ACh controls for each cell. The *n* values were 8, 6, and 7 for α7, α9 and α9 α10, respectively.

after wash-out of *p*CF3-diEPP, strongly increased peak currents induced by ACh were measured in oocytes expressing α9α10 nAChRs (**Figures 5B,C**). In contrast, such effects were not seen in oocytes expressing α7 nAChRs (**Figures 5B,C**). In oocytes expressing α9 nAChRs the responses to ACh after wash-out of *p*CF3-diEPP were reduced (**Figures 5B,C**).

As shown in **Figure 6A**, the application of 30 μM *p*CF3-diEPP to the bath solution initiated a current response in the α7, α9 and α9α10 nAChR-expressing cells during the initial 30 s periods normally used to define the baseline for drug-evoked responses (see section "Materials and Methods"). *p*CF3-diEPP has previously been characterized as a very weak partial agonist for α7 nAChRs effective at inducing large responses only when co-applied with a positive allosteric modulator such as PNU-120596 (Quadri et al., 2017). Therefore, not surprisingly, there was minimal response of the α7-expressing cells, when the bath was switched to a solution containing 30 μM *p*CF3-diEPP (**Figure 6A**, upper trace). However, we noted substantial responses to the cells expressing α9 alone or α9α10 nAChRs (**Figure 6A**, middle and lower traces), suggesting that *p*CF3-diEPP is an activator of these receptors. The ACh applications during the period of *p*CF3-diEPP perfusion were diminished and, at least initially, superimposed on an increased baseline current activated by *p*CF3-diEPP (**Figure 6B**). For the cells expressing α9 nAChRs alone, this shift in baseline, apparently representing a steady-state activation of the receptors, did not decline fully during the 210 s period of data acquisition (**Figures 6A,B**). These data indicate that the steady-state current of the α9 nAChR-expressing cells remained elevated through several of the acquisition periods that followed the switch to the bath containing 30 μM *p*CF3-diEPP.

Ion-Current Stimulation by *p*CF3-diEPP at Human α9 and α9α10 nAChRs

We demonstrated before in *Xenopus laevis* oocytes heterologously expressing human α9α10 nAChR, that application of PC does not provoke ion-currents (Richter et al., 2016). Similarly, *p*CF3-diEPP functions as a very weak partial agonist in *Xenopus laevis* oocytes heterologously expressing α7 nAChRs (Quadri et al., 2017). Here, we performed two-electrode voltage-clamp measurements in *Xenopus laevis* oocytes heterologously expressing either human α9 nAChR or α9α10 nAChR. Of note, co-expression of α10 with α9 in the oocyte will favor the heteromeric composition of functional receptors compared to α9 alone (Elgoyhen et al., 2001; Sgard et al., 2002). Concentration-response studies of ACh or *p*CF3-diEPP were performed and the ion-current responses were normalized to those provoked by 60 μM ACh. ACh and *p*CF3-diEPP concentration-dependently stimulated ion-currents at α9 nAChRs (**Figures 7A,B**). The maximal ACh-induced ion-current (I_{max}) was set to 1 and for *p*CF3-diEPP a relative I_{max} of 0.36 ± 0.02 was determined. The half maximal effective concentration (EC_{50}) of ACh was 26.1 ± 4.2 μM, whereas that of *p*CF3-diEPP was 7.0 ± 1.9 μM. Similar ion-currents were induced at α9α10 nAChRs, with a relative I_{max} of 0.30 ± 0.01 for *p*CF3-diEPP, an EC_{50} for ACh of 29.7 ± 9.9 μM and an EC_{50} of 6.4 ± 0.9 μM for *p*CF3-diEPP (**Figures 7C,D**).

DISCUSSION

We demonstrate that the silent α7 nAChR agonist *p*CF3-diEPP inhibits the expression of pro-inflammatory cytokines by monocytes/macrophages in response to stimulation with the Toll-like receptor agonist LPS, while PC is ineffective. We further provide evidence that *p*CF3-diEPP, similar to PC, efficiently controls the maturation and release of monocytic IL-1β in response to P2X7 receptor stimulation, a mechanism that is mediated by nAChR subunits α7 and α9/α10. Surprisingly, *p*CF3-diEPP functions as a partial agonist and induces ionotropic functions at heterologously expressed human α9 and α9α10 nAChRs when given alone, while efficiently inhibiting ACh-gated ion currents.

Most reports on α7 nAChR-mediated anti-inflammatory effects describe an inhibition of the release of LPS-induced pro-inflammatory cytokines such as IL-6 and TNF-α (Borovikova et al., 2000; Kox et al., 2009; Simard et al., 2013; St-Pierre et al., 2016). Here, we show that PC did not change the LPS-induced cytokine release by murine M1-like BMDMs. In contrast, *p*CF3-diEPP inhibited the expression and release of IL-6 and IL-10 but no significant change was seen for TNF-α. The results of BMDMs from nAChR gene-deficient mice suggested that nAChR subunit α7 but not α9/α10 plays a role in the signaling of *p*CF3-diEPP. In a similar experiment on human whole blood cultures, the release of the pro-inflammatory cytokines IL-6, TNF-α and IL-1β was reduced by *p*CF3-diEPP, whereas the anti-inflammatory cytokine IL-10 was unaffected. The differences between mouse BMDMs and human whole blood cells may be due to differences between species or due to differences between monocytes and macrophages. However, increasing the n-number of the mouse experiments by at least twofold might show, that the secretion of TNF-α is also reduced by *p*CF3-diEPP. Regarding the secretion of IL-1β, it has been shown before, that monocytes secrete small amounts of IL-1β in response to LPS alone, whereas macrophages need a second danger signal to process pro-IL-1β and release mature IL-1β (Netea et al., 2010; Gaidt et al., 2016).

The observation that PC was ineffective in the experiments on murine BMDMs deserves further discussion. We demonstrated before, that PC efficiently inhibits the ATP-induced release of monocytic IL-1β and that this effect is mediated *via* nAChR subunits α7, α9 and α10 (Hecker et al., 2015; Richter et al.,

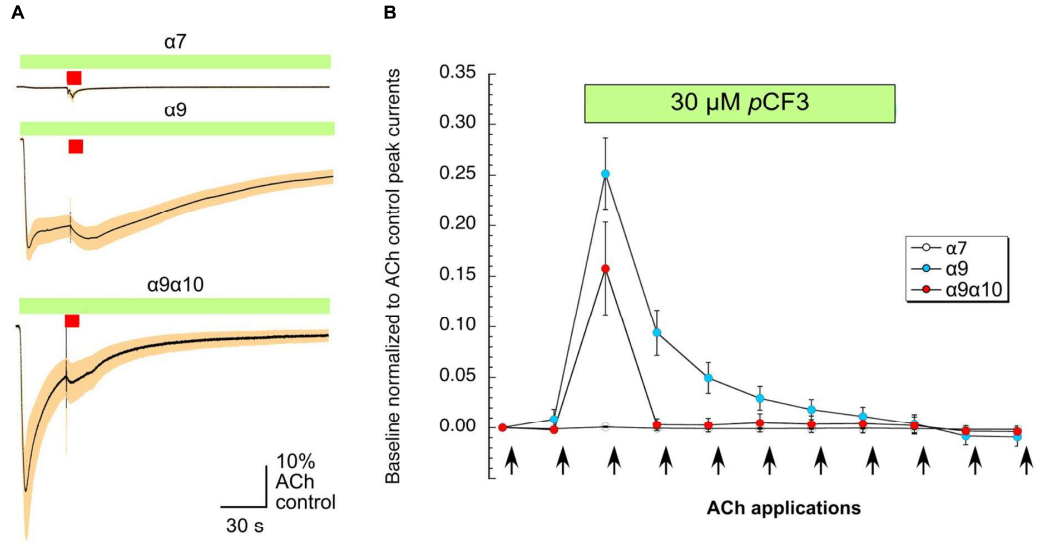

FIGURE 6 | Baseline currents during the applications of 30 μM *p*CF3-diEPP (*p*CF3). Two-electrode voltage-clamp measurements were performed on *Xenopus laevis* oocytes heterologously expressing human α7, α9 or α9α10 nAChRs. **(A)** Averaged responses of oocytes heterologously expressing human α7 nAChRs (upper trace, $n = 6$), α9 nAChRs (middle trace, $n = 7$), or α9α10 nAChRs (lower trace, $n = 7$) to the onset of the bath application of 30 μM *p*CF3 and the co-application with acetylcholine (ACh, 60 μM, red bar). The data from each cell were first normalized to the peak current amplitude of the ACh control response that preceded the bath application of 30 μM *p*CF3. The normalized data were then averaged (black lines) at each of the 10,000 points in the data traces and SEMs calculated and displayed as the tan-colored bands. The scale bar represents 10% of the averaged ACh controls, which were 5.1 ± 1.3 μA, 128 ± 33 9 nA, and 2.95 ± 0.97 μA for α7, α9, and α9α10 nAChR-expressing cells, respectively. **(B)** The shift in baseline was measured over the initial 30 s intervals of subsequent acquisition periods as the absolute value of the average current during that time minus the holding currents prior to the first two ACh (12 s pulses, indicated by arrows) controls. These values were then normalized to the peak currents of the ACh controls in the same cells.

2016; Zakrzewicz et al., 2017). However, when PC was given at different time points before addition of BzATP, its inhibitory function was time-dependently attenuated, which was not the case for *p*CF3-diEPP (**Supplementary Figure 1**). We concluded from these results, that PC is degraded or absorbed within minutes in cell culture and did not test its effects on human whole blood cells. This observation underlines the need of a stable anti-inflammatory nAChR agonist such as *p*CF3-diEPP for therapeutic use.

P2X7 receptor stimulation by extracellular ATP is a thoroughly investigated second signal that is of high clinical interest as it mediates trauma- or surgery-induced release of IL-1β, which is a trigger for systemic life-threatening inflammation (Rathinam et al., 2012; Cekic and Linden, 2016; Rathinam and Fitzgerald, 2016; Bortolotti et al., 2018). Up to now, it was unknown if the ATP-induced release of IL-1β is also regulated by PC in macrophages and if *p*CF3-diEPP exerts similar effects. Here, we investigated the effects of PC and *p*CF3-diEPP on the ATP-induced IL-1β release by human monocytic THP-1 cells and THP-1-derived M1-like macrophages. As expected, monocytic and macrophage-like THP-1 cells secreted IL-1β in response to LPS and BzATP. IL-1β secretion was efficiently inhibited by PC and by *p*CF3-diEPP. In line with previously published data (Hecker et al., 2015; Richter et al., 2016, 2020), PC and *p*CF3-diEPP signaled *via* nAChR subunits α7, α9 and/or α10, which was demonstrated by the use of the general nAChR antagonist mecamylamine (Innocent et al., 2008) and the conopeptides [V11L, V16D]ArIB specifically antagonizing nAChR subunit α7 (Whiteaker et al., 2008; Hone et al., 2009, 2010) or RgIA4 that antagonizes nAChRs composed of subunits α9 and α10 (Christensen et al., 2017; Romero et al., 2017; Grau et al., 2018). We conclude that PC and *p*CF3-diEPP exert similar functions at monocytic and macrophage-like cells. However, it should be mentioned that, in contrast to mecamylamine, there was a large variation of the data, when conopeptides were used. Of note, *p*CF3-diEPP also reduces the BzATP-mediated IL-1β release by freshly isolated LPS-primed human PBMCs at a very low IC_{50} value in the femtomolar range. Hence, *p*CF3-diEPP seems to have a higher affinity toward monocytic nAChRs containing subunits α7, α9 and/or α10 compared to classical α7 nAChR agonists.

It should be noted that previous studies indicated that reduction of inflammatory pain could be achieved by inhibition of α9 nAChRs, such that administration of RgIA4 or related analogs was intrinsically analgesic (Christensen et al., 2017; Romero et al., 2017; Huynh et al., 2019; Zheng et al., 2020, 2021). However, the present work shows that the anti-inflammatory activity of PC and *p*CF3-diEPP can be blocked by RgIA4, suggesting that stimulation of α9 nAChRs is anti-inflammatory. Previous *in vivo* studies, however, indicated that a single dose of RgIA4 had a long-lasting effect on neuropathic pain, although it is known, that RgIA4 has a very short half-life *in vivo* (Christensen et al., 2017; Romero et al., 2017). This would suggest that there may be downstream mediators of RgIA4 effects *in vivo*. In

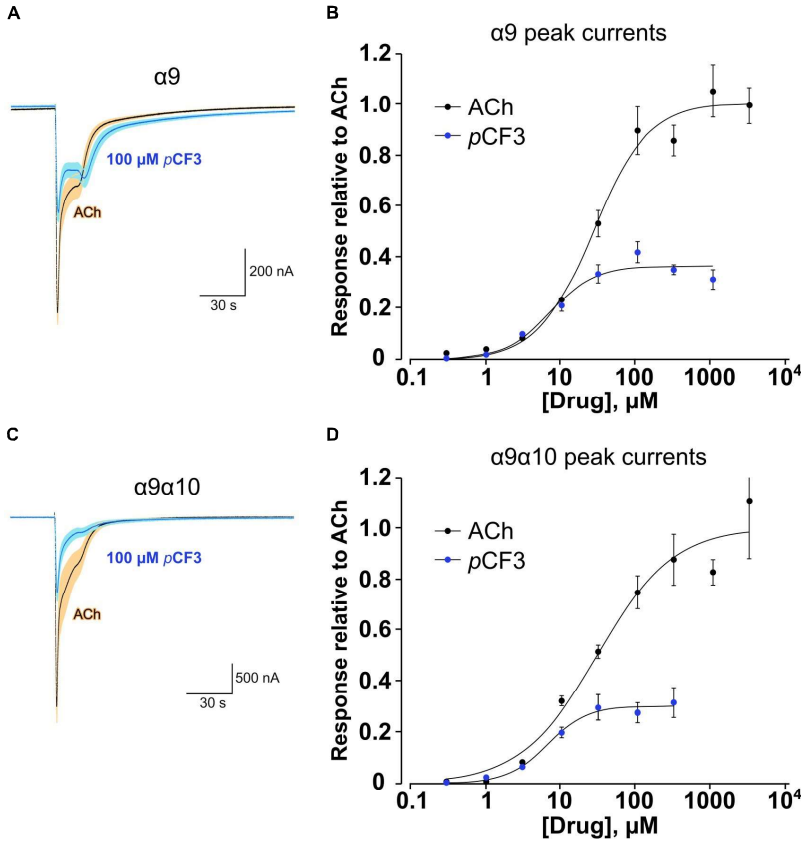

FIGURE 7 | Concentration-response studies for acetylcholine (ACh) and *p*CF3-diEPP (*p*CF3) on heterologously expressed human nAChR subunits α9 and α9α10. Two-electrode voltage-clamp measurements were performed on *Xenopus laevis* oocytes heterologously expressing human α9 or α9α10 nAChR. **(A,C)** Averaged responses of oocytes expressing α9 nAChR (A, $n = 6$) or nAChR α9α10 [**(C)** $n = 5$] to 60 µM ACh in the absence and the presence of 100 µM *p*CF3. Individual cell responses were scaled to the peak amplitude of the ACh controls, then averaged (dark blue line) and plotted with the calculated SEM at each point (light blue band). These are compared to the averaged responses (black lines) and SEMs (tan bands) of the ACh control responses. **(B)** Average peak-current responses to ACh, normalized to the ACh maximum response of cells expressing subunit α9, compared to responses to *p*CF3 ($n = 7$). The *p*CF3 currents were initially measured relative to 60 µM ACh controls obtained prior to the *p*CF3 applications and then adjusted for the ratio of the ACh controls to ACh maximum by multiplying the values by 0.63. With the ACh $I_{Max} = 1$, the EC_{50} was 26.1 ± 4.2 µM (Chisq = 0.017, $R = 0.9942$). For *p*CF3, the I_{Max} was 0.36 ± 0.02 and the EC_{50} was 7.0 ± 1.9 µM (Chisq = 0.007, $R = 0.979$). **(D)** Similar experiments as described in **(B)** were performed on oocytes heterologously expressing α9α10 nAChRs. With the ACh $I_{Max} = 1$, the EC_{50} was 29.7 ± 9.9 µM (Chisq = 0.037, $R = 0.986$). For *p*CF3, the I_{Max} was 0.30 ± 0.01 and EC_{50} was 6.4 ± 0.9 µM (Chisq = 0.001, $R = 0.995$). For all concentration-response studies, the applications of the test compounds (ACh or *p*CF3) at progressively higher concentrations were alternated by repeated application of ACh at the control concentration of 60 µM to confirm that there was no rundown of the ACh controls through the course of the experiment.

contrast, our results indicate that the immediate effect of the conopeptides on the immune cells tested are pro-inflammatory, because they enable the release of pro-inflammatory cytokines in the presence of endogenous cholinergic agonists.

Irrespective of the nAChR agonist used, the nAChR subunit α9 is essential in the cholinergic inhibition of the ionotropic function of the P2X7 receptor. This suggests that *p*CF3-diEPP interacts with α9 nAChRs or α9α10 nAChRs, which turned out to be true. In two-electrode voltage-clamp measurements in *Xenopus laevis* oocytes heterologously expressing human α9 or α9α10 nAChR, *p*CF3-diEPP was a partial agonist that induced about one third of the maximal peak currents stimulated by ACh with a concentration-response relationship resembling that of ACh. This result was unexpected because *p*CF3-diEPP is a poor inducer of ion-currents at α7 nAChR (Quadri et al., 2017).

*p*CF3-diEPP is a silent agonist that favors the desensitized state of α7 nAChRs and, hence, inhibits the ionotropic response to conventional nAChR agonists (Quadri et al., 2017). Here, we investigated if PC exerts similar functions at heterologously expressed human α7, α9 or α9α10 nAChRs. PC had essentially no effect on ACh-induced ion-currents at α7 nAChRs, but it slightly reduced ion-current changes provoked by pulses of ACh on oocytes expressing α9 or α9α10 nAChRs. These mild effects seem to be in contrast to the known strong PC-mediated inhibition of the ionotropic responses to choline (Richter et al., 2016). However, in these previous experiments choline was used instead of ACh (Richter et al., 2016) and the EC_{50} of choline at α9

or α9α10 nAChRs is considerably higher than that of ACh (McIntosh et al., 2009; Boffi et al., 2017; Moglie et al., 2021). PC and conventional nAChR agonists seem to compete for the ligand binding sites at α9 or α9α10 nAChRs and PC functions as a weak to moderate silent agonist or partial antagonist.

The same kind of experiments were performed with 30 μM *p*CF3-diEPP and its known silent agonist function at α7 nAChRs was confirmed. Interestingly, bath application of 30 μM *p*CF3-diEPP also fully inhibited ACh-gated ion-currents at heterologously expressed α9 and α9α10 nAChRs. At the lower concentration of 1 μM, *p*CF3-diEPP was still fully effective at suppressing the transient responses of α9 or α9α10 nAChRs to 60 μM ACh applications, whereas no effects were seen at α7 nAChRs. While being a moderate partial agonist at α9 or α9α10, *p*CF3-diEPP has a higher potential to desensitize these nAChRs compared to α7 nAChRs. It should also be noted that agonistic functions of *p*CF3-diEPP are not detected at concentrations of 1 μM and below, suggesting that *p*CF3-diEPP functions as a desensitizing agent at this low concentration. While the presence of PC or *p*CF3-diEPP in the bath suppressed ACh-evoked responses, upon washout of these compounds the ACh responses, most notably those of the α9/α10 nAChR expressing cells, recovered to levels higher than the initial ACh controls. This suggest that initial ACh responses may have been limited by resting desensitization and that the compounds had the additional effect of altering the equilibrium between pre-desensitized and activatable receptors.

This study has numerous limitations, and without a doubt further studies are needed to characterize the properties of *p*CF3-diEPP in more detail. Different cell types of murine and human origin were investigated, which is of advantage but also a limitation, because not all experiments were performed with all cell types. However, all cell types included in this study express nAChR subunits α7, α9 and α10 (Kawashima et al., 2007; Benfante et al., 2011; Hecker et al., 2015; St-Pierre et al., 2016; Fujii et al., 2017a). The n-number of experiments on BMDMs from gene-deficient animals is too low to draw firm conclusions regarding the regulation of LPS-induced secretion of TNF-α and IL-10, and more nAChR gene-deficient mouse strains need to be tested including those with a single deficiency in nAChR α10 and mice with a triple gene deficiency in nAChR α7α9α10. The concentrations of *p*CF3-diEPP used in this study were based on previous studies, where this compound was characterized as a silent agonist at α7 nAChRs (Quadri et al., 2017). The concentration-response relationship of the *p*CF3-diEPP-mediated control of IL-1β release by human PBMCs and the two-electrode voltage-clamp experiments, in which *p*CF3-diEPP was applied together with ACh in *Xenopus* oocytes, suggest that much lower concentrations of *p*CF3-diEPP are needed to activate metabotropic functions at α9 or α9α10 nAChRs or to desensitize these receptors. More careful analyses are needed to establish the concentration-response relationship for receptor desensitization. Further, more silent agonists of α7 nAChRs have been described (Horenstein and Papke, 2017; Quadri et al., 2017; Godin et al., 2020), which should be tested for their interaction with α9 and α9α10 nAChRs to identify and characterize ideal candidates for preclinical studies. Finally, experiments on LPS-induced cytokine release and BzATP-induced IL-1β release originate from two independent laboratories, which has the disadvantage that different experimental protocols and different murine and human cells were studied. Hence for both experimental settings, further monocytic phagocytes of diverse origin should be investigated. Finally, we did not test if *p*CF3-diEPP induces ion-currents at monocytic phagocytes.

Despite these limitations, *p*CF3-diEPP or related compounds seem to be promising future medicaments These findings raise the possibility of other approaches for the treatment of pain. Positive allosteric modulators (PAMs) that target α7 nAChRs have shown promise (Uteshev et al., 2002; Freitas et al., 2013) as analgesics. Developing PAMs that target receptors containing α9, and/or α10 nAChR subunits, may provide distinct advantages. Chronic use of agonists alone can have the undesired effect of disrupting the endogenous cholinergic tone either through ongoing receptor activation or desensitization. In contrast, PAMs can amplify the effect of endogenous agonists at the therapeutic site or potentiate the effect of exogenously administered agonists. Interestingly, m-bromo PEP, another synthetic silent nAChR agonists based on the diEPP scaffold, protects against EAE (Godin et al., 2020).

Another interesting application might be the prevention of life-threatening trauma-induced inflammation, either as an emergency treatment for accident victims or in the context of major surgery. Although IL-1β is a central pathogenic factor for life-threatening post-operative systemic inflammation that can cause sepsis, therapies targeting the IL-1 system were unsuccessful so far (Bortolotti et al., 2018), most probably because these therapies increase the risk for infectious complications. The *p*CF3-diEPP-mediated control of inflammasome-independent cytokines such as IL-6 or TNF-α seems to be mediated *via* α7 nAChRs and requires relatively high drug concentrations, whereas for the control of the ATP-induced release of IL-1β, the highly *p*CF3-diEPP-sensitive nAChR subunit α9 is mandatory. Low therapeutic concentrations of *p*CF3-diEPP might have the advantage of preventing trauma-induced sterile inflammation, while sparing most pathways that are involved in host-defense against infections.

In conclusion, we provide evidence that *p*CF3-diEPP is a potent agonist at unconventional nAChRs expressed by human monocytic cells. Activation of these receptors can inhibit both, the synthesis of pro-inflammatory cytokines in response to LPS and the inflammasome-dependent maturation and release of IL-1β triggered by P2X7 receptor activation. More research is needed to evaluate the potential of *p*CF3-diEPP to prevent trauma-induced inflammation, inflammatory or autoimmune diseases and neuropathic pain.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Laurentian University Research Ethics Board for Research Involving Human Participants (file number 6012214) and the Ethics Committee of the Medical Faculty Giessen, Giessen, Germany (No. 90/18). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Boys Town National Research Hospital Institutional Animal Care Committee, Omaha, NE, United States (protocol #16-04), the Laurentian University Animal Care Committee, Sudbury, ON, Canada (file number 6013816) and the University of Florida Institutional Animal Care and Use Committee, Gainesville, FL, United States (approval number 202002669). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

KR participated in the research design, performance of experiments, interpretation of the data, and writing of the manuscript. CS conducted the oocyte experiments, participated in the interpretation of the data, and writing of the manuscript. DCR, ESE, PMKW, and VKS participated in performance of the experiments, interpretation of the data, and editing of the manuscript. AH, JL, WP, K-DS, and MR participated in the research design, interpretation of the data, and editing of the manuscript. ARS, VG, RLP, NAH, JMM, and BJM participated in the research design, interpretation of the data, writing and revision of the manuscript. Additionally, RLP conducted all analyses of the oocyte data, prepared the related figures, and wrote the sections related to the oocyte experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2022.779081/full#supplementary-material

REFERENCES

Aickin, M., and Gensler, H. (1996). Adjusting for multiple testing when reporting research results: the bonferroni vs holm methods. *Am. J. Public. Health* 86, 726–728. doi: 10.2105/ajph.86.5.726

Beckmann, J., and Lips, K. S. (2013). The non-neuronal cholinergic system in health and disease. *Pharmacology* 92, 286–302. doi: 10.1159/000355835

Benfante, R., Antonini, R. A., Pizzol, M., Gotti, C., Clementi, F., Locati, M., et al. (2011). Expression of the α7 nAChR subunit duplicate form (CHRFAM7A) is down-regulated in the monocytic cell line THP-1 on treatment with LPS. *J. Neuroimmunol.* 230, 74–84. doi: 10.1016/j.jneuroim.2010.09.008

Boffi, J. C., Marcovich, I., Gill-Thind, J. K., Corradi, J., Collins, T., Lipovsek, M. M., et al. (2017). Differential contribution of subunit interfaces to α9α10 nicotinic acetylcholine receptor function. *Mol. Pharmacol.* 91, 250–262. doi: 10.1124/mol.116.107482

Borovikova, L. V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G. I., Watkins, L. R., et al. (2000). Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 405, 458–462. doi: 10.1038/35013070

Bortolotti, P., Faure, E., and Kipnis, E. (2018). Inflammasomes in tissue damages and immune disorders after trauma. *Front. Immunol.* 9:1900. doi: 10.3389/fimmu.2018.01900

Briggs, C. A., and McKenna, D. G. (1996). Effect of MK-801 at the human α7 nicotinic acetylcholine receptor. *Neuropharmacology* 35, 407–414. doi: 10.1016/0028-3908(96)00006-8

Broz, P., and Dixit, V. M. (2016). Inflammasomes: mechanism of assembly, regulation and signalling. *Nat. Rev. Immunol.* 16, 407–420. doi: 10.1038/nri.2016.58

Bruchfeld, A., Goldstein, R. S., Chavan, S., Patel, N. B., Rosas-Ballina, M., Kohn, N., et al. (2010). Whole blood cytokine attenuation by cholinergic agonists ex vivo and relationship to vagus nerve activity in rheumatoid arthritis. *J. Intern. Med.* 268, 94–101. doi: 10.1111/j.1365-2796.2010.02226.x

Cekic, C., and Linden, J. (2016). Purinergic regulation of the immune system. *Nat. Rev. Immunol.* 16, 177–192. doi: 10.1038/nri.2016.4

Christensen, S. B., Hone, A. J., Roux, I., Kniazeff, J., Pin, J.-P., Upert, G., et al. (2017). RgIA4 potently blocks mouse α9α10 nAChRs and provides long lasting protection against oxaliplatin-induced cold allodynia. *Front. Cell. Neurosci.* 11:219. doi: 10.3389/fncel.2017.00219

De Jonge, W. J., and Ulloa, L. (2007). The alpha7 nicotinic acetylcholine receptor as a pharmacological target for inflammation. *Br. J. Pharmacol.* 151, 915–929. doi: 10.1038/sj.bjp.0707264

Dinarello, C. A. (2018). Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol. Rev.* 281, 8–27. doi: 10.1111/imr.12621

Elgoyhen, A. B., Vetter, D. E., Katz, E., Rothlin, C. V., Heinemann, S. F., and Boulter, J. (2001). alpha10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3501–3506. doi: 10.1073/pnas.051622798

Freitas, K., Ghosh, S., Ivy Carroll, F., Lichtman, A. H., and Imad Damaj, M. (2013). Effects of α7 positive allosteric modulators in murine inflammatory and chronic neuropathic pain models. *Neuropharmacology* 65, 156–164. doi: 10.1016/j.neuropharm.2012.08.022

Fujii, T., Mashimo, M., Moriwaki, Y., Misawa, H., Ono, S., Horiguchi, K., et al. (2017a). Expression and function of the cholinergic system in immune cells. *Front Immunol* 8:1085. doi: 10.3389/fimmu.2017.01085

Fujii, T., Mashimo, M., Moriwaki, Y., Misawa, H., Ono, S., Horiguchi, K., et al. (2017b). Physiological functions of the cholinergic system in immune cells. *J. Pharmacol. Sci.* 134, 1–21. doi: 10.1016/j.jphs.2017.05.002

Gaidt, M. M., Ebert, T. S., Chauhan, D., Schmidt, T., Schmid-Burgk, J. L., Rapino, F., et al. (2016). Human monocytes engage an alternative inflammasome pathway. *Immunity* 44, 833–846. doi: 10.1016/j.immuni.2016.01.012

Galozzi, P., Bindoli, S., Doria, A., and Sfriso, P. (2021). The revisited role of interleukin-1 alpha and beta in autoimmune and inflammatory disorders and in comorbidities. *Autoimmun. Rev.* 20:102785. doi: 10.1016/j.autrev.2021.102785

Galvis, G., Lips, K. S., and Kummer, W. (2006). Expression of nicotinic acetylcholine receptors on murine alveolar macrophages. *JMN* 30:107. doi: 10.1385/JMN:30:1:107

Godin, J.-R., Roy, P., Quadri, M., Bagdas, D., Toma, W., Narendrula-Kotha, R., et al. (2020). A silent agonist of α7 nicotinic acetylcholine receptors modulates inflammation ex vivo and attenuates EAE. *Brain. Behav. Immun.* 87, 286–300. doi: 10.1016/j.bbi.2019.12.014

Gotti, C., Zoli, M., and Clementi, F. (2006). Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends Pharmacol. Sci.* 27, 482–491. doi: 10.1016/j.tips.2006.07.004

Grau, V., Richter, K., Hone, A. J., and McIntosh, J. M. (2018). Conopeptides V11L;V16DArIB and RgIA4: powerful tools for the identification of novel nicotinic acetylcholine receptors in monocytes. *Front. Pharmacol.* 9:1499. doi: 10.3389/fphar.2018.01499

Gross, O., Thomas, C. J., Guarda, G., and Tschopp, J. (2011). The inflammasome: an integrated view. *Immunol. Rev.* 243, 136–151. doi: 10.1111/j.1600-065X.2011.01046.x

Halevi, S., Yassin, L., Eshel, M., Sala, F., Sala, S., Criado, M., et al. (2003). Conservation within the RIC-3 gene family. effectors of mammalian nicotinic acetylcholine receptor expression. *J. Biol. Chem.* 278, 34411–34417. doi: 10.1074/jbc.m300170200

Hecker, A., Küllmar, M., Wilker, S., Richter, K., Zakrzewicz, A., Atanasova, S., et al. (2015). Phosphocholine-modified macromolecules and canonical nicotinic agonists inhibit ATP-induced IL-1β release. *J. Immunol.* 195, 2325–2334. doi: 10.4049/jimmunol.1400974

Hecker, A., Mikulski, Z., Lips, K. S., Pfeil, U., Zakrzewicz, A., Wilker, S., et al. (2009). Pivotal advance: up-regulation of acetylcholine synthesis and paracrine cholinergic signaling in intravascular transplant leukocytes during rejection of rat renal allografts. *J. Leukoc. Biol.* 86, 13–22. doi: 10.1189/jlb.1107722

Hone, A. J., Whiteaker, P., Christensen, S., Xiao, Y., Meyer, E. L., and McIntosh, J. M. (2009). A novel fluorescent alpha-conotoxin for the study of alpha7 nicotinic acetylcholine receptors. *J. Neurochem.* 111, 80–89. doi: 10.1111/j.1471-4159.2009.06299.x

Hone, A. J., Whiteaker, P., Mohn, J. L., Jacob, M. H., and McIntosh, J. M. (2010). Alexa fluor 546-ArIBV11L;V16A is a potent ligand for selectively labeling alpha 7 nicotinic acetylcholine receptors. *J. Neurochem.* 114, 994–1006. doi: 10.1111/j.1471-4159.2010.06819.x

Horenstein, N. A., and Papke, R. L. (2017). Anti-inflammatory silent agonists. *ACS Med. Chem. Lett.* 8, 989–991. doi: 10.1021/acsmedchemlett.7b00368

Huynh, P. N., Giuvelis, D., Christensen, S., Tucker, K. L., and McIntosh, J. M. (2019). RgIA4 accelerates recovery from paclitaxel-induced neuropathic pain in rats. *Mar. Drugs* 18:12. doi: 10.3390/md18010012

Innocent, N., Livingstone, P. D., Hone, A., Kimura, A., Young, T., Whiteaker, P., et al. (2008). Alpha-conotoxin arenatus IB[V11L,V16D] corrected is a potent and selective antagonist at rat and human native alpha7 nicotinic acetylcholine receptors. *J. Pharmacol. Exp. Ther.* 327, 529–537. doi: 10.1124/jpet.108.142943

Johnson, D. S., Martinez, J., Elgoyhen, A. B., Heinemann, S. F., and McIntosh, J. M. (1995). alpha-Conotoxin ImI exhibits subtype-specific nicotinic acetylcholine receptor blockade: preferential inhibition of homomeric alpha 7 and alpha 9 receptors. *Mol. Pharmacol.* 48, 194–199.

Katz, E., Verbitsky, M., Rothlin, C. V., Vetter, D. E., Heinemann, S. F., and Belén Elgoyhen, A. (2000). High calcium permeability and calcium block of the α9 nicotinic acetylcholine receptor. *Hear. Res.* 141, 117–128. doi: 10.1016/S0378-5955(99)00214-2

Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. *Nat. Immunol.* 11, 373–384. doi: 10.1038/ni.1863

Kawashima, K., and Fujii, T. (2003). The lymphocytic cholinergic system and its contribution to the regulation of immune activity. *Life Sci.* 74, 675–696. doi: 10.1016/j.lfs.2003.09.037

Kawashima, K., and Fujii, T. (2004). Expression of non-neuronal acetylcholine in lymphocytes and its contribution to the regulation of immune function. *Front. Biosci.* 9:2063–2085. doi: 10.2741/1390

Kawashima, K., Fujii, T., Moriwaki, Y., Misawa, H., and Horiguchi, K. (2015). Non-neuronal cholinergic system in regulation of immune function with a focus on α7 nAChRs. *Int. Immunopharmacol.* 29, 127–134. doi: 10.1016/j.intimp.2015.04.015

Kawashima, K., Yoshikawa, K., Fujii, Y. X., Moriwaki, Y., and Misawa, H. (2007). Expression and function of genes encoding cholinergic components in murine immune cells. *Life Sci.* 80, 2314–2319. doi: 10.1016/j.lfs.2007.02.036

Koval, L., Lykhmus, O., Zhmak, M., Khruschov, A., Tsetlin, V., Magrini, E., et al. (2011). Differential involvement of α4β2, α7 and α9α10 nicotinic acetylcholine receptors in B lymphocyte activation in vitro. *Int. J. Biochem. Cell Biol.* 43, 516–524. doi: 10.1016/j.biocel.2010.12.003

Kox, M., van Velzen, J. F., Pompe, J. C., Hoedemaekers, C. W., van der Hoeven, J. G., and Pickkers, P. (2009). GTS-21 inhibits pro-inflammatory cytokine release independent of the Toll-like receptor stimulated via a transcriptional mechanism involving JAK2 activation. *Biochem. Pharmacol.* 78, 863–872. doi: 10.1016/j.bcp.2009.06.096

Kummer, W., and Krasteva-Christ, G. (2014). Non-neuronal cholinergic airway epithelium biology. *Curr. Opin. Pharmacol.* 16, 43–49. doi: 10.1016/j.coph.2014.03.001

Lipovsek, M., Fierro, A., Pérez, E. G., Boffi, J. C., Millar, N. S., Fuchs, P. A., et al. (2014). Tracking the molecular evolution of calcium permeability in a nicotinic acetylcholine receptor. *Mol. Biol. Evol.* 31, 3250–3265. doi: 10.1093/molbev/msu258

Lustig, L. R., Peng, H., Hiel, H., Yamamoto, T., and Fuchs, P. A. (2001). Molecular cloning and mapping of the human nicotinic acetylcholine receptor alpha10 (CHRNA10). *Genomics* 73, 272–283. doi: 10.1006/geno.2000.6503

Manson, J., Thiemermann, C., and Brohi, K. (2012). Trauma alarmins as activators of damage-induced inflammation. *Br. J. Surg.* 99, 12–20. doi: 10.1002/bjs.7717

Mantovani, A., Dinarello, C. A., Molgora, M., and Garlanda, C. (2019). Interleukin-1 and related cytokines in the regulation of inflammation and immunity. *Immunity* 50, 778–795. doi: 10.1016/j.immuni.2019.03.012

McIntosh, J. M., Absalom, N., Chebib, M., Elgoyhen, A. B., and Vincler, M. (2009). Alpha9 nicotinic acetylcholine receptors and the treatment of pain. *Biochem. Pharmacol.* 78, 693–702. doi: 10.1016/j.bcp.2009.05.020

Mikulski, Z., Hartmann, P., Jositsch, G., Zasłona, Z., Lips, K. S., Pfeil, U., et al. (2010). Nicotinic receptors on rat alveolar macrophages dampen ATP-induced increase in cytosolic calcium concentration. *Respir. Res.* 11:133. doi: 10.1186/1465-9921-11-133

Moglie, M. J., Marcovich, I., Corradi, J., Carpaneto Freixas, A. E., Gallino, S., Plazas, P. V., et al. (2021). Loss of choline agonism in the inner ear hair cell nicotinic acetylcholine receptor linked to the α10 subunit. *Front. Mol. Neurosci.* 14:639720. doi: 10.3389/fnmol.2021.639720

Morley, B. J., Lysakowski, A., Vijayakumar, S., Menapace, D., and Jones, T. A. (2017). Nicotinic acetylcholine receptors regulate vestibular afferent gain and activation timing. *J. Comp. Neurol.* 525, 1216–1233. doi: 10.1002/cne.24131

Netea, M. G., Simon, A., van de Veerdonk, F., Kullberg, B.-J., van der Meer, J. W. M., and Joosten, L. A. B. (2010). IL-1beta processing in host defense: beyond the inflammasomes. *PLoS Pathog* 6:e1000661. doi: 10.1371/journal.ppat.1000661

Papke, R. L. (2014). Merging old and new perspectives on nicotinic acetylcholine receptors. *Biochem. Pharmacol.* 89, 1–11. doi: 10.1016/j.bcp.2014.01.029

Papke, R. L., and Porter Papke, J. K. (2002). Comparative pharmacology of rat and human alpha7 nachr conducted with net charge analysis. *Br. J. Pharmacol.* 137, 49–61. doi: 10.1038/sj.bjp.0704833

Papke, R. L., and Stokes, C. (2010). Working with opusxpress: methods for high volume oocyte experiments. *Methods* 51, 121–133. doi: 10.1016/j.ymeth.2010.01.012

Pavlov, V. A., Chavan, S. S., and Tracey, K. J. (2018). Molecular and functional neuroscience in immunity. *Annu. Rev. Immunol.* 36, 783–812. doi: 10.1146/annurev-immunol-042617-053158

Pavlov, V. A., Ochani, M., Yang, L.-H., Gallowitsch-Puerta, M., Ochani, K., Lin, X., et al. (2007). Selective alpha7-nicotinic acetylcholine receptor agonist GTS-21

improves survival in murine endotoxemia and severe sepsis. *Crit. Care Med.* 35, 1139–1144. doi: 10.1097/01.CCM.0000259381.56526.96

Peng, H., Ferris, R. L., Matthews, T., Hiel, H., Lopez-Albaitero, A., and Lustig, L. R. (2004). Characterization of the human nicotinic acetylcholine receptor subunit alpha (alpha) 9 (CHRNA9) and alpha (alpha) 10 (CHRNA10) in lymphocytes. *Life Sci.* 76, 263–280. doi: 10.1016/j.lfs.2004.05.031

Quadri, M., Matera, C., Silnović, A., Pismataro, M. C., Horenstein, N. A., Stokes, C., et al. (2017). Identification of α7 nicotinic acetylcholine receptor silent agonists based on the spirocyclic quinuclidine-Δ2 -isoxazoline scaffold: synthesis and electrophysiological evaluation. *Chem. Med. Chem.* 12, 1335–1348. doi: 10.1002/cmdc.201700162

Rathinam, V. A. K., and Fitzgerald, K. A. (2016). Inflammasome complexes: emerging mechanisms and effector functions. *Cell* 165, 792–800. doi: 10.1016/j.cell.2016.03.046

Rathinam, V. A. K., Vanaja, S. K., and Fitzgerald, K. A. (2012). Regulation of inflammasome signaling. *Nat. Immunol.* 13, 333–342. doi: 10.1038/ni.2237

Razani-Boroujerdi, S., Boyd, R. T., Davila-Garcia, M. I., Nandi, J. S., Mishra, N. C., Singh, S. P., et al. (2007). T cells express 7-nicotinic acetylcholine receptor subunits that require a functional TCR and leukocyte-specific protein tyrosine kinase for nicotine-induced Ca^{2+} response. *J. Immunol.* 179, 2889–2898. doi: 10.4049/jimmunol.179.5.2889

Richter, K., Mathes, V., Fronius, M., Althaus, M., Hecker, A., Krasteva-Christ, G., et al. (2016). Phosphocholine – an agonist of metabotropic but not of ionotropic functions of α9-containing nicotinic acetylcholine receptors. *Sci. Rep.* 6:28660. doi: 10.1038/srep28660

Richter, K., Ogiemwonyi-Schaefer, R., Wilker, S., Chaveiro, A. I., Agné, A., Hecker, M., et al. (2020). Amyloid beta peptide (Aβ1-42) reverses the cholinergic control of monocytic IL-1β release. *J. Clin. Med.* 9:2887. doi: 10.3390/jcm9092887

Richter, K., Sagawe, S., Hecker, A., Küllmar, M., Askevold, I., Damm, J., et al. (2018). C-reactive protein stimulates nicotinic acetylcholine receptors to control ATP-mediated monocytic inflammasome activation. *Front. Immunol.* 9:1604. doi: 10.3389/fimmu.2018.01604

Romero, H. K., Christensen, S. B., Mannelli, L., Gajewiak, J., Ramachandra, R., Elmslie, K. S., et al. (2017). Inhibition of α9α10 nicotinic acetylcholine receptors prevents chemotherapy-induced neuropathic pain. *Proc. Natl. Acad. Sci. U.S.A.* 114, E1825–E1832. doi: 10.1073/pnas.1621433114

Sgard, F., Charpantier, E., Bertrand, S., Walker, N., Caput, D., Graham, D., et al. (2002). A novel human nicotinic receptor subunit, alpha10, that confers functionality to the alpha9-subunit. *Mol. Pharmacol.* 61, 150–159. doi: 10.1124/mol.61.1.150

Simard, A. R., Gan, Y., St-Pierre, S., Kousari, A., Patel, V., Whiteaker, P., et al. (2013). Differential modulation of EAE by α9*- and β2*-nicotinic acetylcholine receptors. *Immunol. Cell Biol.* 91, 195–200. doi: 10.1038/icb.2013.1

Skok, V. I. (2002). Nicotinic acetylcholine receptors in autonomic ganglia. *Auton. Neurosci.* 97, 1–11. doi: 10.1016/s1566-0702(01)00386-1

St-Pierre, S., Jiang, W., Roy, P., Champigny, C., LeBlanc, É, Morley, B. J., et al. (2016). Nicotinic acetylcholine receptors modulate bone marrow-derived pro-inflammatory monocyte production and survival. *PLoS One* 11:e0150230. doi: 10.1371/journal.pone.0150230

Tracey, K. J. (2009). Reflex control of immunity. *Nat. Rev. Immunol.* 9, 418–428. doi: 10.1038/nri2566

Uteshev, V. V., Meyer, E. M., and Papke, R. L. (2002). Activation and inhibition of native neuronal alpha-bungarotoxin-sensitive nicotinic ach receptors. *Brain Res.* 948, 33–46. doi: 10.1016/s0006-8993(02)02946-3

Valbuena, S., and Lerma, J. (2016). Non-canonical signaling, the hidden life of ligand-gated ion channels. *Neuron* 92, 316–329. doi: 10.1016/j.neuron.2016.10.016

Verbitsky, M., Rothlin, C. V., Katz, E., and Elgoyhen, A. B. (2000). Mixed nicotinic-muscarinic properties of the alpha9 nicotinic cholinergic receptor. *Neuropharmacology* 39, 2515–2524. doi: 10.1016/s0028-3908(00)00124-6

Wang, H., Liao, H., Ochani, M., Justiniani, M., Lin, X., Yang, L., et al. (2004). Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. *Nat. Med.* 10, 1216–1221. doi: 10.1038/nm1124

Wessler, I., and Kirkpatrick, C. J. (2008). Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. *Br. J. Pharmacol.* 154, 1558–1571. doi: 10.1038/bjp.2008.185

Whiteaker, P., Marks, M. J., Christensen, S., Dowell, C., Collins, A. C., and McIntosh, J. M. (2008). Synthesis and characterization of 125I-alpha-conotoxin ArIBV11L;V16A, a selective alpha7 nicotinic acetylcholine receptor antagonist. *J. Pharmacol. Exp. Ther.* 325, 910–919. doi: 10.1124/jpet.108.136895

Yue, Y., Liu, R., Cheng, W., Hu, Y., Li, J., Pan, X., et al. (2015). GTS-21 attenuates lipopolysaccharide-induced inflammatory cytokine production in vitro by modulating the Akt and NF-κB signaling pathway through the α7 nicotinic acetylcholine receptor. *Int. Immunopharmacol.* 29, 504–512. doi: 10.1016/j.intimp.2015.10.005

Zakrzewicz, A., Richter, K., Agné, A., Wilker, S., Siebers, K., Fink, B., et al. (2017). Canonical and novel non-canonical cholinergic agonists inhibit ATP-induced release of monocytic interleukin-1β via different combinations of nicotinic acetylcholine receptor subunits α7, α9 and α10. *Front. Cell. Neurosci.* 11:491. doi: 10.3389/fncel.2017.00189

Zheng, N., Christensen, S. B., Blakely, A., Dowell, C., Purushottam, L., McIntosh, J. M., et al. (2020). Development of conformationally constrained α-RgIA analogues as stable peptide antagonists of human α9α10 nicotinic acetylcholine receptors. *J. Med. Chem.* 63, 8380–8387. doi: 10.1021/acs.jmedchem.0c00613

Zheng, N., Christensen, S. B., Dowell, C., Purushottam, L., Skalicky, J. J., McIntosh, J. M., et al. (2021). Discovery of methylene thioacetal-incorporated α-RgIA analogues as potent and stable antagonists of the human α9α10 nicotinic acetylcholine receptor for the treatment of neuropathic pain. *J. Med. Chem.* 64, 9513–9524. doi: 10.1021/acs.jmedchem.1c00802

Conflict of Interest: *p*CF3-diEPP is the subject of a United States provisional patent application, filed in the names of the University of Florida, the University of Giessen, and the Northern Ontario School of Medicine. RLP, CS, NAH, VG, KR, and ARS are listed as inventors on that patent. Certain conopeptides, including RgIA4, have been patented by the University of Utah; JMM is an inventor on these patents.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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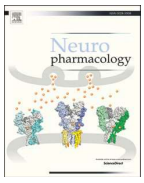

Pharmacological profiles and anti-inflammatory activity of *p*CN-diEPP and *m*CN-diEPP, new alpha9alpha10 nicotinic receptor ligands

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ABSTRACT

Pain due to inflammation can be reduced by targeting the noncanonical nicotinic receptors (NCNR) in cells of the immune system that regulate the synthesis and release of pro- and anti-inflammatory cytokines. Although NCNR do not generate ion channel currents, the pharmacology of ion-channel forms of the receptors can predict drugs which may be effective regulators of the cholinergic anti-inflammatory system (CAS). Agonists of α7 type receptors have been definitively associated with CAS. Receptors containing α9 and α10 subunits have also been implicated. We have recently characterized two small molecules, *p*CN-diEPP and *m*CN-diEPP, as selective α9α10 agonists and antagonists, respectively. We used these drugs, along with nicotine, an α7 agonist and α9α10 antagonist, to probe the mixed populations of receptors that are formed when α7, α9, and α10 are all expressed together in *Xenopus* oocytes. We also evaluated the effects of the CN-diEPP compounds on regulating the ATP-induced release of interleukin-1β from monocytic THP-1 cells, which express NCNR. The compounds successfully identified separate populations of receptors when all three subunits were co-expressed, including a potential population of homomeric α10 receptors. The α9α10 agonist *p*CN-diEPP was the more effective regulator of interleukin-1β release in THP-1 cells. *p*CN-diEPP was also fully effective in a mouse model of inflammatory pain, while *m*CN-diEPP had only partial effects, requiring a higher dosage. The analgetic effects of *p*CN-diEPP and *m*CN-diEPP were retained in α7 knockout mice. Taken together, our results suggest that drugs that selectively activate α9α10 receptors may useful to reduce inflammatory pain through the CAS.

1. Introduction

In light of the present opiate epidemic, which has been precipitated by the widespread use of prescription opiates as pain killers, there is an acute need to develop alternative non-addicting analgetic therapies. The discovery that cells of the immune system have nicotinic acetylcholine receptors (nAChRs) that can be stimulated to decrease the release of pro-inflammatory cytokines (Borovikova et al., 2000) has generated a great deal of interest in the cholinergic anti-inflammatory systems (CAS) as new avenues for treating pain associated with inflammation (Hone and McIntosh, 2023). Many studies have demonstrated the essential role played by homomeric α7 nAChR in CAS (Bencherif et al., 2011; Fujii et al., 2017; Piovesana et al., 2021; Treinin et al., 2017) and, more recently, by α9* nAChR (the asterisk denotes α9 homomeric receptors or, more often, α9 co-assembled with α10 subunits), which have been have identified as alternative targets in CAS (AlSharari et al., 2020; Hone et al., 2018). Although only partially understood, numerous downstream signaling mechanisms are activated in response to nAChR stimulation of immune cells (for review (Richter and Grau, 2023)). On the one hand, stimulation of mainly α7 nAChRs down-regulates the expression of pro-inflammatory mediators on the transcriptional and translational level (for review (Richter and Grau, 2023)). On the other hand, stimulation of nAChRs containing subunits α9, α7, and/or α10 inhibits the function of the ATP-sensitive P2X7 receptor and down-regulates the maturation and release of inflammasome-dependent cytokines including interleukin (IL)-1β (Hecker et al., 2015; Richter et al., 2016; Zakrzewicz et al., 2017). Of note, the P2X7 receptor and IL-1β play an important role in pain (Ren and Illes, 2022).

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Although rapidly desensitizing and with low probability of channel opening (Papke and Horenstein, 2021), α-bungarotoxin-sensitive α7 nAChRs (Amar et al., 1993) have been widely studied as modulators of brain function and considered as potential targets for disorders of the central nervous system such as Alzheimer's disease (Bouzat et al., 2018; Russo et al., 2014) and schizophrenia (Terry and Callahan, 2020). The first functional roles identified for α9* nAChRs were as regulators of auditory function (Elgoyhen et al., 2001; Vetter et al., 1999). Both α7 and α9* nAChRs form functional ligand-gated ion channels in heterologous expression systems such as *Xenopus* oocytes, which has allowed for extensive characterization of their pharmacological properties, especially in the case of α7 nAChRs (Papke and Horenstein, 2021). Early studies of α-bungarotoxin-sensitive α9* nAChRs highlighted several unusual pharmacological features, including the fact that nicotine, the eponymous agonist for the receptor family, was an antagonist rather than an agonist (Verbitsky et al., 2000). One of the most selective ligands of α9* nAChRs is the conopeptide antagonist alpha-RgIA (Azam and McIntosh, 2012), although recently other small molecule antagonists have been proposed to be α9 selective (Bavo et al., 2022).

Consistent with their characterization in heterologous expression systems, α7 and α9* nAChR have been shown to mediate electrical signaling in the brain (Frazier et al., 1998) and inner ear (Fuchs, 1996), respectively. Additionally, the expression of α9 and α10 has been reported in dorsal root sensory ganglia (Lips et al., 2002). However, whether the expression of either α7 or α9* in cells of the immune system that mediate CAS is associated with ion channel activation is highly questioned. These receptors have, rather, been proposed to function as metabotropic receptors (Richter and Grau, 2023) and therefore in those tissues may be considered "noncanonical nicotinic receptors" (NCNR). The mechanism accounting for this different form of receptor function is unknown, and while there is, in general, a concurrence between the pharmacology established with heterologous expression systems and ligands modulating CAS, there are also remarkable differences, such that ligands that produce little or no channel activation have been shown to be able to mediate CAS signaling (Papke et al., 2023; Piovesana et al., 2021): NS6740 in the case of α7 nAChRs (Papke et al., 2015; Thomsen and Mikkelsen, 2012) and phosphocholine in the case of α9* nAChRs (Richter et al., 2016). The conformational dynamics of ligand-gated ion channels involves the induction of both conducting and non-conducting (i.e. desensitized) states. Even the most efficacious of α7 agonists more effectively induce desensitization than channel activation (Papke and Lindstrom, 2020), and some ligands promote desensitization without any channel activation. Such ligands have been referred to as "silent agonists", and their ability to induce desensitized conformations can be confirmed through the use of type II positive allosteric modulators such as PNU-120596 that can reactivate the desensitized receptors (Papke et al., 2023). Numerous silent agonists have been shown to be effective modulators of CAS, and it has been proposed that for the non-conducting forms of the receptors in immune cells, the activation of a metabotropic pathway is associated with non-conducting conformations corresponding to the desensitized states of channel-forming receptors (Horenstein and Papke, 2017; Papke et al., 2015). Based on the metabotropic activity of ligands like phosphocholine, it has been suggested that similar mechanisms account for CAS function of α9* NCNR (Zakrzewicz et al., 2017).

A recent study compared the metabotropic activity of the weak α7 partial agonist *p*CF3-diEPP (Quadri et al., 2018) and phosphocholine in various immune cell preparations (Richter et al., 2022). It was found that the activity of *p*CF3-diEPP could be blocked by either the α7-specific conopeptide [V11L,V16D]ArIB (Whiteaker et al., 2008) or by the α9α10 nAChR-specific conopeptide RgIA4 (Christensen et al., 2017). Consistent with this, it was shown that, in addition to being an weak α7 partial agonist, *p*CF3-diEPP was a more efficacious partial agonist of α9 nAChRs. This inspired a follow-up study of a family of 1,1-diethyl-4-phenylpiperazin-1-ium compounds (diEPPs) that were previously studied for their α7 silent agonist activity (Quadri et al., 2016), which identified several α9-selective agonists and antagonists (Papke et al., 2022).

While α7-mediated CAS activity is associated with receptor activation and/or desensitization (Papke et al., 2023), studies with α9 conopeptide antagonists (AlSharari et al., 2020; Christensen et al., 2017; Huynh et al., 2019; Pacini et al., 2016; Romero et al., 2017) suggested that α9 CAS activity would arise from α9 inhibition *in vivo* (Toma et al., 2020). However, this was inconsistent with the effect of the conopeptide in the cell-based assays, which suggested α9 nAChR agonism was the basis for the CAS effects.

In this paper, we address this controversy by using two closely related compounds, the α9 nAChR agonist *p*CN-diEPP and the α9 nAChR antagonist *m*CN-diEPP (Papke et al., 2022), characterizing their activity with mixed populations of α7 and α9α10 nAChRs in *Xenopus* oocytes, with human monocytic THP-1 cells, and with an animal model of inflammatory pain using both wild-type (WT) and α7-knockout (KO) mice.

Note that, in general, heterologous expression of nAChR takes advantage of the fact that each receptor subtype can be studied in isolation. However, in the *in vivo* and cell-based assays, both α7 and α9α10 nAChRs may be present in the same cells. Therefore, some experiments were conducted in oocytes expressing α7 along with α9 and α10 to validate the usefulness of our probe compounds with mixed receptor populations.

2. Methods

2.1. pCN-diEPP and mCN-diEPP

4-(4-cyanophenyl)-1,1-diethylpiperazin-1-ium (*p*CN-diEPP) and 4-(3-cyanophenyl)-1,1-diethylpiperazin-1-ium (*m*CN-diEPP) were synthesized as previously documented (Quadri et al., 2016). For oocyte experiments 100 mM stock solutions were prepared in dimethyl sulfoxide and stored in −20°, then freshly diluted in Ringer's solution.

2.2. Expression of human nAChR subunits in Xenopus laevis oocytes

Plasmid DNA encoding the human α7 nAChR was obtained from Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). The human resistance-to-cholinesterase 3 (RIC3) clone was obtained from Millet Treinin (Hebrew University, Jerusalem, Israel) and RNA co-injected with α7 to improve the level and speed of receptor expression without affecting the pharmacological properties (Halevi et al., 2003). Plasmid DNA encoding the human α10 nAChR was obtained from J. Michael McIntosh (University of Utah, Salt Lake City, UT). Plasmid DNA encoding the human α9 nAChR and the human receptor-associated protein of the synapse (RAPSYN) with codon optimization for expression in *Xenopus laevis* were obtained from Eurofins Genomics (Ebersberg, Germany). RAPSYN RNA was co-injected with the α9 and α10 to improve expression (Richter et al., 2016). After linearization and purification of the plasmid DNAs, RNAs were prepared using the mMessage mMachine *in vitro* RNA transcription kit (Ambion, Austin, TX).

Frogs were maintained in the Animal Care Service facility of the University of Florida, and all procedures were approved by the University of Florida Institutional Animal Care and Use Committee (approval number 202002669). In brief, the animals were first anesthetized for 15–20 min in 1.5 l frog tank water containing 1 g of MS-222 buffered with sodium bicarbonate. Oocytes were obtained surgically from mature female *Xenopus laevis* (Nasco, Ft. Atkinson, WI) and treated with 1.4 mg/ml type 1 collagenase (Worthington Biochemicals, Freehold, NJ, USA) for 2–4 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 15 mM HEPES, and 12 mg/l tetracycline, pH 7.6) to remove the ovarian tissue and the follicular layers. Stage V oocytes were injected with 4–6 ng α7 RNA and 2–3 ng RIC3 RNA (2:1 ratio) in 50 nl water, or with 12 ng α9 RNA, or along with 12 ng α10 RNA, and 3 ng RAPSYN RNA in 50 nl water. Oocytes were maintained in Barth's solution containing additionally 0.32 mM $Ca(NO_3)_2$ and 0.41 mM $CaCl_2$, and recordings were

carried out 2–20 days after injection.

In order to obtain mixed populations of α7 and α9α10 receptors, RNA for each nAChR subunit and their supporting chaperone proteins (RIC3 for α7 and RAPSYN for α9* receptors) were injected at varying ratios. Since α7 expression is consistently more robust, it was necessary to inject α9 and α10 at higher levels. The injections were as indicated in Table 2.

2.3. Two-electrode voltage-clamp electrophysiology

Two-electrode voltage-clamp experiments were conducted using OpusXpress 6000A (Molecular Devices, Union City CA, USA) (Papke and Smith-Maxwell, 2009). Both the voltage and current electrodes were filled with 3 M KCl. Oocytes were voltage-clamped at −60 mV at RT. The oocytes were perfused with Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, 1 μM atropine, pH 7.2) at 2 ml/min. NaCl, KCl, $CaCl_2$, and HEPES came from Fisher Scientific (Waltham MA, USA), and unless otherwise noted, all other salts and chemicals were obtained from Sigma-Aldrich (St. Louis MO, USA). To evaluate the effects of experimental compounds, responses were compared to control ACh-evoked responses, defined as the average of two initial applications of 60 μM ACh made before test applications. Drug applications were 12 s in duration, followed by 181 s washout periods.

Each episode of data acquisition was a total of 210 s and included an initial 30 s period used to define the baseline for the drug-evoked responses. After 30 s, drugs were applied, and the following 120 s were defined as the drug response period for analysis. The responses were calculated as both peak-current amplitudes and net charge, as previously described (Papke and Papke, 2002). Data were collected at 50 Hz, filtered at 20 Hz, and analyzed by Clampfit (Molecular Devices) and Excel (Microsoft, Redmond WA, USA). Data for concentration-response studies were expressed as averages ± SEM from at least five oocytes for each experiment and plotted with Kaleidagraph 4.5.2 (Synergy Software, Reading PA, USA). The values for the curve fits were generated using the Levenberg-Marquardt algorithm to obtain the best Chi-Square fit to the Hill equation using the Kaleidagraph 4.5.2 plotting program. The errors in Table 1 are the calculated standard errors of the fit parameters based on the goodness of fit.

Multi-cell averages were calculated for comparisons of complex responses. Averages of the normalized data were calculated for each of the 10,322 points in each of the 206.44 s traces (acquired at 50 Hz), as well as the standard errors for those averages. All other data are plotted as the individual replicates normalized to the ACh control responses obtained from the same cells. ANOVA was conducted in Kaleidagraph 4.5.2.

Table 1
Curve fit parameters from Fig. 1.

*p*CN-diEPP activation of α9α10				
I_{max}	*n*	EC_{50}	ChiSq	R
0.94 ± 0.06	1.07 ± 0.2	2.57 ± 0.57 μM	0.012	0.993
*m*CN-diEPP inhibition of α9α10				
n	IC_{50}		ChiSq	R
−1.41 ± 0.16	17.1 ± 1.54 μM		0.0083	0.996
Nicotine activation of α7				
I_{max}	*n*	EC_{50}	ChiSq	R
0.85 ± 0.04	1.45 ± 0.34	14.9 ± 2.8 μM	0.018	0.991
Nicotine inhibition of α9α10				
n	IC_{50}		ChiSq	R
−1.98 ± 0.53	437 ± 73 μM		0.044	0.982

Table 2
RNA values in ratio experiments.

	α7	RIC3	α9	α10	RAPSYN
α7 alone	6 ng	3 ng			
α7, α9α10 1:2	4 ng	2 ng	8 ng	8 ng	2 ng
α7, α9α10 1:3	3.6 ng	1.8 ng	10.7 ng	10.7 ng	2.5 ng
α7, α9α10 1:4	2.48 ng	1.24 ng	12 ng	12 ng	2.7 ng
α9α10 alone			12 ng	12 ng	3.0 ng

2.4. Monocytic THP-1 cells

Monocytic THP-1 cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were cultured in RPMI 1640 medium (Capricorn Ebsdorfergrund, Germany, Cat# RPMI-A) supplemented with 10% FBS from Capricorn (Cat# FBS-16A) under 5% CO_2 atmosphere at 37 °C. To investigate IL-1β release, monocytic cells were resuspended in FBS-free RPMI medium. 0.5×10^6 cells/0.5 ml per well were seeded in 48-well plates (Greiner Bio-One, Frickenhausen, Germany). Cells were primed for 5 h with 1 μg/ml LPS (*E. coli* O26:B6, Merck, Darmstadt, Germany, Cat# L2654) as described previously (Richter et al., 2022). Then the P2X7 receptor agonist BzATP (2′(3′)-O-(4-benzoyl-benzoyl)ATP trieethylammonium salt; Jena Bioscience, Jena, Germany, Cat# NU-1620-5) was added for 40 min in the absence or the presence of different concentrations of cholinergic agonists: ACh (Merck, Cat# A6625), phosphocholine (Merck, Cat# P0378), *p*CN-diEPP, or *m*CN-diEPP. After treatment, cells were spun down (500 g, 8 min, 4 °C) and the supernatants were collected and stored at −20 °C for later IL-1β and lactate dehydrogenase (LDH) measurements.

IL-1β concentrations in cell-free supernatants obtained from experiments on THP-1 cells were measured using the Human IL-1β/IL-1F2 DuoSet enzyme-linked immunosorbent assay (ELISA) from R&D Systems (Minneapolis MN, USA, Cat# DY201) according to the supplier's instructions. To test for cell viability at the end of the cell culture experiments, LDH activity was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison WI, USA; Cat# G1780) according to the supplier's instructions. LDH activities determined in cell-free supernatants are given as percentage of the total LDH activity of lysed control cells.

2.5. Statistical analysis of cytokine data

Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, La Jolla CA, USA). Results obtained in the cell-based assays on BzATP-induced cytokine release were analyzed using SPSS (Version 23, IBM, Armonk, NY, United States) visualized using Inkscape version 0.48.5 r10040 (Free and Open Source Software licensed under the GPL).

The n-numbers (≤6) in the experiments with were too small to determine a normal distribution with certainty. Therefore, non-paramateric test were used. Dependent data sets were analyzed first by the Friedman test. When the P value was below 0.05, the two-tailed Wilcoxon signed-rank test was performed for pairwise comparison.

All numbers (n) of the individual experiments and details on the type of statistical test used for each experiment are indicated in the Results section and Figure legends. No outliers were excluded from the analyses.

2.6. Measurements of inflammatory pain

2.6.1. Animals

Experiments were conducted using adult (10–15 weeks) male and female C57BL/6J mice from Jackson Laboratory (Bar Harbor ME, USA): α7 WT and KO mice on a C57BL/6J background. Mice null for the α7 subunit along with their WT littermates were initially procured from Jackson Laboratory and later bred in an approved animal care facility at

Virginia Commonwealth University. The breeding scheme involved crossing heterozygous mice and backcrossing progeny for at least 12 to 15 generations, to control for irregularities that might occur crossing solely mutant animals, to generate both mutant and WT animals. Then mice were weaned at 21 days of age and subsequently housed in groups of two to five with Teklad corn cob bedding (#7097, Envigo Teklad, Madison WI, USA). Initially, they were maintained in a temperature- and humidity-controlled vivarium space (21 ± 3 °C, 55 ± 10%) on a 12-h light/dark cycle (lights on at 7:00 a.m.) with free access to food (Teklad LM-485 mouse sterilized diet, Harlan Laboratories Inc., Indianapolis IN, USA) and water until needed. Then mice were retrieved from the vivarium and housed (4–5 mice per cage) for the duration of the study in a temperature- and humidity-controlled out-of-vivarium space on the same light/dark cycle. They were given ad libitum food and water. All experiments were performed during the light cycle. This study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University (approval # AM10142) and carried out in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. In total we used 66 male C57BL/6J mice and 63 female C57BL/6J mice, 24 male KO mice and 24 female KO mice. All experimental animals were included in further behavioral testing and none of them showed behavioral disturbances unrelated to the pain induction procedure.

2.6.2. Induction of inflammatory pain by complete Freund's adjuvant (CFA)

We explored the effects of *p*CN-diEPP and *m*CN-diEPP in the CFA test, composed of inactivated and dried *Mycobacterium tuberculosis* and adjuvant, a widely used model of persistent inflammatory pain (Kaliyaperumal et al., 2020). CFA was purchased from Sigma-Aldrich (St. Louis MO, USA). The CFA model is based on hypersensitivity, paw swelling, and nuclear factor-κB–mediated transcription of tumor necrosis factor α involved in the formation of the principal mediators of inflammation (Hartung et al., 2015). Mice were injected intraplantarly with 20 μl of CFA (50%, diluted in mineral oil; Sigma-Aldrich). Mechanical sensitivity (see the measurement of the von Frey test) was measured before and 3 days after CFA injection. *p*CN-diEPP (0.1, 0.3, and 1 mg/kg) and *m*CN-diEPP (0.6, 1, and 3 mg/kg), dissolved in a mixture of 1:1:18 [1 vol ethanol/1 vol Emulphor-620 (Rhone-Poulenc, Inc., Princeton NJ, USA)/18 vol distilled water] or vehicle was injected intraperitoneally (i.p.) on day 3 after CFA injection, and mice were tested for mechanical sensitivity at different time points (1, 3, and 6 h) after drug injection.

2.6.3. Evaluation of mechanical sensitivity

Mechanical sensitivity thresholds were determined according to the method of Chaplan et al. (1994) and as adapted in Bagdas et al. (2015); Bagdas et al. (2015). A series of calibrated von Frey filaments (Stoelting, Wood Dale IL, USA) with logarithmically incremental stiffness ranging from 2.83 to 5.07 expressed as diameter sensitivity (ds) log 10 of 10 x force (in milligrams) was applied to the paw with a modified up-down method (Dixon, 1965). The mechanical threshold was expressed as log 10 of 10 x force (in milligrams), indicating the force of the von Frey hair to which the animal reacted (paw withdrawn, licking, or shaking). All behavioral testing on animals was performed in a blinded manner.

The data obtained were discrete values that were not normally distributed and, hence, not suitable for parametric analysis. The data were therefore first evaluated with the non-parametric Kruskal–Wallis test, a one-way analysis of variance by ranks. A significant Kruskal–Wallis test indicated that at least one sample stochastically dominated the other samples, in this case, the baseline data for all groups. For analyzing the specific sample pairs at the different time points for stochastic dominance, as a second step the groups were tested pairwise with the Mann–Whitney test. The P values calculated by the Mann-Whitney test are provided in the tables or shown in the figures.

2.6.4. Locomotor activity

Mice were placed into individual Omnitech photocell activity cages (28 × 16.5 cm) (Columbus OH, USA) 1 h after administration of either vehicle, *p*CN-diEPP (1 mg/kg, i.p.), or *m*CN-diEPP (3 mg/kg, i.p.). Interruptions of the photocell beams (two banks of eight cells each) were then recorded for the next 60 min. Data are expressed as number of photocell interruptions.

2.6.5. Body temperature

Rectal temperature was measured by a thermistor probe (inserted 24 mm) and digital thermometer (Yellow Springs Instrument Co., Yellow Springs OH, USA). Readings were taken just before and at 60 min after the injection of either vehicle, *p*CN-diEPP (1 mg/kg, i.p.), or *m*CN-diEPP (3 mg/kg, i.p.) (n = 8/group). The difference in rectal temperature before and after treatment was calculated for each mouse. The ambient temperature of the laboratory varied from 21 to 24 °C from day to day.

3. Results and discussion

3.1. Characterization of probe compounds for α7 and α9α10 receptors

We evaluated several compounds for their ability to distinguish α7 from α9α10 nAChR in anticipation of doing experiments with mixed populations of receptors. We used agonists and antagonists that were selective for one nAChR population or another.

3.2. Position of the cyano group determines the activity of CN-diEPPcompounds

*p*CN-diEPP is a partial agonist for α7 nAChRs that produced substantial PNU-120596-sensitive desensitization (Quadri et al., 2016), while *m*CN-diEPP produced no activation and only modest inhibition of α7 nAChR (for structures, see Fig. 1). When characterized on α9 homomeric nAChRs, *p*CN-diEPP was shown to be a full agonist with an EC_{50} of 0.368 ± 0.10 μM, while *m*CN-diEPP was an antagonist with an IC_{50} of 21 ± 2.71 μM (Papke et al., 2022). Similar results were obtained with heteromeric α9α10 receptors (Fig. 1A). *p*CN-diEPP was a very efficiacious agonist with an I_{max} 0.94 ± 0.06 that of ACh and an EC_{50} of 2.57 ± 0.57 μM, while *m*CN-diEPP antagonized the ionotropic responses of α9α10 nAChR to ACh (60 μM) with an IC_{50} of 17.1 ± 1.57 μM (Papke et al., 2022). Curve fit parameters and error estimates are provided in Table 1, and plots of the individual replicates are provided in Supplemental Fig. 1A.

3.3. Nicotine as a probe to distinguish α7 from α9α10 receptors

Nicotine is an efficacious agonist of α7 nAChR (Papke et al., 2007), yet it has been reported to be an antagonist of α9 nAChR ion channel function (Verbitsky et al., 2000), so that nicotine responses may be useful to separate components of mixed receptor populations (Fig. 1B). The I_{max} of nicotine for α7 net-charge response was 85 ± 4% that of ACh, with an EC_{50} of 14.9 ± 2.8 μM. The IC_{50} for the inhibition of α9α10 peak current responses was 437 ± 73 μM (Fig. 1B). The IC_{50} of nicotine at α9α10 nAChR heteromers seems to be high compared to the published IC_{50} of 32 μM at α9 homomers (Verbitsky et al., 2000). However, the previous study used ACh at a concentration of 10 μM, while we used 60 μM ACh for stimulation, which may account at least in part for the different IC_{50} values. Alternatively, it may be that α9 homomers are more sensitive to inhibition by nicotine than are α9α10 heteromers. Curve fit parameters and error estimates are provided in Table 1, and plots of the individual replicates are provided in Supplemental Fig. 1B.

3.4. Probing mixed populations of receptors

While there are likely to be factors *in vivo* that regulate the subunit composition of nAChR formed in specific cells, expression in *Xenopus*

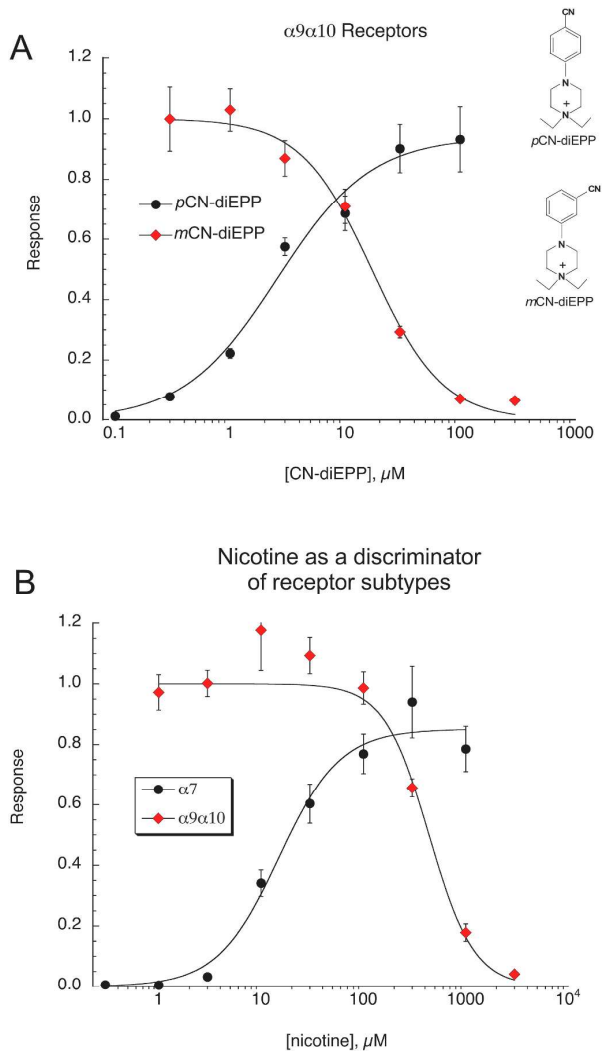

Fig. 1. Concentration-response studies with probe compounds. **A**) Cells co-expressing human α9, α10, and RAPSYN were evaluated for their sensitivity to *p*CN-diEPP as an agonist or *m*CN-diEPP as an antagonist of 60 μM ACh-evoked control responses. Structures are shown as inserts. Plotted are the average peak-current responses of at least 6 cells (6–8) at each concentration (±SEM). Data were fit to the Hill equation with either a positive Hill coefficient for agonist activity or a negative Hill coefficient for antagonist activity. Responses to *p*CN-diEPP are relative to ACh maximum. Individual replicates are plotted in Supplemental Fig. 1A. Responses to co-applications of *m*CN-diEPP are relative to ACh alone. Curve fit parameters are provided in Table 1. **B**) Nicotine as an agonist of α7 and an antagonist for α9α10 receptors. Plotted are the average peak current responses of at least 6 cells (6–8) at each concentration (±SEM). Data were fit to the Hill equation with either a positive Hill coefficient for agonist activity or a negative Hill coefficient for antagonist activity. Responses of α7 nAChR are relative to ACh maximum. Responses of α9α10 nAChR to co-applications of nicotine are relative to responses to ACh alone. Curve fit parameters are provided in Table 1. Individual replicates are plotted in Supplemental Fig. 1B.

oocytes may be more permissive and able to be regulated by mass action. For example, it is a common practice to bias the subunit composition of α4β2-containing nAChR by altering the ratio of α4 and β2 RNA injected (Zwart et al., 2006). Therefore, *Xenopus* oocytes were injected with RNA for α7 alone, α9 together with α10 alone, or α7 in combination with α9 and α10 at various ratios (see Table 2) to determine whether receptors expressed would be mixed populations of typical α7 and α9α10 nAChRs or perhaps contain receptors with mixed α7 and α9* properties.

In addition to having differing responses to the pharmacological probes illustrated in Fig. 1, due to the unique rapid concentration-dependent desensitization of α7 receptors (Papke and Horenstein, 2021), ACh control responses are also distinctly different between α7 and α9* nAChRs (Papke et al., 2022) (Fig. 2A), with α9α10 responses having an initial peak followed by later current, resulting in an increased ratio of net charge-to-peak current. With the higher levels of α9α10 expression, the response wave forms took on the characteristic shape of the α9α10 ACh responses, having the higher ratio of charge-to-peak current. At the lower two ratios of α9α10 to α7, the co-expression α9 and α10 had the effect of decreasing ACh responses compared to α7 expressed alone in both peak and net charge (Fig. 2B; see Supplemental Data, Table 1 for ANOVA). We should be very circumspect in comparing one set of injected *Xenopus* oocytes to another, since both α7 and α9α10 RNA amounts were varied. However, the observation that at the 1:2 and 1:3 ratios, mixed α7 and α9α10 expression seemed to reduce both peak currents and net charge in response to ACh is interesting. It might suggest that α7α9α10 nAChR heteromers could be forming in *Xenopus* oocytes that do not function as ligand-gated ion channels. Alternatively, co-expression of all three subunits may interfere with efficient expression of any one type, or the oocytes' capability to transport receptors to the membrane becomes saturated with the expression of the multiple subunits.

Nicotine allowed for the separation of sensitive α7 responses from insensitive α9α10 nAChR (Fig. 3A). Note that these data were normalized to their respective ACh controls, which factored out the overall lower responses associated with α9α10 co-expression. The two lower ratios of α9α10 co-expression produced a mixed population of nicotine-sensitive and -insensitive receptors, while at the highest ratio of α9α10 co-injection, the cells were insensitive to nicotine, similar to those injected with just α9 and α10 (see Supplemental Data, Table 2A for ANOVA).

As expected, *p*CN-diEPP produced minimal activation of α7 nAChRs and cells injected with the lowest ratio of α9α10, while substantial *p*CN-diEPP responses were observed at the higher ratios of α9α10 expression (Fig. 3B; see Supplemental Data, Table 2B for ANOVA). In contrast to the data with nicotine and *p*CN-diEPP, where intermediate ratios of expression suggested intermediate responses, presumably due to mixed populations of α7 and α9α10 nAChR, it appeared that any level of α7 co-expression provided protection from the profound inhibition by *m*CN-diEPP that was seen in oocytes in which only α9α10 nAChR subunits were expressed (Fig. 3C; see Supplemental Data, Table 2B for ANOVA). These data might indicate that hybrid α7α9α10 nAChR are being formed and that their ionotropic function is somehow protected from the antagonism by *m*CN-diEPP. This is, however, in contrast to the above mentioned suggestion that α7α9α10 nAChR heteromers may lack ionotropic function. This difference may relate to the specific subunit stoichiometries of the heteromeric receptors.

3.5. *Possible activation of α10 homomeric receptors*

While our co-injections of α7, α9, and α10 largely seemed to indicate the formation of separate functional α7 and α9* nAChR (Figs. 2 and 3), it may be the case that co-expression of α9 and α10 will naturally result in multiple populations of receptors: α9 homomers, α9α10 heteromers, and possibly even some α10 homomers, which may be assembled but not readily detected as functional. The possibility that quiescent homomeric α10 nAChR may be converted, at least transiently, into functional receptors is supported by a recent report (Hone and McIntosh, 2022) that quiescent homomeric α10 nAChR can be functionally activated by exposure to certain alkaloids, for example methyllycaconitine (MLA) or strychnine. MLA is often purported to be an α7-selective antagonist, although it has been argued that it behaves more like an inverse agonist

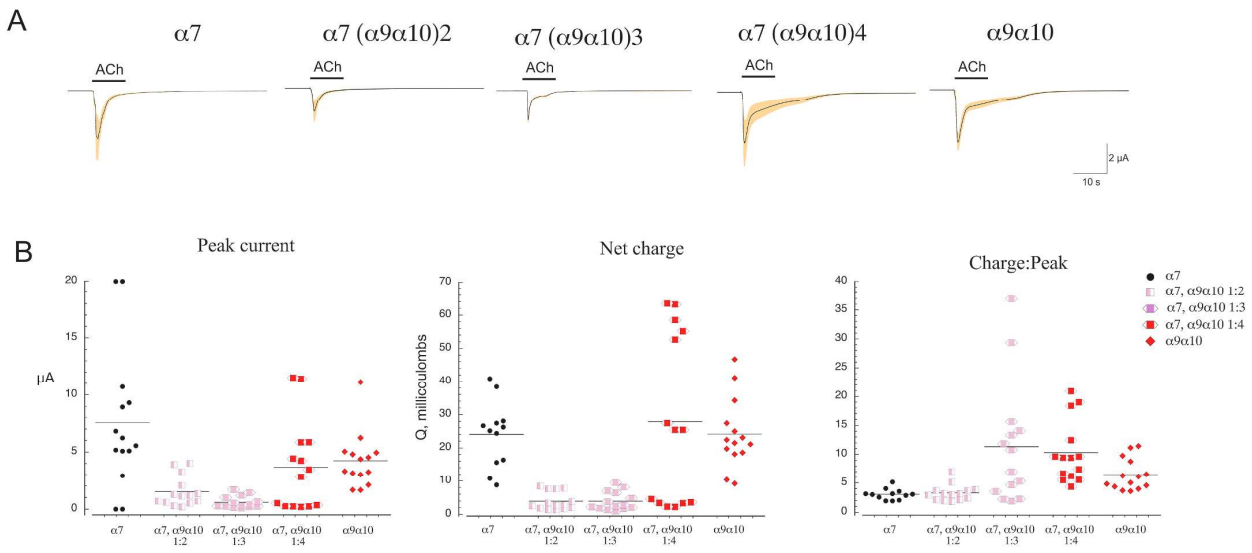

Fig. 2. ACh control responses of cells expressing α7, α9 plus α10, or all three nAChR subunits at the various ratios indicated (see Methods and Table 2 for RNA ratios). **A**) Averaged first 60 μM ACh control responses (dark line) and SEM (tan shaded areas) of 6–7 cells, calculated for each of 10,000 points over 210-s acquisition periods. **B**) Data on individual responses (two responses from each of 6–7 cells). Both peak current amplitude and net charge (Papke and Papke, 2002) over a 120 s interval following the start of the drug application were measured. Additionally, the ratio of peak currents (μA) and net charge (millicoulombs) were calculated and plotted (see Supplemental Data, Table 1 for statistical analyses). Black bars are the averages of the replicates.

than a simple competitive antagonist (Papke et al., 2018; Williams et al., 2011). MLA has been used to implicate an α7-dependent mechanism in some studies of CAS activity (Quadri et al., 2018), where an α9 mechanism has subsequently also been implicated (Richter et al., 2022). We therefore evaluated the ability of MLA co-applications to inhibit the ACh-evoked responses of cells expressing α7, or α9 alone, or α9 co-expressed with α10 (Fig. 4). We measured inhibition of both peak currents and net charge, with net charge probably being the better measurement (Papke and Papke, 2002). All three populations of receptors were inhibited by sub-micromolar concentrations of MLA (Net-charge IC_{50}s were 0.12 ± 0.05, 0.19 ± 0.02, and 0.24 ± 0.13 μM for α7, α9, and α9α10 expressing cells, respectively, Fig. 4A). We noticed, however, that the MLA applications had a potentiating effect on successive ACh responses of the cells expressing both α9 and α10 nAChR subunits, but not for cells expressing α7 or α9 alone (Fig. 4B and C; see Supplemental Data, Table 3 for ANOVA). At all concentrations greater than 300 nM, the ACh responses of α9α10-expressing cells were greater than those expressing either α7 or α9 alone and greater for the highest concentration of MLA than for the lowest. This would be consistent with an activation of a previously quiescent population of homomeric α10 nAChRs by MLA. Interestingly, we saw that although 30 μM nicotine did not activate currents in α9α10-expressing cells, it did have a similar potentiating effect on subsequent responses to ACh (Fig. 4D). This is consistent with the hypothesis that if there were homomeric α10 receptors present, ion currents in response to ACh would be facilitated if a certain number of the five potential ligand binding sites of homomeric α10 nAChRs remained occupied by an antagonist. An alternative explanation might be a MLA- or nicotine-mediated induction of the α10 receptor expression on the cell surface. In the light of the short time between pretreatment and the application of the nAChR agonist, the latter explanation seems to be less probable. More in-depth analyses are warranted to clarify this interesting aspect and to test if it is relevant to the *in vivo* situation.

3.6. The impact of pCN-diEPP and mCN-diEPP on the ATP-mediated release of IL-1β by monocytic cells

It was shown before that ACh and phosphocholine function as unconventional agonists at monocytic nAChRs containing α7 and/or α9/α10 subunits and as potent inhibitors of the BzATP-induced maturation and release of IL-1β in monocytic cells (Richter et al., 2016; Zakrzewicz et al., 2017). To test if *p*CN-diEPP and *m*CN-diEPP exert similar effects on monocytic cells, human monocytic THP-1 cells were primed with LPS (1 μg/ml) for 5 h, followed by stimulation with the P2X7 receptor agonist BzATP (100 μM) for another 40 min in the presence or the absence of ACh (10 μM), phosphocholine (200 μM), *p*CN-diEPP (100 μM), or *m*CN-diEPP (100 μM) (Fig. 5). Thereafter, the concentration of released IL-1β was measured in cell culture supernatants by ELISA. As expected, untreated cells and cells primed with LPS did not release relevant amounts of IL-1β, whereas stimulation with BzATP resulted in elevated IL-1β levels released by monocytic THP-1 cells, in the range of 84 pg/ml to 236 pg/ml (Fig. 5A). The BzATP-induced release of IL-1β was significantly inhibited by phosphocholine, ACh, and *p*CN-diEPP, while for *m*CN-diEPP only a tendency ($P = 0.08$, Friedman test followed by the two-tailed Wilcoxon signed-rank test) towards a mild inhibition was seen. The effects of *p*CN-diEPP and *m*CN-diEPP on the IL-1β release were repeated and confirmed in a second independent set of experiments (Fig. 5B). When both experiments were taken together (Fig. 5A and B), the weak inhibitory effects of *m*CN-diEPP on the BzATP-induced release of IL-1β were statistically significant ($P = 0.014$, $n = 12$ each, Friedman test followed by the two-tailed Wilcoxon signed-rank test). The biological relevance of such subtle changes in IL-1β release are, however, questionable.

These results are in line with our expectations that stimulation of α9* nAChRs in monocytic cells metabotropically inhibits the ionotropic function of the ATP-sensitive P2X7R and, hence, the release of IL-1β (Richter et al., 2016; Zakrzewicz et al., 2017). However, we cannot predict which nAChR subunits are involved in the effects of *p*CN-diEPP. They might indeed be mediated via α9α10 nAChR, similar to the unconventional nAChR agonists glycerophosphocholine or lysophosphatidylcholine (Zakrzewicz et al., 2017). In addition, α7 nAChR subunits

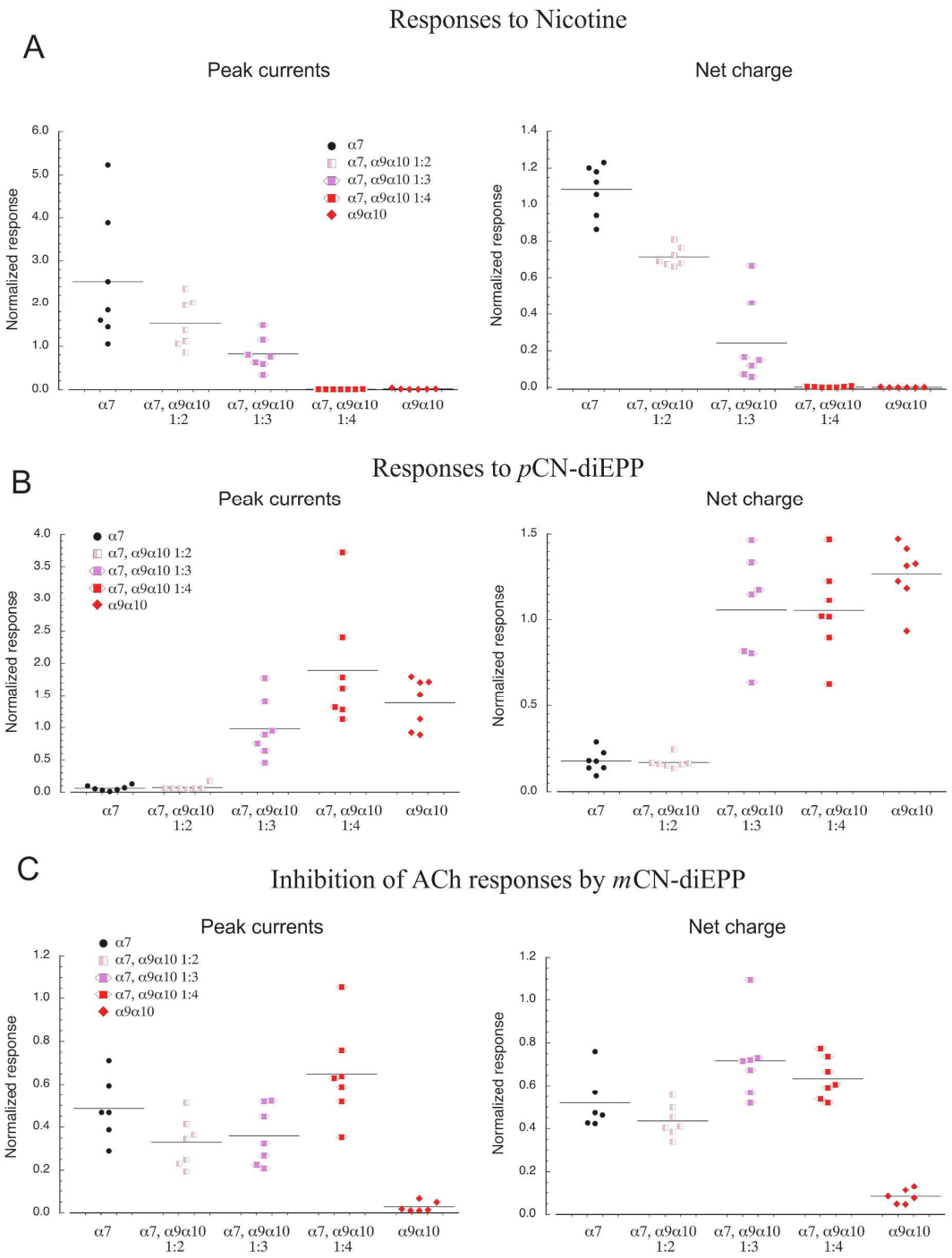

Fig. 3. The pharmacological separation of receptors in mixed populations. **A**) Detection of (30 μM) nicotine-sensitive α7 responses. **B**) Detection of (30 μM) *p*CN-diEPP-sensitive responses, primarily representing α9α10 receptors. **C**) Inhibition of 60 μM ACh-evoked responses by co-application of 30 μM *m*CN-diEPP. In all panels, data are shown for both peak currents and net charge (n = 6–7 for each condition, see Supplemental Data, Table 2 for statistical analyses). Black bars are the averages of the replicates.

might be involved, as we have shown before that α7 nAChR silent agonists can inhibit the BzATP-induced release of IL-1β as well (Richter et al., 2022). As *p*CN-diEPP was characterized as a very weak but strongly desensitizing agonist at α7 nAChRs (Quadri et al., 2016), properties that are close to silent agonists, it might also induce metabotropic functions of this receptor. More research including the use of α7-and α9-specific nAChR antagonists and gene silencing experiments might address these questions.

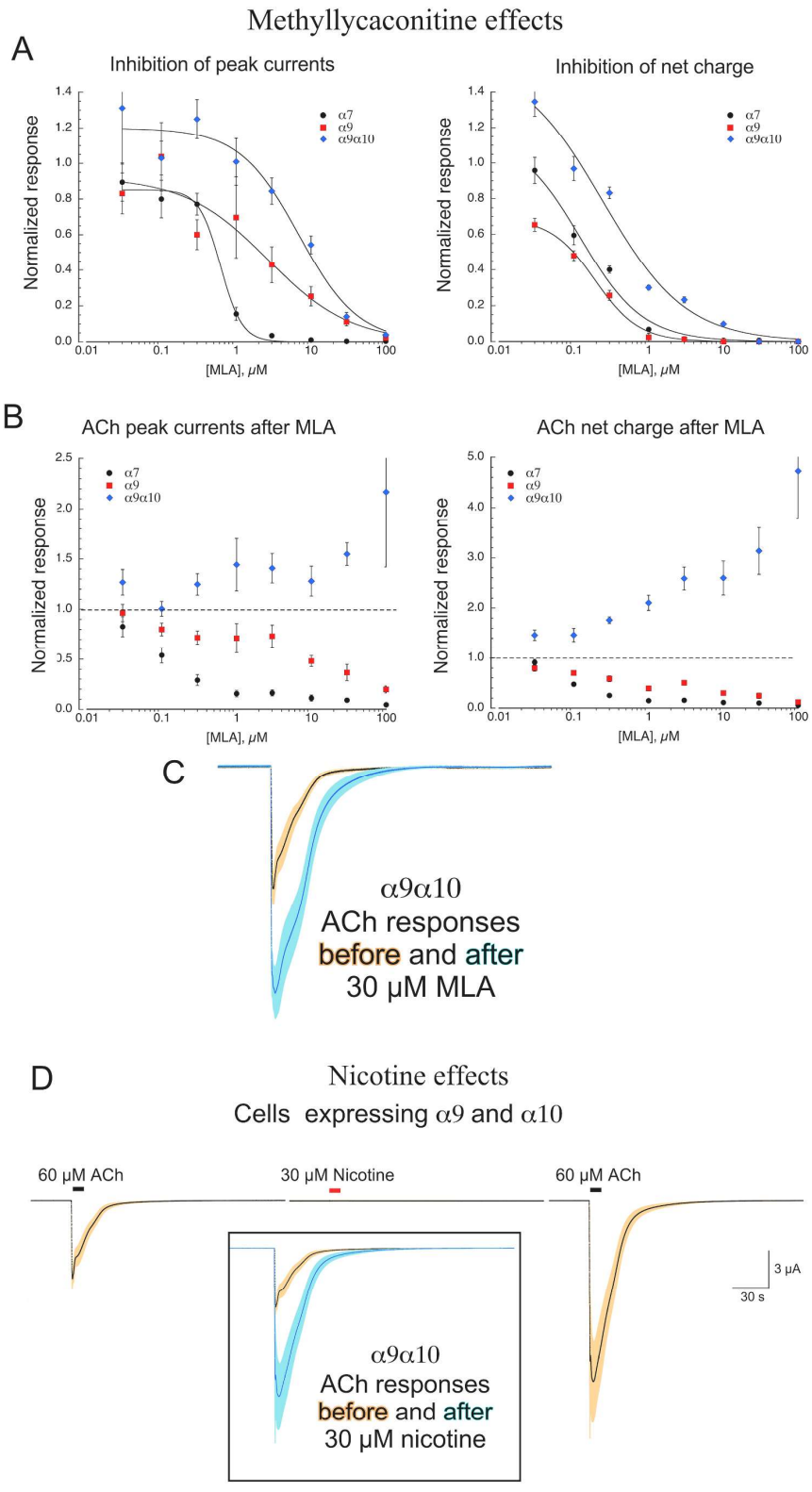

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Fig. 4. Probing α9α10-expressing cells for the possible activation of α10 homomeric receptors with methyllycaconitine (MLA). **A**) Evaluation of MLA antagonist activity. MLA was co-applied with 60 μM ACh to cells expressing α7, α9, or both α9 and α10. Normalized responses were measured relative to the average of two initial ACh controls on both peak currents (left panel) and net charge (right panel). Each cell was tested with a single concentration of MLA,. Data plotted are the averaged normalized responses of 5–8 cells at each concentration (±SEM). The IC_{50} values for the inhibition of peak currents were 0.62 ± 0.05, 2.64 ± 1.75, and 6.87 ± 1.9 μM for α7, α9, and α9α10, respectively. The IC_{50} values for the inhibition of net charge were 0.12 ± 0.05, 0.19 ± 0.02, and 0.24 ± 0.13 μM for α7, α9, and α9α10, respectively (See Supplemental Data Fig. 2A for dot plots of replicates.). **B**) Effects of MLA co-application on subsequent responses to ACh. Following the co-application of MLA and ACh (represented in panel A), an additional application of 60 μM ACh was made, and those responses (peak currents, left panel and net charge in right panel) were normalized to the average of the two initial ACh control responses (see Supplemental Data Fig. 2B for dot plots of replicates). **C**) Averaged traces of the ACh responses of cells expressing α9 and α10 (n = 6) before (black line with tan background, representing the SEM) and after (dark blue line with light blue SEM) the application of 30 μM MLA. **D**) Averaged responses of cells expressing α9 and α10 (n = 7) before, during, and after the application of 30 μM nicotine (Fig. 3A). The insert shows the overlay of the before and after responses, with the currents after nicotine in blue.

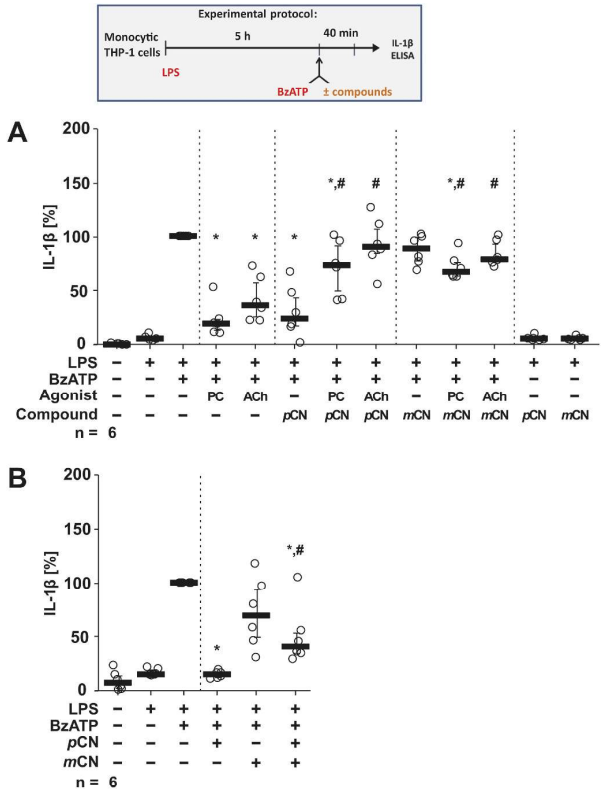

Fig. 5. The impact of *p*CN-diEPP (*p*CN) and *m*CN-diEPP (*m*CN) on the ATP-mediated release of interleukin-1β (IL-1β) by human monocytic THP-1 cells. THP-1 cells were primed with lipopolysaccharide (LPS; 1 μg/ml, 5 h). Then the P2X7 receptor agonist BzATP ((2'/3'-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate, tri(triethylammonium) salt) was added for another 40 min to trigger IL-1β release, which was measured by ELISA. **A**) The BzATP (100 μM) -induced release of IL-1β was investigated in the presence and absence of the compounds *p*CN (100 μM) or *m*CN (100 μM), as well as phosphocholine (PC, 200 μM) or acetylcholine (ACh, 10 μM). PC, ACh, and *p*CN inhibited the BzATP-induced release of IL-1β, while *m*CN alone had no effect. **B**) The inhibitory effect of *p*CN on the BzATP-induced release of IL-1β was partially reversed in the presence of *m*CN. In each experiment, the IL-1β concentrations obtained after stimulation with BzATP + solvent were set to 100%, and all other values were calculated accordingly. Data are presented as individual data points; the bar represents the median, and whiskers encompass the 25th to 75th percentile. *P ≤ 0.05, different from LPS-primed cells stimulated with BzATP alone. #P ≤ 0.05 significantly different from samples where BzATP plus corresponding agonist were given. Statistics are based on the Friedman test followed by the two-tailed Wilcoxon signed-rank test.

As expected, the α9α10 nAChR antagonist *m*CN-diEPP reversed the inhibitory effect of ACh, phosphocholine (Fig. 5A), and *p*CN-diEPP (Fig. 5A and B). Surprisingly, *p*CN-diEPP also antagonized the effects of ACh and phosphocholine (Fig. 5A). We conclude that both *p*CN-diEPP and *m*CN-diEPP can exert agonistic and antagonistic functions in this experimental setting. This observation adds another conundrum to the specific characteristics of NCNRs, and suggests that the ionotropic effects of a certain ligand at conventional nAChRs do not fully predict the effects induced at NCNRs.

As shown in Supplementary Figs. 2A and B, LDH values remained below 14% of the total release, irrespective of the experiment performed, which suggests that, at least in the short term, *p*CN-diEPP and *m*CN-diEPP are not overtly toxic to mononuclear phagocytes.

3.7. pCN-diEPP fully attenuates CFA-induced inflammatory pain in an α7 nAChR-independent manner

Animals treated with CFA showed greatly reduced mechanical pain threshold compared to vehicle-treated controls (P = 0.001, Supplemental Data, Table 4 6). Pain thresholds were elevated by treatments with *p*CN-diEPP (Fig. 6A and Supplemental Data, Table 4). Note that these data were not well fit to nomal distributions, especially under control conditions where the majority of responses were the same high threshold value of 3.63 g. Therefore, the data in Fig. 6A are the median scores for each condition (along with the 25–75% ranges). The individual responses are shown in Supplemental Fig. 3. Statistical analysis was conducted using a Kruskal-Wallis test followed by two-tailed Mann–Whitney U tests at each time point following *p*CN-diEPP treatment. There were significant effects for all the doses tested at the 1 h time point (P = 0.001, Supplemental Data, Table 4), and for the two highest dosages at the 3 h time point (P = 0.014 and 0.006, Table 6), with no significant effects at the 6 h time point.

These data are in line with the common notion that full and silent agonists at α7 nAChRs exert anti-inflammatory functions *in vivo*, and hence are also expected to reduce inflammatory pain. Therefore, the next logical step was to investigate if the α7 nAChR mediates the effect of *p*CN-diEPP.

In a separate experiment (Fig. 6B), we tested CFA-treated α7 WT and KO mice with *p*CN-diEPP (1 mg/kg) or vehicle and evaluated their mechanical hypersensitivity 3 days after CFA. Our experiments indicated that the antinociceptive effect of *p*CN-diEPP was independent of α7 nAChRs since the effects of 1 mg/kg *p*CN-diEPP were essentially the same in both WT and α7 nAChR KO animals.

As *p*CN-diEPP functioned as a full agonist at α9α10 nAChRs, our data strongly suggest that the reduction of CFA-induced pain was mediated via the activation of α9* nAChRs. However, for a firm conclusion, experiments on α9α10 nAChR KO mice are warranted. It would be of high clinical relevance to test *p*CN-diEPP in experimental neuropathic pain and to treat animals suffering from CFA-induced inflammatory pain with the conopeptide RgIA4. In contrast to a conopeptide, small molecules such as *p*CN-diEPP generally have a higher probability of entering the clinical arena.

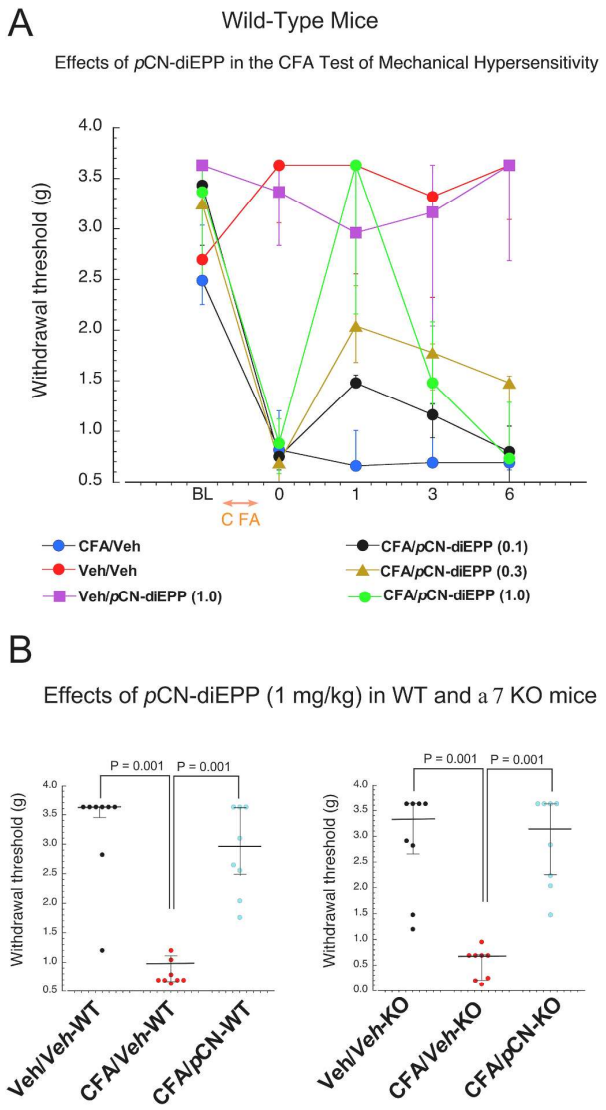

Fig. 6. The effects of systemic *p*CN-diEPP in the CFA-induced chronic inflammatory pain model. **A**) Antiallodynic effects after intraperitoneal administration of various doses of *p*CN-diEPP (0.1, 0.3, and 1 mg/kg) in WT mice. The mechanical paw withdrawal thresholds were determined 3 days after intraplantar injection of CFA (100%) at 1, 3, and 6 h after the drug administration (n = 8/group, 50% male and 50% female). *p*CN-diEPP fully reversed the mechanical hypersensitivity in a dose-dependent manner. Replicate data are provided in Supplementary Fig. 3. Statistical results based on the Friedman test followed by the two-tailed Wilcoxon signed-rank test are provided in Supplemental Data, Table 4 **B**) The antiallodynic effects of systemic *p*CN-diEPP in the CFA model was not lost in the α7 KO mice. Antiallodynic effects after intraperitoneal administration of *p*CN-diEPP (1 mg/kg) in WT and in alpha7 KO mice. The mechanical paw withdrawal thresholds were determined 3 days after intraplantar injection of CFA (100%) at 1 h after the drug administration (n = 6–8/group, 50% male and 50% female). Plotted are the median scores under each condition, whiskers encompass the 25th to 75th percentile. P values (Table 6) as determined by the Friedman test followed by the two-tailed Wilcoxon signed-rank test are indicated in the figure.

3.8. *mCN-diEPP only partially attenuates CFA-induced inflammatory pain*

The highest concentrations of *m*CN-diEPP, 3 mg/kg, produced a modest reduction of CFA-induced mechanical hypersensitivity 1 and 3 h after injection (Fig. 7A, Supplemental Data Table 5). The effect of that dose dissipated 6 h after injection. In sham-treated mice 3 mg/kg *m*CN-

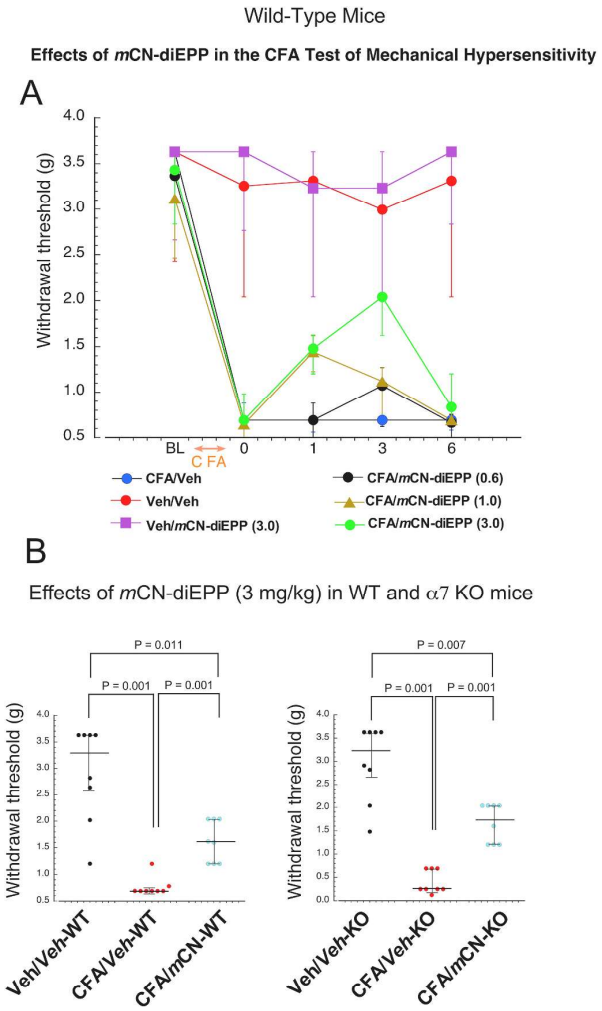

Fig. 7. The effects of systemic *m*CN-diEPP in the CFA-induced chronic inflammatory pain model. **A**) Antiallodynic effects after intraperitoneal administration of various doses of *m*CN-diEPP (0.6, 1, and 3 mg/kg) in WT mice. The mechanical paw withdrawal thresholds were determined 3 days after intraplantar injection of CFA (100%) at 1, 3, and 6 h after the drug administration (n = 8/group, 50% male and 50% female). Plotted are the median scores under each condition bracketed by the 25–75% ranges. Replicate data are provided in Supplementary Fig. 4. Statistical results based on Friedman test followed by the two-tailed Wilcoxon signed-rank test are provided in Supplemental Data, Table 5. **B**). Antiallodynic effects of systemic *m*CN-diEPP in the CFA model were observed in the alpha7 KO mice. Antiallodynic effects after intraperitoneal administration of *m*CN diEPP (3 mg/kg) in WT and in alpha7 KO mice. The mechanical paw withdrawal thresholds were determined 3 days after intraplantar injection of CFA (100%) at 1 h after the drug administration (n = 8/group, 50% male and 50% female). Plotted are the median scores under each condition, whiskers encompass the 25th to 75th percentile. P values (Supplemental Data, Table 5) as determined by the Friedman test followed by the two-tailed Wilcoxon signed-rank test are indicated in the figure.

diEPP did not alter von Frey responses. As with Fig. 6A, data presented are the median scores for each condition (along with the 25–75% ranges). The individual responses are shown in Supplemental Fig. 4.

In a separate experiment, we tested CFA-treated α7 nAChR WT and KO mice with *m*CN-diEPP (3 mg/kg) or vehicle and evaluated their mechanical hypersensitivity 3 days after CFA (Fig. 7B). At the 1 h time point, *m*CN-diEPP showed a significant effect of treatment in both WT and the α7 KO mice, although in both cases the treatments did not fully reverse the effects of CFA treatment. This result suggests that α7 nAChR subunits are not involved in the weak antinociceptive effects of *m*CN-diEPP. It rather suggests that for some cellular mediators of CAS, α9 nAChR antagonism may have some limited efficacy. However, compared to *p*CN-diEPP, *m*CN-diEPP seems to be a far less promising candidate for clinical application.

We also confirmed that neither the highest dose tested of *p*CN-diEPP (1 mg/kg) nor the highest dose tested of *m*CN-diEPP (3 mg/kg) had any significant effects on locomotor activity or body temperature of mice compared to vehicle-treated animals (see Supplemental Fig. 5 and associated statistical anlysis in Supplemental Data Table 6).

4. Conclusions

Numerous cell types have been implicated to be engaged in the NCNR-mediated modulation of CAS, including various leukocyte lineages, macrophages, and microglia. It seems likely that in whole-animal studies, multiple different cell types may contribute to the anti-inflammatory activity of any particular drug. Much of the work characterizing the activity of the NCNR that regulate the CAS has focused on α7 due to a partial correspondence between the pharmacology of α7 ion channel pharmacology and efficacy in models of CAS, with the large caveat that α7 channel desensitizers may have greater efficacy in CAS than ion-channel activators (Papke and Lindstrom, 2020; Papke et al., 2023; Piovesana et al., 2021). Several *in vivo* studies using α9*-selective conopeptides have also implicated α9 nAChRs as potential modulators of CAS (AlSharari et al., 2020; Christensen et al., 2017; Huynh et al., 2019; Pacini et al., 2016). Cell-based assays like those reported in this study implicate both α7 and α9* nAChRs as regulators of CAS (Richter et al., 2016; Zakrzewicz et al., 2017), but with the caveat that the α9*-selective conopeptide RgIA4 blocks the effects of other ligands that activate α9* receptors (Richter et al., 2022). Therefore, it remains a challenge to sort out exactly which receptor subtypes contribute to the NCNR that affect inflammation, particularly in whole-animal experiments, where all the nAChR subtypes will be present and expressed at different levels in various cell types.

For some ligands (PNU-282987, GAT-107, and NS6740) that were effective with *in vivo* models of inflammatory pain, an α7 nAChR−dependent mechanism was confirmed by demonstrating that effects were lost in α7 nAChR KO animals (Bagdas et al., 2016; Donvito et al., 2017; Papke et al., 2015). However, such data do not support the hypothesis that α7 nAChR expression alone is sufficient for CAS activity. In the present study, we demonstrate that for the α9 nAChR agonist *p*CN-diEPP, α7 expression is not necessary for CAS activity in the CFA model of inflammatory pain. The retention of full activity of *p*CN-diEPP in α7 nAChR KO animals strongly implicates an α9* nAChR-dependent mechanism. Likewise, the milder anti-nociceptive effects of *m*CN-diEPP also seemed to be independent of α7 nAChR subunits.

In some studies (Costa et al., 2012; Garai et al., 2018; Patel et al., 2017; Pinheiro et al., 2020; Quadri et al., 2018; Toma et al., 2019), block by MLA has been proposed to demonstrate an α7-dependent mechanism. However, due to the α9* nAChR sensitivity to MLA (Fig. 4), the interpretation of those results should be called into question.

The mystery remains concerning the degree to which agonist and/or antagonist properties of canonical ion channel function of the α-bungarotoxin-sensitive nAChR can really be predictive of the ability of the same ligands to regulate the CAS function of NCNR. On this point, it should be kept in mind that even the most efficacious of agonists do not just promote ion channel activation, which in any case is usually just transient, particularly in the case of α7 nAChR. Agonists stabilize ligand-dependent non-conducting states as well. This has led to the hypothesis that signal transduction by NCNR is associated with the induction of non-conducting conformations (Papke and Lindstrom, 2020; Papke et al., 2023), consistent with the CAS activity of the strongly desensitizing α7 nAChR silent agonist NS6740 (Papke et al., 2015; Thomsen and Mikkelsen, 2012) and the nonconventional α9 nAChR agonist phosphocholine (Richter et al., 2016).

The ultimate development of new therapeutic agents will require study of basic ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties. A further consideration is brain permeation if microglia, which are potential mediators of inflammation in neurodegenerative diseases are to be regarded as targets. Due to their quaternary ammonium, the diEPP compounds are unlikely to have good brain penetration, a limitation that might be overcome with tertiary amine analogs. However, in general, peripheral sensitization in nociceptors is essential for the development of inflammatory pain, so our finding of the α9-dependent CAS activity of *p*CN-diEPP highlights the utility of targeting of α9* nAChR for the development of non-opiate analgetics.

CRediT authorship contribution statement

Katrin Richter: Designed and conducted experiments and contributed to writing the manuscript. **Sara M. Herz:** Conducted experiments. **Clare Stokes:** Designed and conducted experiments and contributed to writing the manuscript. **M. Imad Damaj:** Designed experiments, analyzed data and contributed to writing the manuscript. **Veronika Grau:** Designed experiments and contributed to writing the manuscript. **Roger L. Papke:** Designed experiments, analyzed data, and played the major role in writing the manuscript.

Declaration of competing interest

The authors declare that they have no conflicts of interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2023.109717.

References

AlSharari, S.D., Toma, W., Mahmood, H.M., Michael McIntosh, J., Imad Damaj, M., 2020. The alpha9alpha10 nicotinic acetylcholine receptors antagonist alpha-conotoxin RgIA reverses colitis signs in murine dextran sodium sulfate model. Eur. J. Pharmacol. 883, 173320.

Amar, M., Thomas, P., Johnson, C., Lunt, G.G., Wonnacott, S., 1993. Agonist pharmacology of the neuronal alpha 7 nicotinic receptor expressed in Xenopus oocytes. FEBS Lett. 327, 284–288.

Azam, L., McIntosh, J.M., 2012. Molecular basis for the differential sensitivity of rat and human alpha9alpha10 nAChRs to alpha-conotoxin RgIA. J. Neurochem. 122, 1137–1144.

Bagdas, D., AlSharari, S.D., Freitas, K., Tracy, M., Damaj, M.I., 2015. The role of alpha5 nicotinic acetylcholine receptors in mouse models of chronic inflammatory and neuropathic pain. Biochem. Pharmacol. 97, 590–600.

Bagdas, D.A., Wilkerson, J.L., Kulkarni, A., Toma, W., AlSharari, S., Gul, Z., Lichtman, A. H., Papke, R.L., Thakur, G.A., Damaj, M.I., 2016. The alpha7 nicotinic receptor dual allosteric agonist and positive allosteric modulator GAT107 reverses nociception in mouse models of inflammatory and neuropathic pain. Br. J. Pharmacol. 173, 2506–2520.

Bavo, F., Pallavicini, M., Pucci, S., Appiani, R., Giraudo, A., Eaton, B., Lucero, L., Gotti, C., Moretti, M., Whiteaker, P., Bolchi, C., 2022. From 2-triethylammonium ethyl ether of 4-stilbenol (MG624) to selective small-molecule antagonists of human alpha9alpha10 nicotinic receptor by modifications at the ammonium ethyl residue. J. Med. Chem. 65, 10079–10097.

Bencherif, M., Lippiello, P.M., Lucas, R., Marrero, M.B., 2011. Alpha7 nicotinic receptors as novel therapeutic targets for inflammation-based diseases. Cell. Mol. Life Sci. 68, 931–949.

Borovikova, L.V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G.I., Watkins, L.R., Wang, H., Abumrad, N., Eaton, J.W., Tracey, K.J., 2000. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405, 458–462.

Bouzat, C., Lasala, M., Nielsen, B.E., Corradi, J., Esandi, M.D.C., 2018. Molecular function of alpha7 nicotinic receptors as drug targets. J. Physiol. 596, 1847–1861.

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., Yaksh, T.L., 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53, 55–63.

Christensen, S.B., Hone, A.J., Roux, I., Kniazeff, J., Pin, J.P., Upert, G., Servent, D., Glowatzki, E., McIntosh, J.M., 2017. RgIA4 potently blocks mouse alpha9alpha10 nAChRs and provides long lasting protection against oxaliplatin-induced cold allodynia. Front. Cell. Neurosci. 11, 219.

Costa, R., Motta, E.M., Manjavachi, M.N., Cola, M., Calixto, J.B., 2012. Activation of the alpha-7 nicotinic acetylcholine receptor (alpha7 nAchR) reverses referred mechanical hyperalgesia induced by colonic inflammation in mice. Neuropharmacology 63, 798–805.

Dixon, W.J., 1965. The up-and-down method for small samples. J. Am. Stat. Assoc. 60, 967–978.

Donvito, G., Bagdas, D., Toma, W., Rahimpour, E., Jackson, A., Meade, J.A., AlSharari, S., Kulkarni, A.R., Ivy Carroll, F., Lichtman, A.H., Papke, R.L., Thakur, G. A., Imad Damaj, M., 2017. The interaction between alpha 7 nicotinic acetylcholine receptor and nuclear peroxisome proliferator-activated receptor-alpha represents a new antinociceptive signaling pathway in mice. Exp. Neurol. 295, 194–201.

Elgoyhen, A.B., Vetter, D.E., Katz, E., Rothlin, C.V., Heinemann, S.F., Boulter, J., 2001. alpha10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. Proc. Natl. Acad. Sci. U.S.A. 98, 3501–3506.

Frazier, C.J., Rollins, Y.D., Breese, C.R., Leonard, S., Freedman, R., Dunwiddie, T.V., 1998. Acetylcholine activates an alpha-bungarotoxin-sensitive nicotinic current in rat hippocampal interneurons, but not pyramidal cells. J. Neurosci. 18, 1187–1195.

Fuchs, P.A., 1996. Synaptic transmission at vertebrate hair cells. Curr. Opin. Neurobiol. 6, 514–519.

Fujii, T., Mashimo, M., Moriwaki, Y., Misawa, H., Ono, S., Horiguchi, K., Kawashima, K., 2017. Expression and function of the cholinergic system in immune cells. Front. Immunol. 8, 1085.

Garai, S., Raja, K.S., Papke, R.L., Deschamps, J.R., Damaj, M.I., Thakur, G.A., 2018. B-973, a novel alpha7 nAChR ago-PAM: racemic and asymmetric synthesis, electrophysiological studies, and in vivo evaluation. ACS Med. Chem. Lett. 9, 1144–1148.

Halevi, S., Yassin, L., Eshel, M., Sala, F., Sala, S., Criado, M., Treinin, M., 2003. Conservation within the RIC-3 gene family. Effectors of mammalian nicotinic acetylcholine receptor expression. J. Biol. Chem. 278, 34411–34417.

Hartung, J.E., Eskew, O., Wong, T., Tchivileva, I.E., Oladosu, F.A., O'Buckley, S.C., Nackley, A.G., 2015. Nuclear factor-kappa B regulates pain and COMT expression in a rodent model of inflammation. Brain Behav. Immun. 50, 196–202.

Hecker, A., Kullmar, M., Wilker, S., Richter, K., Zakrzewicz, A., Atanasova, S., Mathes, V., Timm, T., Lerner, S., Klein, J., Kaufmann, A., Bauer, S., Padberg, W., Kummer, W., Janciauskiene, S., Fronius, M., Schweda, E.K., Lochnit, G., Grau, V., 2015. Phosphocholine-modified macromolecules and canonical nicotinic agonists inhibit ATP-induced IL-1beta release. J. Immunol. 195, 2325–2334.

Hone, A.J., McIntosh, J.M., 2022. Alkaloid ligands enable function of homomeric human alpha10 nicotinic acetylcholine receptors. Front. Pharmacol. 13, 981760.

Hone, A.J., McIntosh, J.M., 2023. Nicotinic acetylcholine receptors: therapeutic targets for novel ligands to treat pain and inflammation. Pharmacol. Res. 190, 106715.

Hone, A.J., Servent, D., McIntosh, J.M., 2018. alpha9-containing nicotinic acetylcholine receptors and the modulation of pain. Br. J. Pharmacol. 175, 1915–1927.

Horenstein, N.A., Papke, R.L., 2017. Anti-inflammatory silent agonists. ACS Med. Chem. Lett. 8, 989–991.

Huynh, P.N., Giuvelis, D., Christensen, S., Tucker, K.L., McIntosh, J.M., 2019. RgIA4 accelerates recovery from paclitaxel-induced neuropathic pain in rats. Mar. Drugs 18.

Kaliyaperumal, S., Wilson, K., Aeffner, F., Dean Jr., C., 2020. Animal models of peripheral pain: biology review and application for drug discovery. Toxicol. Pathol. 48, 202–219.

Lips, K.S., Pfeil, U., Kummer, W., 2002. Coexpression of alpha 9 and alpha 10 nicotinic acetylcholine receptors in rat dorsal root ganglion neurons. Neuroscience 115, 1–5.

Pacini, A., Micheli, L., Maresca, M., Branca, J.J., McIntosh, J.M., Ghelardini, C., Di Cesare Mannelli, L., 2016. The alpha9alpha10 nicotinic receptor antagonist alpha-conotoxin RgIA prevents neuropathic pain induced by oxaliplatin treatment. Exp. Neurol. 282, 37–48.

Papke, R.L., Andleeb, H., Stokes, C., Quadri, M., Horenstein, N.A., 2022. Selective agonists and antagonists of alpha9 versus alpha7 nicotinic acetylcholine receptors. ACS Chem. Neurosci. 13, 624–637.

Papke, R.L., Bagdas, D., Kulkarni, A.R., Gould, T., AlSharari, S., Thakur, G.A., Damaj, I. M., 2015. The analgesic-like properties of the alpha7 nAChR silent agonist NS6740 is associated with nonconducting conformations of the receptor. NeuroPharm 91, 34–42.

Papke, R.L., Dwoskin, L.P., Crooks, P.A., 2007. The pharmacological activity of nicotine and nornicotine on nAChRs subtypes: relevance to nicotine dependence and drug discovery. J. Neurochem. 101, 160–167.

Papke, R.L., Horenstein, N.A., 2021. Therapeutic targeting of alpha7 nicotinic acetylcholine receptors. Pharmacol. Rev. 73, 1118–1149.

Papke, R.L., Lindstrom, J.M., 2020. Nicotinic acetylcholine receptors: conventional and unconventional ligands and signaling. Neuropharmacology 168, 108021.

Papke, R.L., Papke, J.K.P., 2002. Comparative pharmacology of rat and human alpha7 nAChR conducted with net charge analysis. Br. J. Pharmacol. 137, 49–61.

Papke, R.L., Quadri, M., Gulsevin, A., 2023. Silent agonists for alpha7 nicotinic acetylcholine receptors. Pharmacol. Res. 190, 106736.

Papke, R.L., Smith-Maxwell, C., 2009. High throughput electrophysiology with Xenopus oocytes. Comb. Chem. High Throughput Screen. 12, 38–50.

Papke, R.L., Stokes, C., Damaj, M.I., Thakur, G.A., Manther, K., Treinin, M., Bagdas, D., Kulkarni, A.R., Horenstein, N.A., 2018. Persistent activation of alpha7 nicotinic ACh receptors associated with stable induction of different desensitized states. Br. J. Pharmacol. 175, 1838–1854.

Patel, H., McIntire, J., Ryan, S., Dunah, A., Loring, R., 2017. Anti-inflammatory effects of astroglial alpha7 nicotinic acetylcholine receptors are mediated by inhibition of the NF-kappaB pathway and activation of the Nrf2 pathway. J. Neuroinflammation 14, 192.

Pinheiro, N.M., Miranda, C., Santana, F.R., Bittencourt-Mernak, M., Arantes-Costa, F.M., Olivo, C., Perini, A., Festa, S., Caperuto, L.C., Tiberio, I., Prado, M.A.M., Martins, M. A., Prado, V.F., Prado, C.M., 2020. Effects of VAChT reduction and alpha7nAChR stimulation by PNU-282987 in lung inflammation in a model of chronic allergic airway inflammation. Eur. J. Pharmacol. 882, 173239.

Piovesana, R., Salazar Intriago, M.S., Dini, L., Tata, A.M., 2021. Cholinergic modulation of neuroinflammation: focus on alpha7 nicotinic receptor. Int. J. Mol. Sci. 22.

Quadri, M., Bagdas, D., Toma, W., Stokes, C., Horenstein, N.A., Damaj, M.I., Papke, R.L., 2018. The antinociceptive and anti-inflammatory properties of the alpha7 nAChR weak partial agonist p-CF3N,N-diethyl-N'-phenylpiperazine. J. Pharmacol. Exp. Therapeut. 367, 203–214.

Quadri, M., Papke, R.L., Horenstein, N.A., 2016. Dissection of N,N-diethyl-N'-phenylpiperazines as alpha7 nicotinic receptor silent agonists. Bioorg. Med. Chem. 24, 286–293.

Ren, W.J., Illes, P., 2022. Involvement of P2X7 receptors in chronic pain disorders. Purinergic Signal. 18, 83–92.

Richter, K., Grau, V., 2023. Signaling of nicotinic acetylcholine receptors in mononuclear phagocytes. Pharmacol. Res. 191, 106727.

Richter, K., Mathes, V., Fronius, M., Althaus, M., Hecker, A., Krasteva-Christ, G., Padberg, W., Hone, A.J., McIntosh, J.M., Zakrzewicz, A., Grau, V., 2016. Phosphocholine - an agonist of metabotropic but not of ionotropic functions of alpha9-containing nicotinic acetylcholine receptors. Sci. Rep. 6, 28660.

Richter, K., Papke, R.L., Stokes, C., Roy, D.C., Espinosa, E.S., Wolf, P.M.K., Hecker, A., Liese, J., Singh, V.K., Padberg, W., Schluter, K.D., Rohde, M., McIntosh, J.M., Morley, B.J., Horenstein, N.A., Grau, V., Simard, A.R., 2022. Comparison of the anti-inflammatory properties of two nicotinic acetylcholine receptor ligands, phosphocholine and pCF3-diEPP. Front. Cell. Neurosci. 16, 779081.

Romero, H.K., Christensen, S.B., Di Cesare Mannelli, L., Gajewiak, J., Ramachandra, R., Elmslie, K.S., Vetter, D.E., Ghelardini, C., Iadonato, S.P., Mercado, J.L., Olivera, B. M., McIntosh, J.M., 2017. Inhibition of alpha9alpha10 nicotinic acetylcholine receptors prevents chemotherapy-induced neuropathic pain. Proc. Natl. Acad. Sci. U. S.A. 114, E1825–E1832.

Russo, P., Bufalo, A.D., Frustaci, A., Fini, M., Cesario, A., 2014. Beyond acetylcholinesterase inhibitors for treating alzheimer's disease: 7-nAChR agonists in human clinical trials. Curr. Pharmaceut. Des. 20, 6014–6021.

Terry Jr., A.V., Callahan, P.M., 2020. alpha7 nicotinic acetylcholine receptors as therapeutic targets in schizophrenia: update on animal and clinical studies and strategies for the future. Neuropharmacology 170, 108053.

Thomsen, M.S., Mikkelsen, J.D., 2012. The alpha7 nicotinic acetylcholine receptor ligands methyllycaconitine, NS6740 and GTS-21 reduce lipopolysaccharide-induced TNF-alpha release from microglia. J. Neuroimmunol. 251, 65–72.

Toma, W., Kyte, S.L., Bagdas, D., Jackson, A., Meade, J.A., Rahman, F., Chen, Z.J., Del Fabbro, E., Cantwell, L., Kulkarni, A., Thakur, G.A., Papke, R.L., Bigbee, J.W., Gewirtz, D.A., Damaj, M.I., 2019. The alpha7 nicotinic receptor silent agonist R-47 prevents and reverses paclitaxel-induced peripheral neuropathy in mice without tolerance or altering nicotine reward and withdrawal. Exp. Neurol. 320, 113010.

Toma, W., Ulker, E., Alqasem, M., AlSharari, S.D., McIntosh, J.M., Damaj, M.I., 2020. Behavioral and molecular basis of cholinergic modulation of pain: focus on nicotinic acetylcholine receptors. Curr Top Behav Neurosci 45, 153–166.

Treinin, M., Papke, R.L., Nizri, E., Ben-David, Y., Mizrachi, T., Brenner, T., 2017. Role of the alpha7 nicotinic acetylcholine receptor and RIC-3 in the cholinergic anti-inflammatory pathway. Cent. Nerv. Syst. Agents Med. Chem. 17, 90–99.

Verbitsky, M., Rothlin, C.V., Katz, E., Elgoyhen, A.B., 2000. Mixed nicotinic-muscarinic properties of the alpha9 nicotinic cholinergic receptor. Neuropharmacology 39, 2515–2524.

Vetter, D.E., Liberman, M.C., Mann, J., Barhanin, J., Boulter, J., Brown, M.C., Saffiote-Kolman, J., Heinemann, S.F., Elgoyhen, A.B., 1999. Role of alpha9 nicotinic ACh receptor subunits in the development and function of cochlear efferent innervation. Neuron 23, 93–103.

Whiteaker, P., Marks, M.J., Christensen, S., Dowell, C., Collins, A.C., McIntosh, J.M., 2008. Synthesis and characterization of 125I-alpha-conotoxin ArIB[V11L;V16A], a

selective alpha7 nicotinic acetylcholine receptor antagonist. J. Pharmacol. Exp. Therapeut. 325, 910–919.
Williams, D.K., Wang, J., Papke, R.L., 2011. Investigation of the molecular mechanism of the Alpha7 nAChR positive allosteric modulator PNU-120596 provides evidence for two distinct desensitized states. Mol. Pharmacol. 80, 1013–1032.
Zakrzewicz, A., Richter, K., Agne, A., Wilker, S., Siebers, K., Fink, B., Krasteva-Christ, G., Althaus, M., Padberg, W., Hone, A.J., McIntosh, J.M., Grau, V., 2017. Canonical and novel non-canonical cholinergic agonists inhibit ATP-induced release of monocytic interleukin-1beta via different combinations of nicotinic acetylcholine receptor subunits alpha7, alpha9 and alpha10. Front. Cell. Neurosci. 11, 189.
Zwart, R., Broad, L.M., Xi, Q., Lee, M., Moroni, M., Bermudez, I., Sher, E., 2006. 5-I A-85380 and TC-2559 differentially activate heterologously expressed alpha4beta2 nicotinic receptors. Eur. J. Pharmacol. 539, 10–17.