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Das inflammatorische Tumormikromilieu in der Pathogenese des Lungenkarzinoms: Rolle bakterieller Pathogenitätsfaktoren und aktivierter Leukozyten

Habilitationsschrift
zum Erreichen der Lehrbefähigung
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INHALTSVERZEICHNIS

Teil I

VORWORT

Diese Habilitationsschrift fasst Auszüge experimenteller Untersuchungen zusammen, die im Rahmen meiner wissenschaftlichen Tätigkeit im Laborbereich von Herrn Prof. Dr. Dr. F. Grimminger entstanden sind. Die räumlichen und apparativen Voraussetzungen zur Durchführung dieser Untersuchungen sind im Wesentlichen von Herrn Prof. Dr. Dr. F. Grimminger, Herrn Prof. Dr. Ulf Sibelius und Herrn Prof. Dr. W. Seeger geschaffen worden. Förderungen dieser Untersuchungen bestanden durch die Deutsche Forschungsgemeinschaft, das Bundesministerium für Forschung und Bildung, das Universitätsklinikum Gießen Marburg (UKGM) sowie durch die Von Behring-Röntgen-Stiftung.

Einige dieser Untersuchungen entstanden in enger Kooperation mit Wissenschaftlerinnen und Wissenschaftlern der Justus-Liebig-Universität (JLU) Gießen und externer Universitäten. An der JLU Gießen waren die Kooperationspartner Herr Prof. Dr. U. Grandel, Herr Dr. L. Kiss, Herr Prof. Dr. J. Lohmeyer, Herr Prof. Dr. R. Schermuly, Herr Prof. Dr. N. Weissmann, Herr Dr. J. Wilhelm (alle Medizinische Klinik JLU Gießen), und Herr Prof. Dr. U. Sachs (Institut für Klinische Immunologie, Transfusionsmedizin und Hämostaseologie). Des Weiteren bestanden Kooperationen mit Herrn Prof. Dr. R. M. Bohle (Klinik für Allgemeine und Spezielle Pathologie des Universitätsklinikums des Saarlandes, Homburg/Saar), Herrn Prof. Dr. W. Gross und Frau Dr. E. Csernok (Klinik für Rheumatologie und Immunologie Bad Bramstedt), Herrn Prof. Dr. T Goldmann und Frau Dr. D. Lang (Histologie, Forschungszentrum Borstel), Herrn Prof. Dr. Dr. T. Hartung (Johns Hopkins University, Baltimore, USA), Herrn Prof. Dr. L. Fink (Überregionale Gemeinschaftspraxis für Pathologie, Wetzlar), Herrn Prof. Dr. S. Morath (EU Kommission, Joint Research Centre), Herrn Prof. Dr. R. Savai (Max-Planck-Institut für Herz- und Lungenforschung Bad Nauheim und Zentrum für Lungengesundheit, JLU Gießen), und Frau PD. Dr. S. Subtil (Klinik für Strahlentherapie und Radioonkologie, Philipps-Universität Marburg).

Diese Habilitationsschrift enthält Inhalte mehrerer unter meiner Betreuung abgeschlossener humanmedizinischer, biologischer und veterinärmedizinischer Doktorarbeiten (S. van Bürck, M. Y. Gökyildirim, K. Franz, B. Himmel, S. Hoffmann, M. Ludwig, S. Linsel, C. Reinert, E. Schütz).

Der erste Teil dieser Habilitationsschrift gibt einen Überblick über den wissenschaftlichen Hintergrund, der der Entwicklung der Fragestellung zugrunde liegt. Zudem werden im ersten Teil alle wesentlichen Ergebnisse der Arbeit zusammengefasst und im Kontext des aktuellen wissenschaftlichen Kenntnisstandes diskutiert. Die wesentlichen Ergebnisse und Diskussionspunkte sind in einer zusammenfassenden Darstellung wiedergegeben. Der individuelle Beitrag der jeweils genannten Wissenschaftlerinnen und Wissenschaftlern ist den Originalpublikationen zu entnehmen. Zur Veranschaulichung ist der erste Teil mit Abbildungen versehen, die sich zum Großteil, teilweise in modifizierter Form, aus den beigelegten Originalarbeiten rekrutieren. Auf eine detaillierte Schilderung der verwendeten Methodik wurde aus Gründen der Übersichtlichkeit verzichtet. Sie wird aus den anliegenden Originalarbeiten ersichtlich. Der zweite Teil besteht aus den in der Anlage beigefügten Originalarbeiten zum Thema. Dabei handelt es sich um bereits veröffentlichte Originalarbeiten (Anlagen 1–9). Diese stellen einen Auszug meiner wissenschaftlichen Tätigkeit dar, die im anliegenden Publikationsverzeichnis abgebildet ist.

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I. Einführung: Die Tumor-assoziierte Inflammation im Lungenkarzinom – Übersicht und Entwicklung der Fragestellung

Das Lungenkarzinom – Ätiologie, Epidemiologie, Klassifikation und aktuelle therapeutische Ansätze

Das Lungenkarzinom stellt die häufigste Krebstodesursache in der westlichen Welt dar (1). Trotz stetiger Fortschritte in Diagnostik und Therapie liegt die 5-Jahres-Gesamtüberlebensrate lediglich bei 15–20 Prozent (2).

Der wichtigste Risikofaktor für die Entwicklung eines Lungenkarzinoms ist das Inhalieren von Tabakrauch, wobei das Risiko mit der im Laufe des Lebens konsumierten Menge ansteigt. So sind in Deutschland innerhalb der diagnostizierten Lungenkarzinome 90 Prozent bei Männern und mindestens 60 Prozent bei Frauen auf aktives Rauchen zurückzuführen (3). Weitere inhalative Noxen stellen beispielsweise Radon, Asbest, Feinstäube etc. dar (4, 5). Eine klassische Komorbidität des Lungenkarzinoms, die ebenfalls mit inhalativer Tabakexposition einhergeht, ist die chronisch obstruktive Lungenerkrankung (6). Diese ist durch eine chronische pulmonale Inflammationsreaktion mit klinischer Exazerbation durch bakterielle pulmonale Infekte gekennzeichnet (7).

Nach klassischer histologischer Einteilung werden nicht kleinzellige Lungenkarzinome (NSCLC: non-small cell lung cancer) von kleinzelligen Lungenkarzinomen (SCLC: small cell lung cancer) unterschieden. 85 Prozent aller Lungenkarzinome weltweit sind den NSCLC, darunter als Hauptvertreter den Adeno- und Plattenepithelkarzinomen, zuzuordnen (2). In Deutschland stellt das Adenokarzinom den größten Anteil aller histologischen Subtypen neben den selteneren Plattenepithelkarzinomen und großzelligen Karzinomen dar. Daneben existieren die kleinzelligen Lungenkarzinome, die den neuroendokrinen Tumoren zuzuordnen sind (8).

Die Therapie des NSCLC erfolgt stadienabhängig und häufig multimodal, wobei die klassischen Therapiesäulen des NSCLC die Operation, Chemotherapie, Immuntherapie und Bestrahlung darstellen. Die Radiotherapie besteht üblicherweise in einer Photonenbestrahlung, deren zelluläre Zielstruktur die DNA bildet (9). Der Haupteffekt der Photonenbestrahlung ist die Induktion nicht oder fehlerhaft reparierter DNA-Doppelstrangbrüche mit konsekutiver Induktion des klonogenen Zelltods. Ein wesentliches Charakteristikum des klonogenen Zelltods ist der endgültige Verlust der Teilungsfähigkeit der betroffenen Zelle trotz initialer morphologischer und metabolischer Intaktheit.

Als Grundlage der modernen Tumortherapie werden molekulare Charakteristika der Lungenkarzinome wie das Vorhandensein genetischer Aberrationen herangezogen. Therapierbare genetische Veränderungen liegen bei etwa 20 Prozent der Tumoren vor (10). Die häufigste dieser Treibermutationen stellt die Mutation des epidermal growth factor receptor (EGFR) dar. EGFR ist ein transmembranöser Rezeptor der HER-Rezeptorfamilie mit einer intrazellulären Tyrosinkinasedomäne, der in inaktiviertem Zustand als Monomer vorliegt. Nach Bindung der physiologischen Liganden epidermal growth factor (ECF) und transforming growth factor alpha (TGF-α) kommt es zur Homo- oder Heterodimerisierung der

Rezeptoren mit konsekutiver Autophosphorylierung der intrazellulären Tyrosinkinasedomänen und der Aktivierung spezifischer Signaltransduktionswege, die letztendlich in einer Tumorproliferation münden (Abb. 1) (11). Interessanterweise kann der EGFR auch durch inflammatorische Mediatoren, wie z. B. das Chemokin Interleukin (IL)-8 oder Prostaglandin E_2 (PGE_2) aktiviert werden; dieses Phänomen wird als Transaktivierung bezeichnet (12, 13). Liegt eine EGFR-Mutation vor, hat dies auch Implikationen für die Therapie: Hier kommen in der First-Line-Therapie des Lungenkarzinoms Inhibitoren der EGFR-Tyrosinkinase wie Afatinib als Zweitgenerationsinhibitor oder Osimertinib als Drittgenerationsinhibitor zum Einsatz (14).

Eine Problematik bei der Anwendung dieser etablierten Therapien stellt die im Verlauf der Erkrankung häufig auftretende Resistenzentwicklung dar. Molekulare Grundlagen einer Strahlenresistenz stellen häufig eine beschleunigte Reparatur der durch die Bestrahlung induzierten DNA-Doppelstrangbrüche dar (15). Diese Resistenzmechanismen können unter anderem durch inflammatorische Mediatoren induziert werden (16, 17). Auch bei der Anwendung zielgerichteter Therapien beim Vorliegen von EGFR-Mutationen, die häufig primär durch eine Deletion im Exon 19 oder die Punktmutationen L858R im Exon 21 gekennzeichnet sind, können sich Resistenzen gegen etablierte EGFR-Tyrosinkinase-Inhibitoren entwickeln (18).

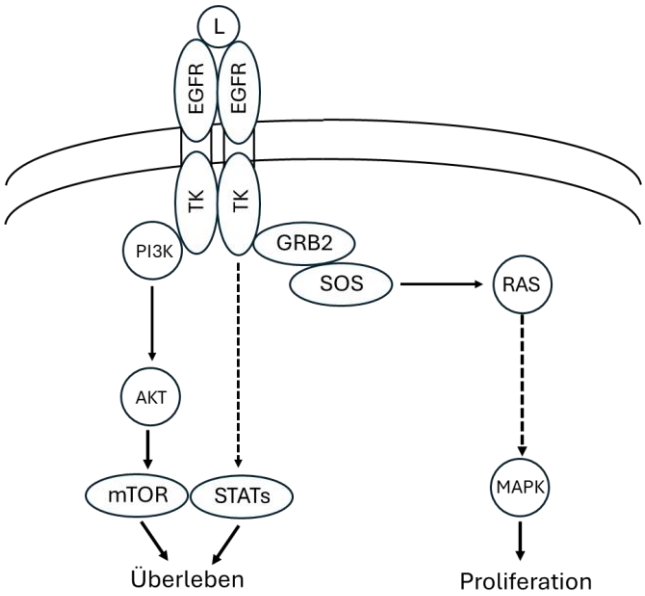

Abbildung 1: Die EGFR-induzierte Signaltransduktion

EGFR wird durch einen extrazellulären Liganden (L) wie EGF oder TGF-a aktiviert. Die Bildung von Homodimeren mit weiteren EGF-Rezeptoren oder Heterodimeren mit anderen Rezeptoren der HER-Familie führt zur Phosphorylierung der intrazellulären Tyrosinkinasedomäne und Aktivierung der PI3K/AKT/mTOR und der RAS/RAF/MEK/MAPK-abhängigen Signaltransduktion. EGF: epidermal growth factor, GRB2: growth factor receptor-bound protein 2, TK: tyrosin-kinase-domain, mTOR: mammalian target of rapamycin, MAPK: mitogen-activated protein kinase, PI3K: Phosphatidylinosol-3-kinase, SOS: Son of Sevenless, STAT: signal transducer and activator of transcription.

Eine weitere wichtige molekularpathologische Information stellt der Grad der intratumoralen Expression des „programmed cell death ligand“ (PD-L1) dar. PD-L1 interagiert mit auf T-Lymphozyten und dendritischen Zellen exprimierten „programmed cell death protein 1“ (PD-1). Diese Interaktion führt zu einer „Immunparalyse“ im Sinne einer ausbleibenden spezifischen Tumorabwehr. Der Einsatz neutralisierender Antikörper gegen PD-1 oder PD-L1 führt zur Demaskierung der Tumorzellen und einer Reaktivierung des spezifischen Immunsystems und Abtötung der Tumorzellen (19, 20).

Über das PD-1/PD-L1-System hinaus weisen Lungentumoren eine Vielzahl immunologischer Charakteristika auf, die aber noch nicht vollumfänglich eingeordnet werden können, um daraus prädiktive Marker für eine Therapieansprache abzuleiten. Ein prädiktives „immunoscoring“ könnte einen weiteren, wesentlichen Fortschritt zur Entwicklung zielgerichteter, individualisierter Tumortherapien darstellen. Hierbei ist unter anderem die Tumor-assoziierte Inflammation im Tumormikromilieu zu berücksichtigen.

Charakteristika der Tumor-assoziierten Inflammation

Schon Virchow erkannte, dass Tumoren klassischerweise von einer lokalen Inflammationsreaktion umgeben sind (21). Wurde diese Inflammation lange als antitumorigene Abwehrreaktion des Immunsystems angesehen, geht die Interpretation heute in der Mehrheit von protumorigenen Effekten des inflammatorischen Tumormikromilieus (TME nach „tumormicroenvironment“) aus (22). Die pathogenetische Bedeutung der Tumor-assoziierten Inflammation wird durch deren Definition als „seventh hallmark of cancer“ unterstrichen (23). Charakterisiert wird diese endogene, durch den Tumor selbst ausgelöste Inflammation durch die Präsenz humoraler und zellulärer Faktoren im Tumormikromilieu, die wiederum selbst protumorigene und pro-proliferative Eigenschaften haben.

Humorale Komponenten des TME im Lungenkarzinom

Unter den humoralen Faktoren sind im Lungenkarzinom proinflammatorische Zytokine wie TNF, IL-1, IL-6, IL-8, Wachstumsfaktoren wie EGF oder pro-angiogene Metabolite wie „vascular endothelial growth factor“ (VEGF) zu nennen (24). Auch dem pleiotropen Zytokin „transforming growth factor-beta“ (TGF-ß) kommt eine wichtige pathogenetische Rolle im NSCLC zu (25). Daneben wird anti-inflammatorischen Zytokinen wie IL-4, IL-10 und IL-23 eine protumorigene Rolle im Sinne einer Modulation des spezifischen Immunantwort gegen die als „fremd“ zu erkennenden Tumorzellen zugeschrieben (24). Eine besondere pathogenetische Rolle könnte unter anderem IL-8 im Lungenkarzinom spielen.
IL-8 ist durch seine chemotaktischen Eigenschaften wesentlich an der Rekrutierung von myeloiden Zellen an einen Entzündungsort beteiligt. Es existieren zwei zelluläre G-Protein-gekoppelte Rezeptoren für IL-8, CXCR1 und CXCR2 (26). Neben myeloiden Zellen sind auch endo- und epitheliale Zellen zur Synthese von IL-8 befähigt, die unter anderem durch TNF oder IL-1 aktiviert wird. IL-8 induziert in vitro in Lungenkarzinomzellen eine Proliferation (27), während es in vivo nach Neutralisation von IL-8 bzw. Depletion des IL-8 Rezeptors CXCR2 zur Inhibition des Tumorwachstum in einem murinen Lungenkarzinommodell kommt (28, 29). Im

humanen Lungenkarzinom existiert eine klare Korrelation zwischen der Expression von IL-8 und der Prognose der Erkrankung (30). In diesen Untersuchungen korrelierte auch die Angiogenese mit der IL-8-Transkription und in vitro wurde gezeigt, dass IL-8 die TNF-mediierte Angiogenese induziert (31). So scheinen diesem Chemokin auch pro-angiogenetische Eigenschaften zuzukommen.

Weitere Mediatoren, die im Lungenkarzinom überexprimiert sind, stellen die Eicosanoide dar. Sie werden durch Cyclooxygenasen (COX), Cytochrom-P450-Epoxygenasen und Lipoxygenasen aus dem Substrat Arachidonsäure (AA) synthetisiert. Diese Omega-6-Fettsäure entsteht durch die Phospholipase A_2 (PLA_2)-mediierte Freisetzung des Substrates aus zellulären Membranen. AA wird von der COX zu Thromboxan und Prostaglandinen verstoffwechselt, während durch eine Aktivierung der Lipoxygenasen das Chemotaxin Leukotrien (LT)B_4, die Hydroxyeicosatetraensäuren sowie die vasoaktiven Cysteinyl-Leukotriene entstehen (Abb. 2) (32).

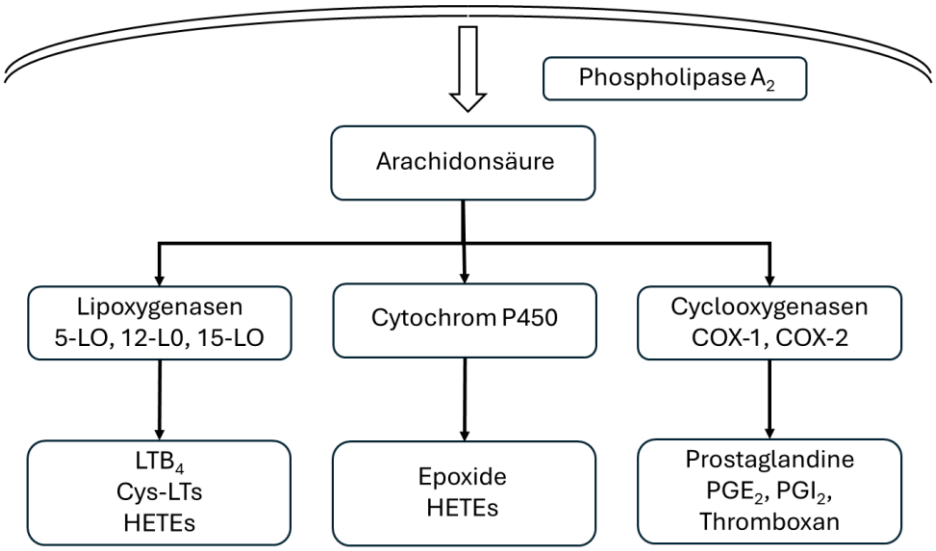

Abbildung 2: Lipidmediatoren der Arachidonsäure

Nach Phopspholipase-A_2-mediierter Freisetzung der Arachidonsäure (AA) aus der nukleären Membran kann AA durch drei enzymatische Wege zu bioaktiven Lipiden, den sog. Eicosanoiden metabolisiert werden. Die Lipoxygenasen metabolisieren AA zu Leukotrien B_4 (LTB_4), den vasoaktiven Cysteinyl-Leukotrienen (Cys-LTs) und den Hydroxytetraensäuren (HETEs). Cytochrom P450 metabolisiert AA ebenfalls zu HETEs und den Epoxiden. Die Cyclooxygenasen (COX) COX-1 und COX-2 verstoffwechseln AA zu Prostaglandinen (PG), wie PGE_2 und PGI_2 und Thromboxan (adaptiert nach 32).

Die Cyclooxygenase existiert in zwei Isoformen, einer konstitutiv exprimierten COX-1 und einer induzierbaren COX-2. Vor allem unter inflammatorischen Bedingungen werden Prostaglandine und Thromboxan zunehmend durch die COX-2 synthetisiert (33). Diesem Metabolismus scheint ebenfalls eine bedeutende Rolle in der Pathogenese des Lungenkarzinoms zuzukommen (34). Im humanen Adenokarzinom der Lunge ist die COX-2 im Vergleich zu gesundem Gewebe verstärkt exprimiert, zudem korreliert eine erhöhte Expression des Enzyms im Tumorgewebe mit einer schlechteren Prognose der Erkrankung (35). Eine Überexpression der COX-2 in vitro induziert die zelluläre Proliferation von NSCLC-

Zelllinien (36). In Tiermodellen des Lungenkarzinoms zeigen COX-2-Inhibitoren protektive Effekte auf die Tumorigenese (37).

Arachidonsäure kann neben der COX auch von den Lipoxygenasen (LO) zu Leukotrienen verstoffwechselt werden. Je nach Positionierung einer Hydroperoxylgruppe in den entsprechenden Metaboliten unterscheidet man zwischen 5-, 12-, und 15-LO. Die breitesten Evidenzen für eine mögliche Rolle bei der Tumorprogression liegen für die 5-LO und deren Metabolit LTB_4 vor, weswegen im Folgenden lediglich auf diesen Stoffwechselweg eingegangen wird. 5-LO wird nach Phosphorylierung vom Zytoplasma an den Zellkern transloziert (38). Für die weitere Metabolisierung freier AA ist die Interaktion mit dem 5-Lipoxygenase-aktivierenden Protein (FLAP) essenziell (39). Der in erster Linie von Granulozyten und Makrophagen synthetisierte Mediator LTB_4 löst als Chemotaxin die Rekrutierung inflammatorischer Zellen ins TME aus (40). Tumorzellen unterschiedlichster Entitäten, unter anderem Lungenkarzinomzellen exprimieren den LTB_4 Rezeptor BLT_2 (41–43), weswegen deren Aktivierung durch LTB_4 möglich scheint. Im Lungenkarzinom liegen Hinweise für eine pathogenetische Rolle der 5-LO, LTB_4 und dessen Rezeptoren vor (43–45).

Zelluläre Komponenten des TME

Neben diesen humoralen Komponenten ist das TME im Gegensatz zu gesundem Lungengewebe von einer spezifischen Stromareaktion mit zellulärer Infiltration von Fibroblasten, Endothelzellen und myeloiden Zellen gekennzeichnet (22, 46). Interessanterweise hängt diese myeloide Infiltration vom Tumorstadium ab (47), sodass dieser zumindest eine prädiktive, vermutlich aber auch pathogenetische Bedeutung zukommt (48, 49). Neben einer spezifischen Infiltration lymphoider und dendritischer Zellen dominieren in vielen Tumoren Makrophagen und polymorphkernige neutrophile Granulozyten (PMN) das zelluläre Infiltrat, sodass die Begriffe Tumor-assoziierte Makrophagen (TAMs) und Tumor-assoziierte Neutrophile (TANs) geprägt wurden (50). Makrophagen werden je nach Polarisierung, die z. B. anhand des spezifischen Zytokinmusters und der Expression von bestimmten Oberflächenmarkern definiert wird, in einen antitumorigenen M1-Typ und in einen protumorigenen M2-Typ unterteilt (50) Die Rolle von PMN ist in diesem Kontext weniger klar definiert; können sie doch Tumorzellen über ihre direkte zytotoxische Aktivität wie der Sekretion freier Sauerstoffradikale und proteolytischer Enzyme abtöten. Der Freisetzung von Sauerstoffradikalen, der sog. „Respiratory Burst“ nach Aktivierung der granulozytären NADPH-Oxidase, könnte aber auch protumorigene Eigenschaften im Sinne einer Genotoxizität zukommen (51). Auch können die in den Granula enthaltenen Serinproteasen, wie Elastase, Matrix-Metalloproteinase-9, Proteinase-3 und Cathepsin G nach deren Freisetzung, der sog. Degranulierung über die Degradierung der extrazellulären Matrix die Migration von Tumorzellen und Metastasierung erleichtern und darüber hinaus genotoxische Effekte ausüben (52, 53). Eine relativ neu entdeckte, ebenfalls zum zytotoxischen Potenzial beitragende Eigenschaft ist die Bildung sog. „neutrophil extracellular traps“ (NETs). NETs sind Netzwerke aus abgebauter neutrophiler DNA, die mit neutrophilen Proteasen verschmelzen und nach Ruptur der Plasmamembran in den Extrazellularraum gelangen und so ihr antimikrobielles Potenzial entfalten (54). Die Fähigkeit von NETs, in einem Tiermodell ruhende

Tumorzellen zum Wachstum und zur Metastasierung zu stimulieren, wirft ein bedeutendes Licht auf diese Granulozyten-abhängigen Strukturen in der Pathogenese des NSCLC (55).

Über diese Sekretion präformierter zytotoxischer Substanzen oder NET-Bildung hinaus haben PMN auch die Kapazität zur De-novo-Synthese proinflammatorischer Mediatoren. Insbesondere verfügen sie über eine ausgeprägte 5-LO-Aktivität (56). Des Weiteren sezernieren PMN proinflammatorische Zytokine wie TNF, IL-1 oder IL-8 (57). Da PMN neben den Makrophagen häufig das TME dominieren (48), könnten Granulozyten-abhängige Mediatoren entscheidend zur Modifikation der peritumoralen Inflammation beitragen.

Die bakterielle Infektion als Komplikation des Lungenkarzinoms

Während die Tumor-assoziierte Inflammation bisher als rein endogenes Geschehen betrachtet wurde, könnten exogene Faktoren diese modifizieren oder sogar aggravieren. Unter den exogenen Faktoren sind an erster Stelle pulmonale Infektionen zu nennen, die bei den meisten Patienten im Verlauf der Erkrankung auftreten. Bereits 1990 beschrieben Perlin et al. einen Zusammenhang zwischen bakteriellen Infektionen und einer Verschlechterung der Prognose im Lungenkarzinom (58). Berghmanns et al. charakterisierten die in Lungenkarzinom vorkommenden Keime, wobei neben den dominierenden grammnegativen Erregern wie *Haemophilus influenzae* und *Moraxella catarrhalis* in 25 Prozent der Fälle auch grampositive Keime gefunden wurden, worunter *Staphylococcus aureus (S. aureus)* und *Streptococcus pneumoniae* führend waren (59). Neuere Untersuchungen bestätigten die Relevanz des pulmonalen Mikrobioms als Kofaktor in der Pathogenese des Lungenkarzinoms (60–61).

Zellwandbestandteile gramnegativer und grampositiver Keime stellen die Prototypen bakterieller Pathogenitätsfaktoren dar. Prominentester Vertreter sind die in der äußeren Bakterienmembran verankerten Lipopolysaccharide (LPS) oder Endotoxine sowie deren grampositiven Äquivalente Peptidoglykane oder Lipoteichonsäuren (LTA) (62, 63). LPS und LTA können im Rahmen der Zellteilung oder Bakteriolyse in das Tumormikromilieu freigesetzt werden.

Die Bindung an eukaryonte Zellen erfolgt an hoch konservierte Rezeptorstrukturen, wie das Glycosylphosphatidylinositol (GPI)-verankerte CD14-Molekül (64). CD14 hat keine transmembranöse Domäne und interagiert mit spezifischen, ebenfalls hoch konservierten Rezeptorkomplexen, den sogenannten „toll-like“-Rezeptoren (TLRs). LPS bindet spezifisch an TLR4, während TLR2 in den meisten Zielzellen den präferenziell von LTA engagierten Rezeptorkomplex darstellt (65, 66). Für die Aktivierung von TLR2 ist zusätzlich eine Heterodimerisierung mit TLR1 oder TLR6 nötig (67). Die Rezeptorliganden-Interaktion induziert eine komplexe Signalkaskade unter Einbeziehung der intrazellulären Adaptoren MyD88 oder TRIF, die in der Aktivierung spezifischer Transkriptionsfaktoren wie NFκB, AP-1 oder IRFs mündet, was wiederum die Synthese inflammatorischer Mediatoren aktiviert (Abb. 3) (68).

Interessanterweise exprimieren nicht nur myeloide Zellen, sondern auch epitheliale Zellen des Lungenkarzinoms TLRs. Die Expression von TLR4 korreliert dabei mit einer erhöhten Malignität im Sinne eines höheren Gradings des Tumors (69). TLR2 mRNA konnte im

Lungenkarzinomgewebe in vivo detektiert werden (70). So scheint eine direkte Interaktion von NSCLC-Zellen und den bakteriellen Pathogenitätsfaktoren LPS und LTA möglich. Tatsächlich gelang Nejman et al. kürzlich ein intrazellulärer Nachweis von LPS und LTA sowohl in Tumorzellen als auch in Immunzellen des NSCLC (71).

Die Konsequenzen dieser Interaktion sind jedoch bis dato noch unklar. Vor diesem Hintergrund erfolgt die Ableitung der Fragestellungen im folgenden Abschnitt.

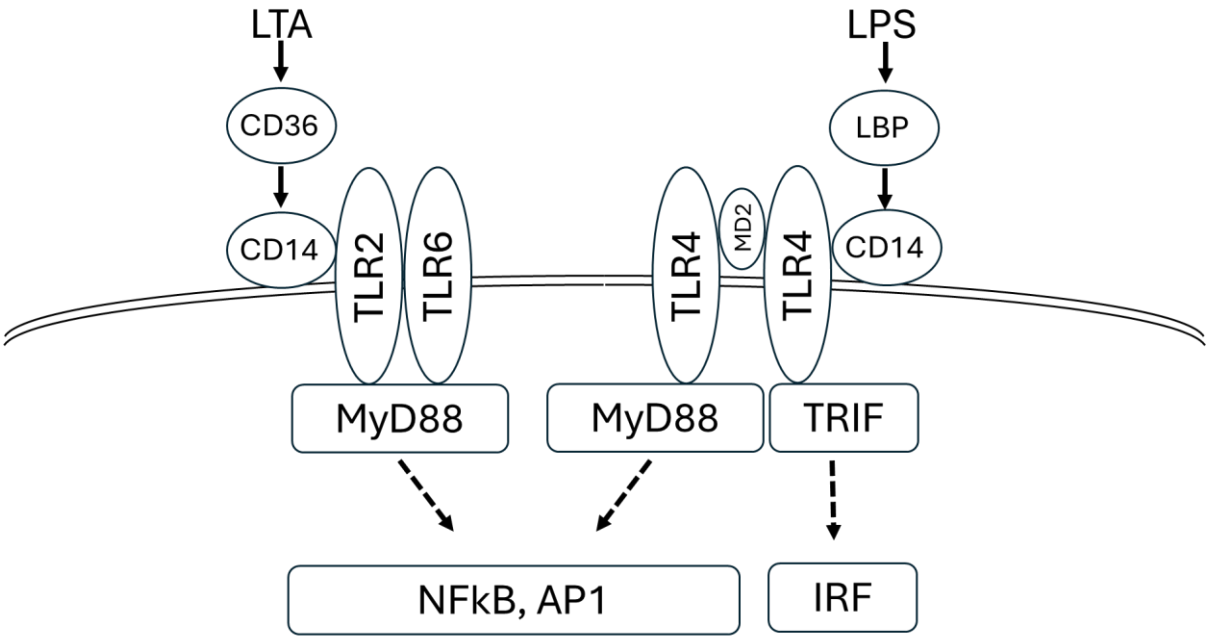

Abbildung 3: TLR2- und TLR4-abhängige Signaltransduktion (vereinfachte Darstellung)

Nach Komplexbildung von LTA mit löslichem CD36 bzw. LPS mit dem Serumprotein LBP binden die bakteriellen Pathogenitätsfaktoren an das GPI-verankerte Glykoprotein CD14. Die Ligation der LTA/CD14-Komplexe an TLR2 bzw. der LPS/CD14-Komplexe and TLR4 induziert eine Rezeptordimerisierung und Aktivierung. TLR2 bildet dabei aktive Heterodimere mit TLR6, wobei TLR4-Homodimere in Interaktion mit MD-2 entstehen. Nach Rezeptoraktivierung von TLR4 kommt es zur Induktion zweier unterschiedlicher intrazellulärer Signaltransduktionswege: TRIF-abhängig werden IRFs aktiviert, während die MyD88-abhängige Signaltransduktion in der Aktivierung der Transkriptionsfaktoren NFkB und AP-1 resultiert. Die Aktivierung von TLR2 induziert primär die MyD88-abhängigen Signaltransduktion.

Abkürzungen: CD: cluster of differentiation, LBP: LPS-binding-protein, GPI: Glycosylphosphatidylinositol, IRF: Interferon regulated factors, TRIF: Toll/IL-1 receptor domain-containing adapter inducing INF-ß, MyD88: myeloid differentiation primary response 88.

Ableitung der Fragestellung

Wie bereits dargestellt, ist die endogene Tumor-assoziierte Inflammation kein simples Epiphänomen, sondern scheint kausal an der Tumorigenese und Tumorproliferation beteiligt zu sein. Sie ist charakterisiert durch das Auftreten inflammatorischer humoraler und zellulärer Komponenten im Tumormikromilieu. Entscheidende zelluläre Komponenten sind neben den Tumor-assoziierten Makrophagen die Tumor-assoziierten neutrophilen Granulozyten. Neben dieser durch den Tumor selbst induzierten, endogenen Inflammation könnten exogene Faktoren, wie bakterielle Infektionen ebenfalls zu einer Tumorproliferation beitragen. Im Rahmen bakterieller Infektionen werden PMN als primäre Effektorzellen rekrutiert. Vor diesem Hintergrund wurde die Arbeitshypothese aufgestellt, dass die isolierten bakteriellen Zellwandbestandteile LPS und LTA direkt über die Interaktion mit Tumorzellen oder indirekt über die Aktivierung von PMN die Tumorproliferation begünstigen. Darüber hinaus sollte überprüft werden, ob bakterielle Pathogenitätsfaktoren eine Strahlentherapieresistenz hervorrufen.

Folgende Fragen sollten beantwortet werden:

1) Welche Konsequenz hat eine direkte Interaktion der bakteriellen Pathogenitätsfaktoren LPS und LTA auf die Tumorproliferation an Modellen des NSCLC?
   Welche Tumor-intrinsischen Mechanismen werden aktiviert?
   Kommt es zur Ausbildung einer Therapieresistenz?
2) Werden die granulozytäre Zytokin- und Leukotriensynthese durch bakterielle Pathogenitätsfaktoren aktiviert?
   Welche Mechanismen sind hierbei involviert?
   Existieren Regulationsmechanismen der Granulozytenaktivierung?
3) Wie beeinflussen bakterielle Pathogenitätsfaktoren das Verhalten von NSCLC-Zellen und PMN in Ko-Kultur?
   Kommt es zu einer reziproken Aktivierung inflammatorischer Mechanismen?
   Welchen Effekt haben PMN auf die Tumorproliferation von NSCLC-Zellen?

Die im Folgenden dargestellten Ergebnisse der experimentellen Untersuchungen zum Effekt der bakteriellen Pathogenitätsfaktoren auf Modelle des NSCLC und des inflammatorischen Tumormikromilieus mit dem Fokus auf neutrophile Granulozyten geben die Kernbefunde der bearbeiteten Projekte wieder. Selbstverständlich sind alle Ergebnisse berücksichtigt, die die Einordnung in einen wissenschaftlichen Gesamtkontext ermöglichen. Bewusst wurde auf eine detaillierte methodische Darstellung verzichtet. Diese findet sich in den beigelegten Originalarbeiten.

II. Vorstellung und Diskussion der bearbeiteten Projekte

Effekte bakterieller Pathogenitätsfaktoren auf experimentelle Modelle des NSCLC

Eine zentrale Fragestellung der hier vorliegenden Arbeit geht von der Hypothese aus, dass die bakteriellen Pathogenitätsfaktoren LPS und LTA direkt mit Zellen des nicht kleinzelligen Lungenkarzinoms interagieren und möglicherweise eine direkte pro-proliferative Reaktion sowie eine Therapieresistenz der Tumorzellen hervorrufen. Dieser Hypothese liegen einerseits die epidemiologischen Studien von Perlin und Berghmans (58, 59) zugrunde, die eine negative Korrelation zwischen dem Auftreten bakterieller Infekte und der Prognose des Lungenkarzinoms nahelegen. Zudem wurde bereits gezeigt, dass TLR4, der eine spezifische Ligation und nachfolgende Aktivierung der Signaltransduktion für LPS ermöglicht, in humanem Lungenkarzinomgewebe überexprimiert wird und mit einer negativen Prognose des NSCLC assoziiert ist (69). Bezüglich der Expression des für Lipoteichonsäuren relevanten TLR2 existieren lediglich Evidenzen in vivo (70), weswegen diese zusätzlich an NSCLC-Zellen charakterisiert werden sollte.

Bakterielle Pathogenitätsfaktoren induzieren eine Tumorproliferation im NSCLC in vitro, ex vivo und in vivo

Ein Kernbefund der vorgelegten Untersuchungen besteht darin, dass die pathogenetisch relevanten Strukturbestandteile LPS und LTA in verschiedenen Modellen des NSCLC eine Tumorproliferation induzieren (Anlagen 1, 2). Hierbei fanden immortalisierte Lungenkarzinomzellen aus Adenokarzinomen und Plattenepithelkarzinomen Verwendung; zudem wurde die Kernaussage, dass LPS eine Tumorproliferation induziert, auch an humanen Lungenkarzinomgeweben und in einem Tiermodell des NSCLC überprüft. Dieser breite Ansatz erlaubt die Ableitung eines pathogenetischen Grundprinzips.

Inkubiert man humane NSCLC-Zellen (A549-Zellen, Adenokarzinom) mit steigenden Konzentrationen zweier isolierter LPS-Stämme von Escherichia-coli (E. coli), so kommt es dosisabhängig zu einer Steigerung der zellulären Proliferation (Abb. 4). Auch hoch aufgereinigte Lipoteichonsäuren von S. aureus lösen eine ähnliche pro-proliferative Reaktion an A549-Zellen und auch an der Plattenepithelkarzinomzelllinie H226 aus (Abb. 5). Die Expression des TLR2 als Voraussetzung für die spezifische Interaktion mit LTA wurde an beiden Zelllinien nachgewiesen (Tabelle 1).

Eingesetzt wurde LPS des breit verwendeten E. coli-Stammes 0111:B4 sowie ein hoch aufgereinigtes LPS des E. coli-Stammes F515, das freundlicherweise von der Arbeitsgruppe von Prof. Otto Holst (Strukturbiochemie, Forschungsinstitut Borstel) zur Verfügung gestellt wurde. Die Proliferation wurde einerseits direkt durch automatisierte Zellzählung quantifiziert, andererseits kam als weitere Methode die Messung der metabolischen Aktivität (MTS-Aktivität) zum Einsatz.

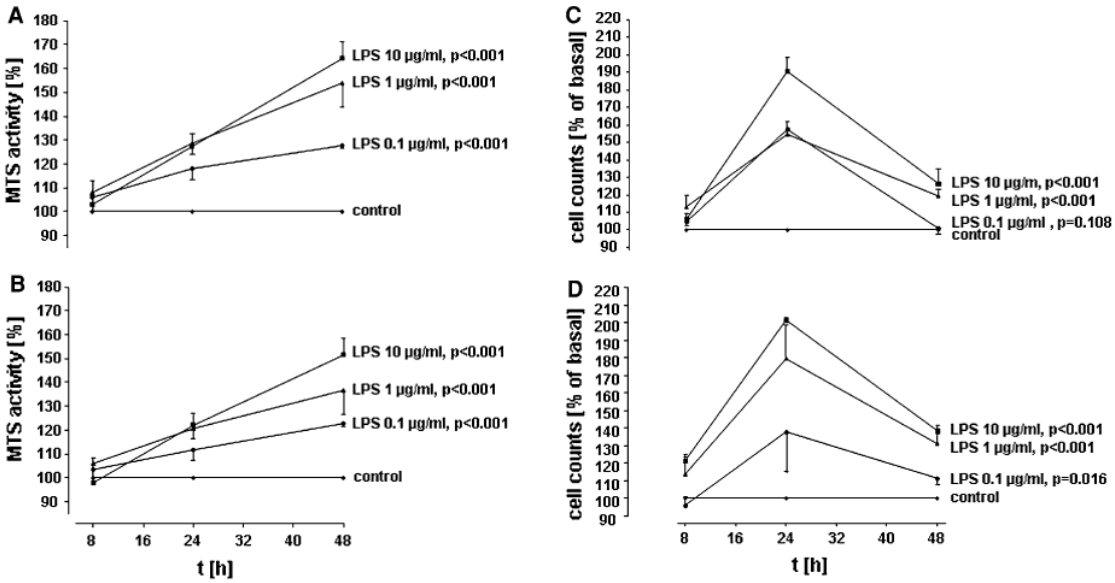

Abbildung 4: Zeit- und dosisabhängige Aktivierung der Proliferation von A549-Zellen durch LPS von E. coli 0:111B4 (A, C) oder LPS von E. coli F515 (B, D). Messung durch MTS-Test (A, B) oder direkte Zellzählung (C, D). Angabe aller Werte prozentual zur unstimulierten Kontrolle (horizontale Linie: 100 %) (Anlage 1).

Beide LPS-Stämme führten zu einer gleichermaßen ausgeprägten, zeit- und dosisabhängigen zellulären Proliferation bis zu einer nahezu Verdopplung in beiden Assays innerhalb von 48 Stunden.

Die durch LTA ausgelöste zelluläre Proliferation wurde ebenfalls an A549-Zellen evaluiert und zusätzlich an der Plattenepithelkarzinomlinie H226 bestätigt. Die zelluläre Proliferation nach Stimulation mit LTA unterschied sich sowohl in quantitativer als auch in qualitativer Sicht vom Effekt des LPS. Rein quantitativ betrachtet, war die LTA-induzierte Proliferation etwas schwächer ausgeprägt. Augenfällig war zudem der fehlende dosisabhängige Effekt. Alle eingesetzten LTA-Konzentrationen (0.01–1 µg/ml) erzielten eine gleichermaßen ausgeprägte Proliferationssteigerung um 30–50 Prozent. Dieser Befund steht im Einklang zu Untersuchungen zum Effekt zum LTA auf humane Prostatakarzinomzellen (72). Interessanterweise führt bereits der Einsatz dieser niedrigen LTA-Konzentrationen zu einem pro-proliferativen Effekt, der sich durch Zugabe höherer Toxindosen nicht weiter steigern ließ. Dies erinnert an das von Adrian et al. beschriebene „all or nothing“-Prinzip in der Physiologie (73), das besagt, dass eine zelluläre Reaktion initiiert wird, sobald eine kritische Rezeptorsättigung erreicht ist. Die fehlende Steigerung der Proliferation durch höhere LTA-Konzentrationen könnte jedoch auch daran liegen, dass LTA in höheren Konzentrationen pro-apoptotische Effekte auslöst, und so den pro-proliferativen Effekt konterkariert, wie auch von Tian an humanen Osteoblasten beschrieben (74).

Tabelle 1: Expression von TLR2-mRNA an A549- und H226-Zellen (Anlage 2).

	A549 [Ct]	H226 [Ct]
TLR2 mRNA	27.69 ± 0.43	27.70 ± 0.39
PBGD mRNA	21.41 ± 0.18	21.94 ± 0.13

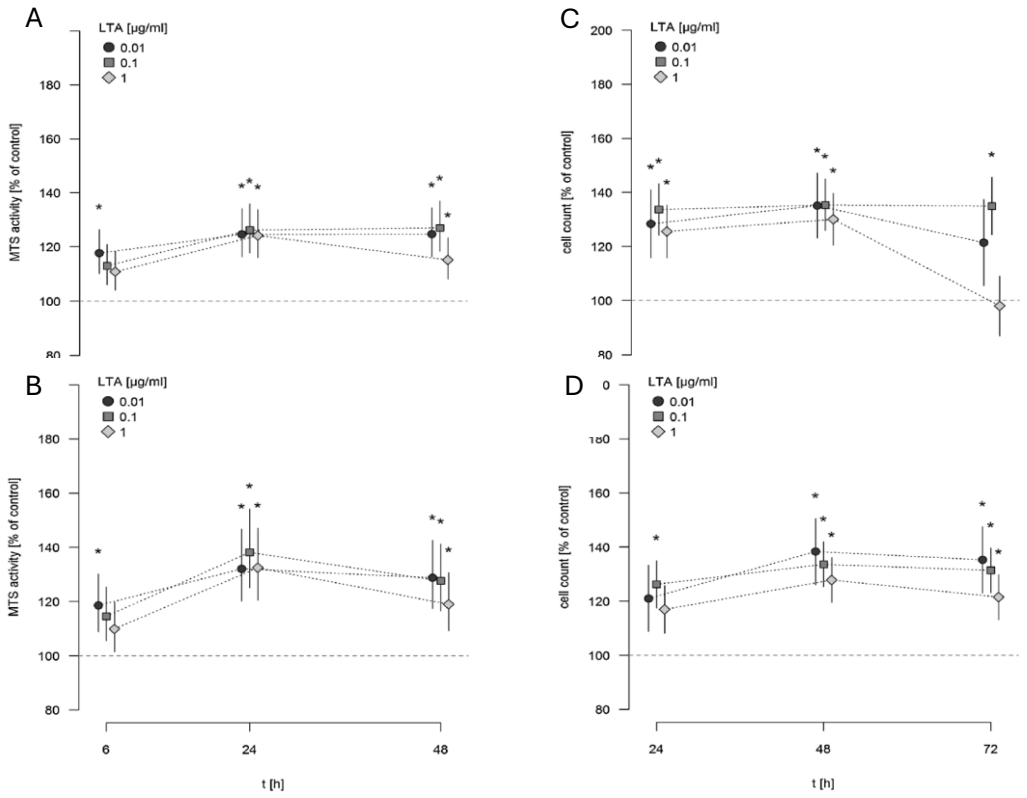

*Abbildung 5: Aktivierung der Proliferation von A549- (A, C) und H226-Zellen (B, D) durch LTA von S. aureus. Messung durch MTS-Test (A, B) oder direkte Zellzählung (C, D). Angabe aller Werte prozentual zur unstimulierten Kontrolle (horizontal gepunktete Linie: 100 %). * p < 0.05 vs. Kontrolle (modifiziert nach Anlage 2).*

Die Tatsache, dass zwei verschiedene, hoch aufgereinigte Stämme von *E. coli* einen ähnlichen pro-proliferativen Effekt auf NSCLC-Zellen in vitro auslösen, spricht gegen ein simples Artefakt oder eine Kontamination der verwendeten LPS-Präparationen. Ebenso wurden hoch aufgereinigte Lipoteichonsäuren verwendet, die nach der Methode von Morath (64) isoliert wurden. Auch die Beobachtung, dass solch geringe Konzentrationen von LTA bereits einen deutlichen pro-proliferativen Effekt hatten, spricht gegen eine Kontamination mit beispielsweise Endotoxin.

Die Spezifität der LPS- bzw. LTA-induzierten Proliferationsreaktion wurde darüber hinaus durch den Einsatz neutralisierender Antikörper gegen die entsprechenden toll-like -Rezeptoren überprüft, wobei bekannt ist, dass TLR4 unter Ligation von CD14 den Rezeptor für LPS darstellt, während LTA an TLR2 bindet (65–67). Man beachte, dass der LPS-induzierte, pro-proliferative Effekt an A549-Zellen durch Neutralisation von CD14 und TLR4 aufgehoben wurde, während Antikörper gegen TLR2 keinen Effekt hatten (Abb.6). Umgekehrt ist die LTA-induzierte zelluläre Proliferation an A549-Zellen durch Einsatz eines TLR2-Antikörpers, nicht jedoch durch Inhibition von TLR4 aufgehoben (Abb. 8).

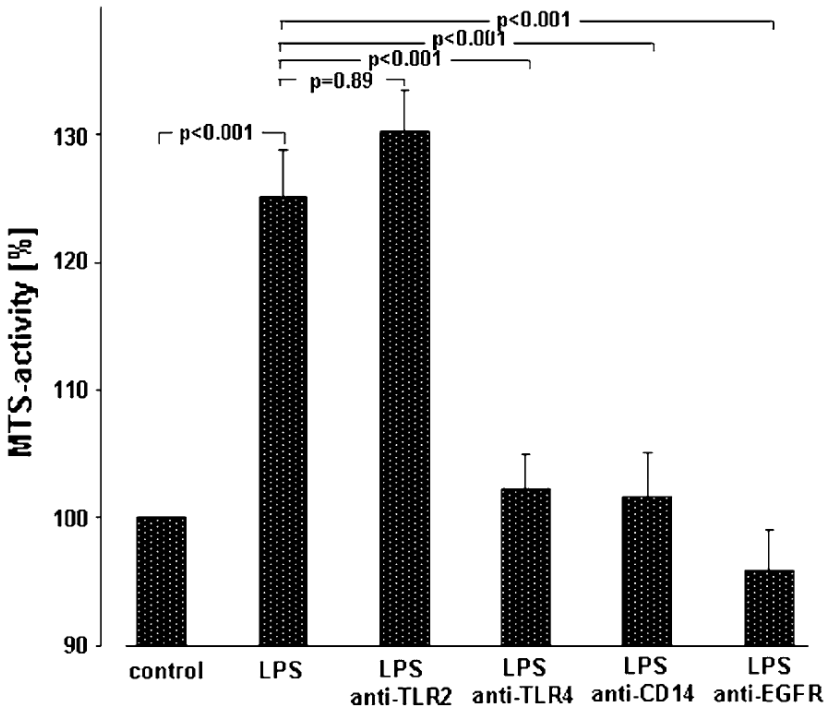

Abbildung 6: Mechanismen der LPS-induzierten Proliferation von A549-Zellen. Aktivierung von A549-Zellen mit 10 µg/ml LPS in An- oder Abwesenheit neutralisierender Antikörper gegen TLR2 (TL2.1, 10 µg/ml), TLR4 (HTA125, 10 µg/ml) CD14 (MY-4, 5 µg/ml) und EGFR (Cetuximab, 10 µg/ml). Darstellung der Proliferation anhand der MTS-Aktivität prozentual zur unbehandelten Kontrolle (100 %) (Anlage 1).

Zum Zeitpunkt der Veröffentlichung waren dies die ersten Arbeiten, die pro-proliferative Effekt von LPS bzw. LTA auf verschiedene Modelle des NSCLC zeigten. Mittlerweile existieren für proliferative Effekte der LPS-induzierten Aktivierung von TLR4 recht breite Evidenzen. So wurden in Modellen des Brust-, Leber-, und Ovarialkarzinoms sowie in einem Tiermodell des Lungenkarzinoms pro-proliferative Effekte von Endotoxin beschrieben (75–78). Aktuelle Untersuchungen an humanen NSCLC-Zelllinien bestätigen die hier beschriebenen Daten (79, 80). Im Gegensatz dazu ist noch wenig über grampositive Pathogenitätsfaktoren bekannt. Jedoch wurde ein Zusammenhang zwischen der Aktivierung von TLR2 und Tumorwachstum im Mundbodenkarzinom beschrieben (81). Zudem wurde eine Rolle für TLR2 in der Pathogenese des Mamma- und Kolonkarzinoms postuliert (82). Aktuelle Studien bestätigen die hier gezeigten pro-proliferativen Effekte von LTA in NSCLC-Zelllinien vitro (80, 83). Zudem wurde gezeigt, dass in Patienten mit Lungenkarzinom durch grampositive Erreger ausgelöste Pneumonien über eine Aktivierung von TLR2 zur Tumorprogression beitragen (84), was eine mögliche Relevanz der hier erhobenen Daten in vivo vermuten lässt. Interessanterweise ist in TLR2- bzw. TLR4-Knock-out-Mäusen in einem Lungenkarzinommodell die durch die Inflammation getriggerte Tumorpromotion unterbunden, was die Relevanz der hier dargestellten Befunde unterstreicht (85).

Die Tumorproliferation wird durch Aktivierung inflammatorischer Mediatoren ausgelöst

Die Bindung von CD14 und TLR4 scheinen also entscheidende initiale Schritte auch bei der Tumorzellaktivierung durch LPS zu sein. Unter den „downstream“ gelegenen Mediatoren

wurde im Endotoxin-Modell ein Fokus auf COX-abhängige Prostanoide, insbesondere PGE_2 gesetzt, da diesen eine zentrale Rolle in der Tumorproliferation im NSCLC zukommt.

Abb. 7A zeigt, dass durch pharmakologische Inhibition der COX, insbesondere auch der induzierbaren Isoform COX-2, der LPS-mediierte pro-proliferative Effekt aufgehoben ist. Zudem wird COX-2-mRNA sowohl in den Zellen als auch im humanen Lungentumorgewebe induziert (Abb. 7B). Nach Stimulation von A549-Zellen mit LPS in Anwesenheit des COX-Substrates Arachidonsäure (AA) akkumuliert PGE_2 im Zellüberstand. Untersuchungen von Lau et al. bestätigten die Notwendigkeit der Substratsubstitution zur effektiven Induktion des COX-Metabolismus in NSCLC-Zellen (86). Interessanterweise wurde die Präsenz freier Arachidonsäure im Tumormikromilieu des Lungenkarzinoms nachgewiesen (87). Somit scheint die Bildung von COX-2-abhängigen Metaboliten auch in vivo durch Lungenkarzinomzellen möglich.

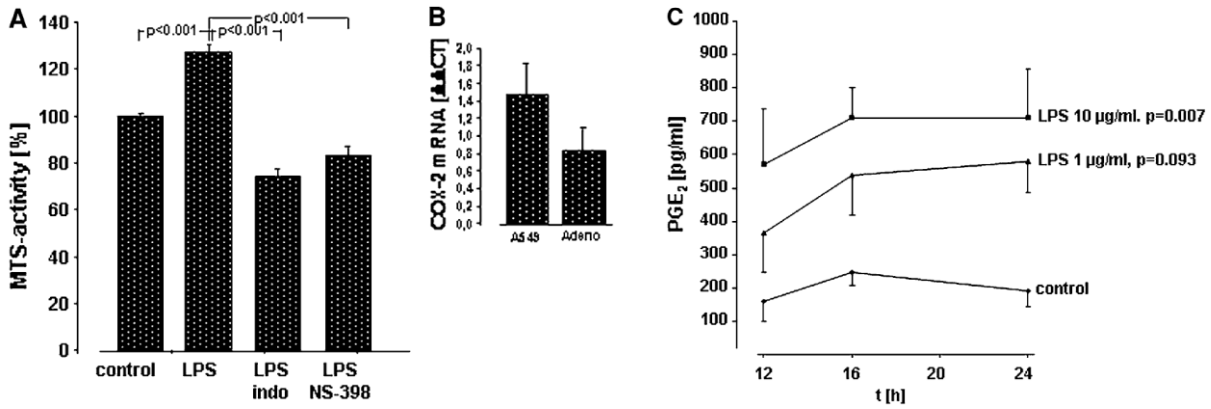

Abbildung 7: Aktivierung des COX-Metabolismus an A549-Zellen und in Adenokarzinomgewebe durch LPS. A) Inhibition der LPS-induzierten Proliferation von A549-Zellen durch den unspezifischen COX-Inhibitor Indometacin (Indo, 100 µM) oder den spezifischen COX-2-Inhibitor NS398 (10 µM) in LPS-stimulierten (10 µg/ml) A549-Zellen. Angabe der MTS-Aktivität prozentual zur unstimulierten Kontrolle (100 %). B) Regulation der COX-2 mRNA nach Stimulation mit LPS (10 µg/ml) in A549-Zellen (A549) oder im humanen Adenokarzinomgewebe. $\Delta\Delta$ CT bezogen auf die unstimulierten Kontrollen. C) Zeit- und dosisabhängige Freisetzung von PGE_2 nach Stimulation von A549-Zellen mit LPS (10 µg/ml) oder Kontrollmedium (control) in Gegenwart freier Arachidonsäure (5 µM) (Anlage 1).

PGE_2 ist mit hoher Wahrscheinlichkeit der entscheidende COX-2-abhängige protumorigene Mediator in unserem System. Erstens wurden weder PGI_2 noch TxB_2 induziert (Anlage 1). Zweitens zeigen vorläufige Daten unserer Arbeitsgruppe einen inhibitorischen Effekt von PGE_2-Rezeptorantagonisten auf die LPS-induzierte Tumorproliferation (s. Kap. V, Abstract 7). Drittens stellt PGE_2 quantitativ den Hauptmetabolit der COX im humanen Lungenkarzinomgewebe wie auch in NSCLC-Zelllinien dar (87, 88). Viertens kann eine Tumorproliferation durch exogenes PGE_2 in vitro in eigenen Untersuchungen (Anlage 9) und in Mausmodellen (89) ausgelöst werden. Des Weiteren wurde an einem Brustkrebsmodell gezeigt, dass LPS eine pulmonale Metastasierung über die Aktivierung der PGE_2-Rezeptoren (EP) EP2-EP4 induziert, was im Einklang zu unseren Überlegungen steht (90).

Als weiteren Oberflächenligand scheint dem EGF-Rezeptorsytem eine Rolle bei der Endotoxin-abhängigen Zellproliferation zuzukommen, da diese in Anwesenheit des neutralisierenden EGFR-Antikörpers Cetuximab erheblich attenuiert war (Abb. 6). Wie es zu einer Aktivierung des EGF-Rezeptors durch Endotoxin kommt, ist nicht geklärt; eine attraktive Hypothese stellt die sogenannte Transaktivierung von EGFR durch autokrin synthetisiertes PGE_2 dar, welches das Potenzial zu einer Transaktivierung von EGFR besitzt (13). Auch IL-8 kann eine solche Transaktivierung hervorrufen (12).

Neuere Publikationen legen zudem eine direkte Abhängigkeit der TLR4-mediierten Zellaktivierung vom EGFR nahe (91, 92). So konnte die LPS-abhängige Zytokinsynthese in einem murinen Modell der Sepsis durch Erlotinib, einem EGFR-Tyrosinkinase-Inhibitor, aufgehoben werden (91).

Selbstverständlich können neben den COX-abhängigen Metaboliten weitere inflammatorische Mediatoren, die durch bakterielle Pathogenitätsfaktoren induziert werden, in die Tumorproliferation involviert sein. Unter den Zytokinen wird hierbei neben TGF-ß (25) den Interleukinen IL-1, IL-6 und IL-8 eine prominente Rolle zugeschrieben (24). Eine zeit- und dosisabhängige Aktivierung der Synthese von IL-8 in A549-Zellen durch Stimulation mit LTA, die ebenfalls von der Ligation des TLR2 abhängig war, wurde bestätigt (Abb. 9). IL-8 war ebenso wie TLR2 funktionell in die durch LTA-induzierte Tumorzellproliferation involviert, was die inhibitorischen Effekte einer Neutralisation von IL-8 auf die durch LTA induzierte zelluläre Proliferation demonstrieren (Abb. 8). Im Einklang mit diesen Befunden übt IL-8 an mehreren Modellen des Lungenkarzinoms pro-proliferative Effekte in vitro und in vivo aus (26–29). Molekular könnte die auto- und parakrine Aktivierung der Chemokin-Rezeptoren CXCR1 und CXC2, die beide in A549-Zellen exprimiert werden, eine pro-proliferative Reaktion auslösen (93). Ebenfalls könnte IL-8 seine proliferativen Effekte, über die bereits beschriebene Transaktivierung des tumoreigenen EGFR induzieren (12). Eine funktionelle Rolle von IL-8 bei der LPS-induzierten zellulären Proliferation an Modellen des NSCLC wurde hier zwar nicht explizit untersucht; diese könnte aber durchaus ebenfalls von pathologischer Relevanz sein. In Tumorzellen unterschiedlicher Entitäten wurden nach Stimulation mit LPS pro-proliferative und anti-apoptotische Effekte für dieses Chemokin in Abhängigkeit einer Aktivierung des TLR4 beschrieben (94–96).

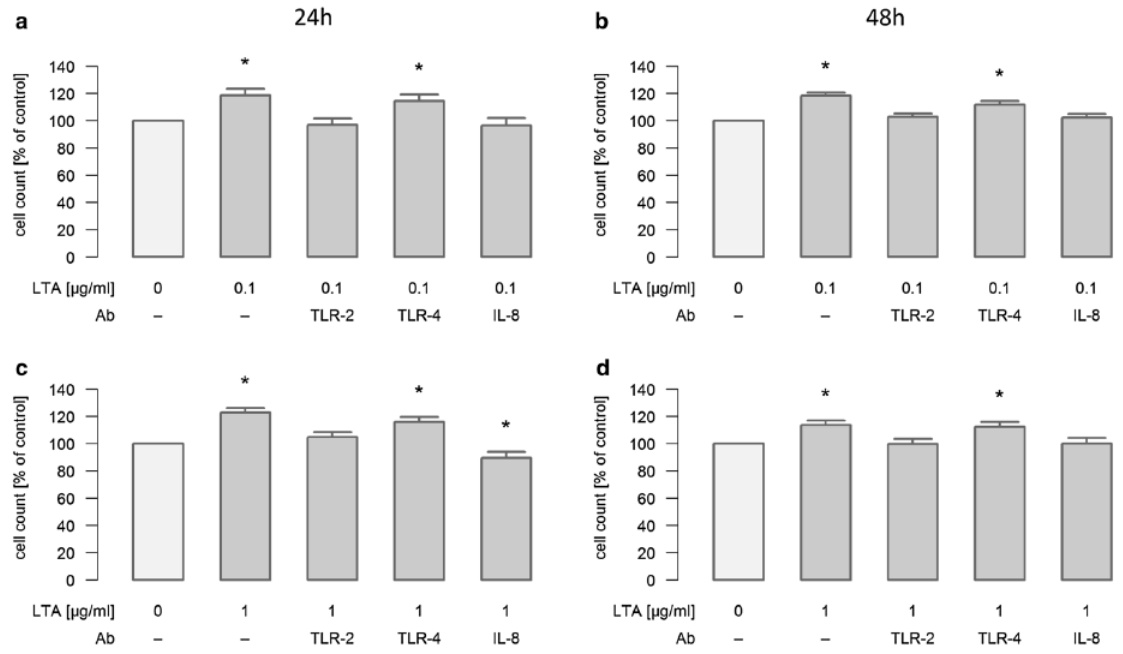

*Abbildung 8: Mechanismen der LTA-induzierten Proliferation von A549-Zellen. Stimulation von A549-Zellen mit 0.1 µg/ml LTA (a, b) oder 1 µg/ml LTA (c, d) in An- oder Abwesenheit neutralisierender Antikörper (Ab) gegen TLR2 (TL2.1, 10 µg/ml), TLR4 (HTA124, 10 µg/ml) und IL-8 (5 µg/ml). Messung der Proliferation durch automatisierte Zellzählung. Angabe der Zellzahl prozentual zur unbehandelten Kontrolle. *p < 0.05 vs. Kontrolle (modifiziert nach Anlage 2).*

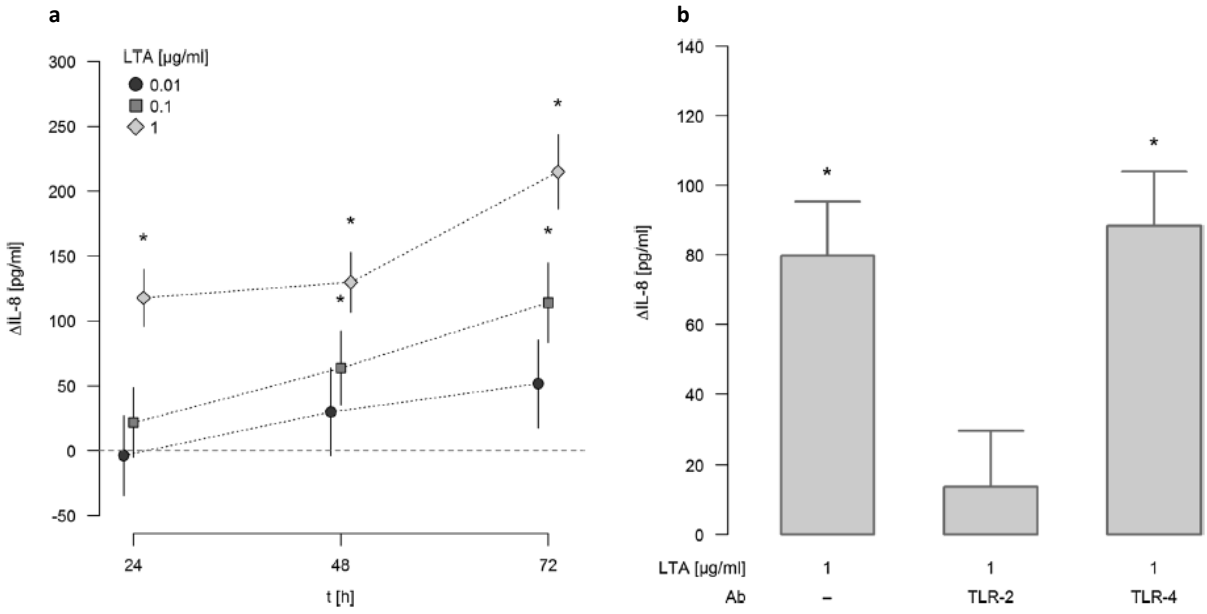

Abbildung 9: Zeit- und dosisabhängige Freisetzung von IL-8 nach Stimulation von A549-Zellen mit LTA – Rolle von TLR2. Stimulation von A549-Zellen mit 0.01–1 µg LTA über 24–72 h (a). bzw. 1 µg/ml LTA (b) für 48 h in Ab- oder Anwesenheit neutralisierender Antikörper gegen TLR-2 (TL2.1, 10 µg/ml) oder TLR-4 (HTA 125, 10 µg/ml) (B). Angabe von IL-8 als ΔIL-8, als Differenz zwischen LTA-stimulierter Freisetzung und Freisetzung aus der unstimulierten Kontrolle (modifiziert nach Anlage 2).

Um die in vitro erhobenen Effekte für Endotoxin auf deren Relevanz in vivo zu überprüfen, wurde sowohl ein Modell humaner Gewebekulturen von Lungenkarzinomproben als auch ein murines Xenograft-Modell herangezogen (Anlage 1).

Im humanen Adenokarzinomgewebe kommt es nach Stimulation mit LPS in etwa zu einer Verdopplung der Expression des Proliferationsmarkers Ki-67. Von einer basalen Expression von 7,5 Prozent positiver Zellen konnte nach LPS-Exposition eine Expression von 15 Prozent erzielt werden.

Eine repräsentative immunhistochemische Färbung ist in Abbildung 10 dargestellt.

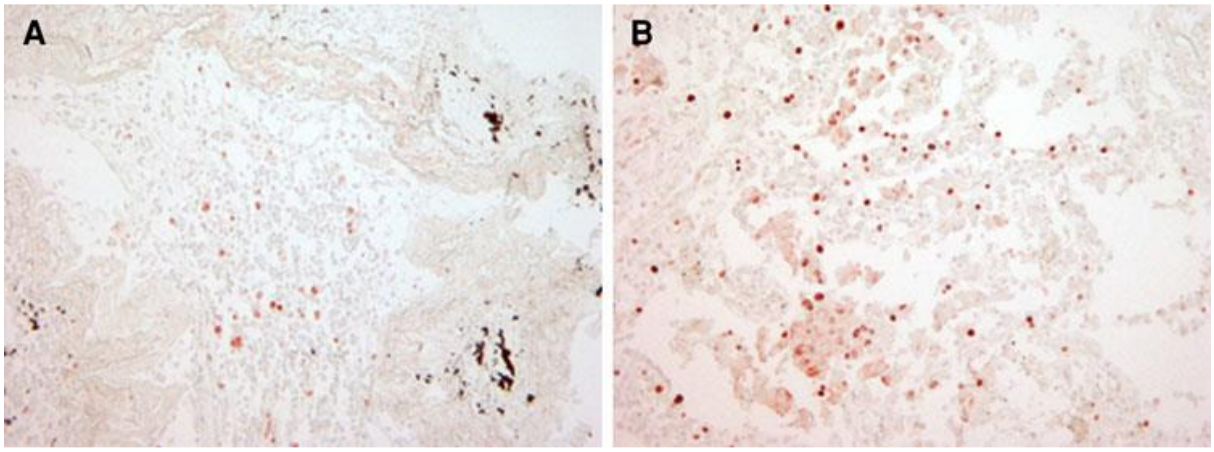

Abbildung 10: Expression von Ki-67 im humanen NSCLC-Gewebe nach Stimulation mit LPS. Humanes Adenokarzinomgewebe der Lunge wurde für 16 h in Ab- (A) oder Anwesenheit (B) von LPS (10 µg/ml) kultiviert. Die Expression von Ki-67 wurde immunhistochemisch dargestellt. Vergrößerung 200x (Anlage 1).

In einem murinen Tumor-Xenograft-Modell (BALBc/c nu/nu Maus) kommt es nach subkutaner Implantation von A549-Zellen zu einer Tumorbildung am Injektionsort, deren Ausdehnung mit digitalen Messschiebern quantifiziert werden kann. Die Tumorzellen wurden vor Implantation entweder in LPS-haltigem Medium oder in Kontrollmedium inkubiert. Nach Implantation LPS-stimulierter Tumorzellen kam es zu einem schnelleren Anstieg der Tumorvolumina verglichen mit Tumorzellen, die lediglich mit Kontrollmedium versetzt wurden. Die Vergrößerung der Tumorvolumina in der mit LPS behandelten Gruppe war bereits am Tag 4 nach Implantation evident und hielt über den Beobachtungszeitraum von zwölf Tagen an. Diese Effekte waren von einer gesteigerten Expression des Proliferationsmarkers Ki-67 in LPS-exponierten Tumoren begleitet (Abb. 11).

Selbstverständlich bilden die genannten kurzzeitigen Effekte von Endotoxin sowie von Lipoteichonsäuren auf die Tumorzellproliferation in vitro, ex vivo und in vivo nicht das komplexe Tumorgeschehen im humanen Lungenkarzinom ab. Jedoch wurde kürzlich in einem murinen orthotopen Tumormodell ein über 180 Tage anhaltender protumorigener Effekt repetitiver LPS-Inhalationen gezeigt (97).

Die Tatsache, dass Endotoxin auch in den zuletzt genannten Langzeitmodellen in Mäusen einen nachhaltigen pro-proliferativen Effekt ausübt, bestätigt die biologische Relevanz der hier dargestellten pro-proliferativen Effekte von Endotoxin auf die Tumorbiologie in vivo. Ebenfalls konnte in Tiermodellen gezeigt werden, dass eine intratracheale Injektion von LTA über einen TLR2-abhängigen Mechanismus die Adhäsion und hepatische Metastasierung von

NSCLC-Zellen induziert. So findet auch die biologische Relevanz der hier in vitro erhobenen pro-proliferativen Effekte von Lipoteichonsäuren in einem Tiermodell Bestätigung (84).

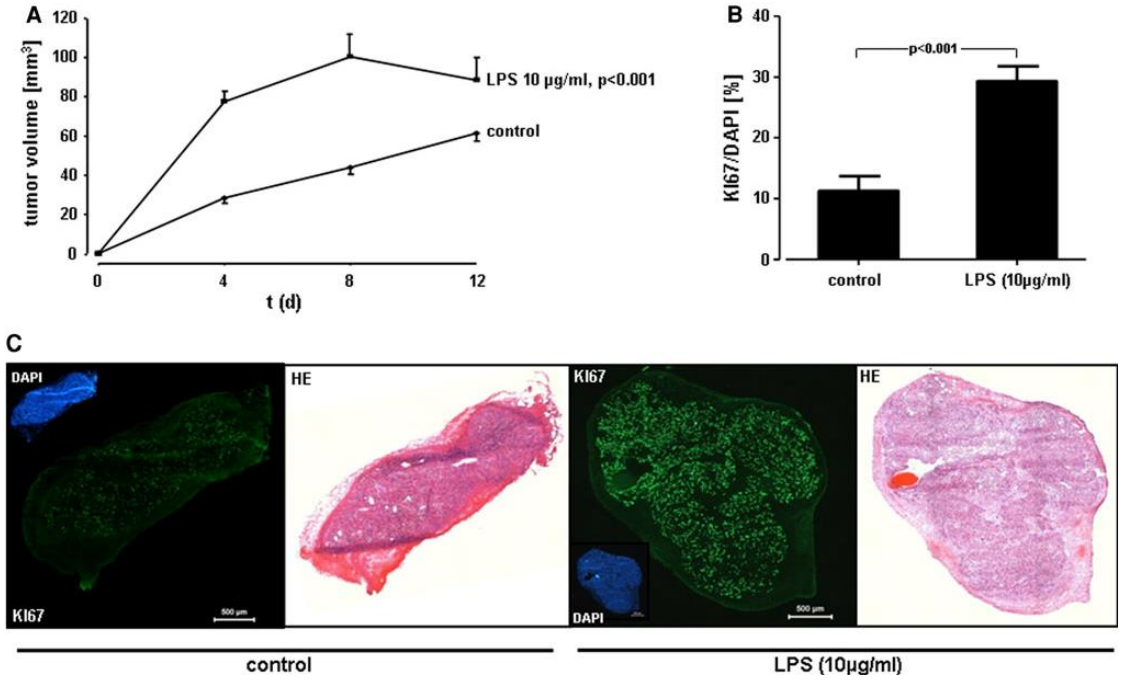

Abbildung 11: Pro-proliferativer Effekt von LPS in vivo (Anlage 1). A) Tumorwachstum in vivo: Inkubation von A549-Zellen mit 10 µg/ml LPS oder Kontrollmedium. Subkutane Injektion in acht Wochen alte BALBc nu/nu-Mäuse. Quantifizierung der Tumorvolumina [mm³] zu den angegebenen Zeitpunkten mit digitalen Messschiebern. B) Immunfluoreszenztechnische Analyse der Ki-67-Expression in Kryokonservaten explantierter Tumore, die wie unter A beschrieben, behandelt wurden. Ki-67 ist prozentual zu DAPI angegeben. C) Repräsentative Abbildung einer Immunfluoreszenz- und HE-Färbung.

Bakterielle Pathogenitätsfaktoren induzieren eine Strahlenresistenz an NSCLC-Zelllinien

Bakterielle Infekte limitieren die Prognose des NSCLC. Dies könnte neben einer direkten Induktion der Tumorzellproliferation auch in einer Therapieresistenz begründet sein. Etwa 60 Prozent aller Patienten mit Lungenkrebs erhalten im Verlauf der Erkrankung eine Strahlentherapie in kurativer oder palliativer Intention (1, 98).

Vor diesem Hintergrund wurde der Effekt von Endotoxin auf die Effizienz der Strahlentherapie untersucht. Um eine mögliche Rolle des EGFR darzustellen, fanden die EGFR-Wildtyp-Zelllinie A549, die EGFR-mutierte Zelllinie H1975 sowie die EGFR-defiziente Zelllinie H520 Verwendung. Diese Zellen wurden vor einer Photonenbestrahlung mit unterschiedlichen Konzentrationen von Endotoxin inkubiert. Nach der Bestrahlung wurde die Fähigkeit der Zellen, Kolonien zu bilden, was den Goldstandard für die Quantifizierung der Strahlensensibilität darstellt, gemessen. Hier induzieren Endotoxine eine Strahlentherapieresistenz in A549- und H1975-Zellen, nicht jedoch in EGFR-defizienten H520-Zellen (Anlage 3). Zudem war die durch Endotoxin-induzierte Strahlenresistenz in Anwesenheit eines EGFR-Inhibitors aufgehoben (Abb. 12). Passend dazu zeigte sich ebenfalls die Induktion der zellulären Proliferation durch LPS (Abb. 6) als abhängig von einer EGFR-Aktivierung. Vor dem Hintergrund der Vermutung, dass der EGF-Rezeptor eventuell einen kritischen Ko-Rezeptor für Endotoxin darstellen könnte (91, 92), lässt sich die Abhängigkeit

der LPS-induzierten Proliferationsreaktion und der Strahlentherapieresistenz vom EGFR erklären. Im Allgemeinen ist die Rolle des EFGR bei der Strahlenresistenz gut belegt (99–101). Insbesondere induziert eine Aktivierung des Rezeptors eine vermehrte Reparatur von DNA-Doppelstrangbrüchen, deren Induktion ein Grundprinzip der Photonenwirkung darstellt (9). Therapeutisch kommen EGFR-Tyrosinkinase-Inhibitoren in der First-Line-Therapie des NSCLC zum Einsatz, wenn aktivierende Mutationen des EGFR vorliegen (10, 11). Der Problematik der Resistenzentwicklung auf diese Tyrosinkinase-Inhibitoren im Laufe der Therapie kann durch Einsatz von Inhibitoren der dritten und vierten Generation begegnet werden. In eigenen Untersuchungen zeigte der Drittgenerations-EGFR-Inhibitor Osimertinib auch bei einer seltenen EGFR-Mutation durchaus langanhaltende Wirkung (102).

Neben der Rolle des EGFR konnte in den Untersuchungen zur LPS-induzierten Strahlenresistenz die in diesem Zusammenhang involvierte Signaltransduktion näher charakterisiert werden und eine zentrale Rolle der phosphorylierten (p) Form des Transkriptionsfaktors „cAMP response element binding“ (CREB) bei der Induktion der Strahlentherapieresistenz herausgearbeitet werden, da pCREB nach LPS Behandlung an bestrahlten Zellen heraufreguliert wird (Anlage 3) und ein CREB-binding Protein-Inhibitor (CBPI) sowohl die LPS-induzierte Strahlentherapieresistenz inhibiert als auch die durch Bestrahlung und LPS verursachten DNA-Doppelstrangbrüche vervielfacht (Abb. 13). Hier kommt es sogar zu einer Strahlensensibilisierung, da die DNA-Doppelstrangbrüche um ein Mehrfaches der bestrahlten Kontrolle gesteigert werden. Passend zu diesen Befunden wurde die Aktivierung von CREB mit einer Ausbildung einer Strahlenresistenz bei Tumoren unterschiedlicher Entitäten in Verbindung gebracht (103). Vorläufige eigene Untersuchungen zeigen, dass auch Lipoteichonsäuren eine Strahlenresistenz in NSCLC auslösen, was ein pathogenetisches Grundprinzip vermuten lässt. Auch scheint LPS die Effektivität einer Cisplatin-haltigen Therapie in vorläufigen Untersuchungen zu inhibieren (s. Kap. V, Abstract 8).

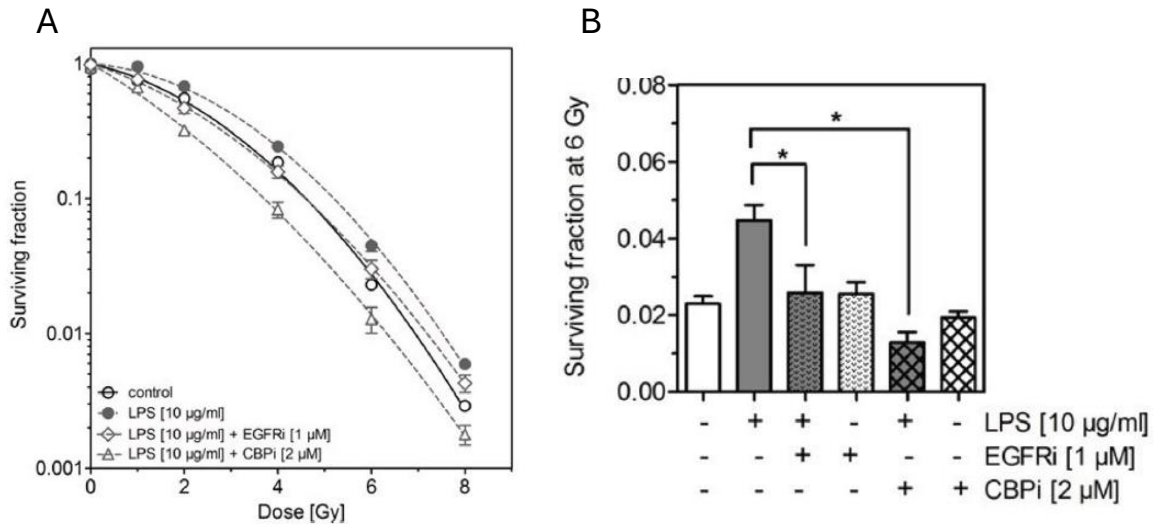

*Abbildung 12: LPS-induzierte Strahlenresistenz an H1975-Zellen – funktionelle Rolle von EGFR und pCREB. Stimulation von H1975-Zellen mit LPS vor Photonen-Bestrahlung in An -oder Abwesenheit eines EGFR-Inhibitors (EGFR-i, AG1478, 1 µM) oder eines CREB-Inhibitors (CBPi, CREB-binding protein inhibitor ICG-001, 2 µM). A) Überlebenskurven, B) Survival bei 6 Gy. *p < 0.05 (modifiziert nach Anlage 3).*

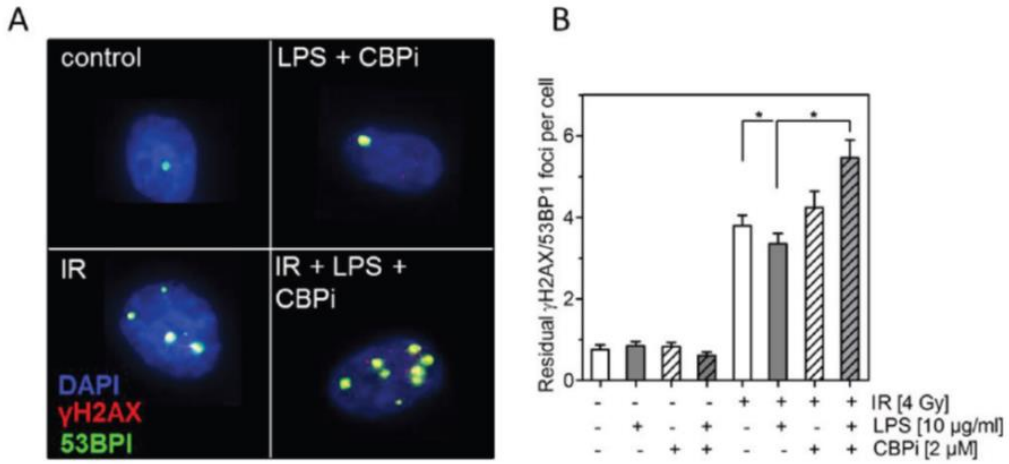

*Abbildung 13: Strahlensensibilisierung und Induktion von DNS-Doppelstrangbrüchen nach CREB-Inhibition. Bestrahlung von H1975-Zellen mit 4Gy. Quantifizierung der residuellen γ-H2AX/BP53 Foci nach 24 h. A) Repräsentative Immunfluoreszenz mit Darstellung der γ-H2AX/BP53 Foci in unbehandelten (control), bestrahlten (IR) und bestrahlten, LPS-behandelten Zellen in Anwesenheit des CREB-Inhibitors (IR+LPS+CBPi) B) Zählung der residuellen γ-H2AX/BP53 Foci von mindestens 200 Zellkernen der unbestrahlten, bestrahlten und mit LPS (10 µg/ml) behandelten H1975-Zellen in An- oder Abwesenheit des CREB-Inhibitors CBPI. *p < 0.05 bezogen auf die jeweilige, durch Balken angegebene Gruppe (modifiziert nach Anlage 3).*

Zusammenfassend üben die bakteriellen Pathogenitätsfaktoren Lipopolysaccharid und deren grampositive Äquivalente Lipoteichonsäuren einen spezifischen, CD14- und TLR-mediierten pro-proliferativen Effekt auf verschiedene NSCLC-Zelllinien aus. Die Aktivierung der Tumorzellproliferation ist über inflammatorische Mediatoren, wie COX-2-abhängige Eicosanoide und IL-8 getriggert. Wesentliche pro-proliferative Effekte konnten in einem Ex-

vivo-Assay an humanem Lungenkarzinomgewebe sowie in einem Mausmodell reproduziert werden. Zudem löst LPS eine Strahlentherapieresistenz aus. Diese ist abhängig von EGFR und dem Transkriptionsfaktor CREB. Somit können bakterielle Pathogenitätsfaktoren durch eine Aktivierung tumorintrinsicher Rezeptoren und inflammatorischer Mediatoren eine Steigerung der Tumorzellproliferation und Induktion einer Therapieresistenz in NSCLC auslösen. Diese Mechanismen könnten die Prognose des NSCLC limitieren.

Bakterielle Pathogenitätsfaktoren als Auslöser inflammatorischer granulozytärer Reaktionen

Bei einer bakteriellen pulmonalen Infektion repräsentieren neutrophile Granulozyten (PMN) die erste Verteidigungslinie des Körpers. Sie sind befähigt, das vaskuläre System nach Adhärenz zu durchwanden und an einen Inflammationsherd zu migrieren. Dies wird einerseits induziert durch exogene Faktoren, wie N-formylierte Oligopeptide, die aus der bakteriellen Zellwand liberiert werden und als potente Chemotaxine agieren. Andererseits werden im Rahmen der Infektion endogene chemotaktische Substanzen liberiert. Hierzu zählt z. B. das CXC-Chemokin IL-8, das nach Interaktion von bakteriellen Pathogenitätsfaktoren mit PMN, Monozyten oder Zellen epithelialen Ursprungs, u. a. auch Tumorzellen (s. Anlagen 2, 4–6, 8) synthetisiert werden kann. Auch das von myeloiden Zellen synthetisierte LTB_4 stellt ein potentes Chemotaxin dar (32, 40). Der Aktivierung von PMN durch bakterielle Pathogenitätsfaktoren könnte daher eine entscheidende Rolle im inflammatorischen Tumormikromilieu unter infektiösen Bedingungen zukommen, zumal diese Zellen quantitativ eine Hauptkomponente des TME darstellen (48). Vor diesem Hintergrund wurde im Folgenden auf die Interaktion zwischen bakteriellen Pathogenitätsfaktoren und neutrophilen PMN eingegangen. Charakterisiert werden sollte die Aktivierung inflammatorischer Reaktionen durch die bakteriellen Pathogenitätsfaktoren Lipopolysaccharide und Lipoteichonsäuren, wobei der Fokus auf die Synthese chemotaktischer Substanzen wie IL-8 und LTB_4 gelegt wurde.

Bakterielle Pathogenitätsfaktoren aktivieren die granulozytäre Zytokinsynthese

Inkubiert man humane PMN mit steigenden Konzentrationen hoch aufgereinigter Lipoteichonsäuren, so zeigt sich eine klare, dosisabhängige Induktion der Synthese von IL-8 von bis zu etwa 10 ng/ml (Abb. 14A, Anlage 4). Diese beruhte auf einer Aktivierung der De-novo-Proteinbiosynthese, wie der inhibitorische Effekt des Translationsinhibitors Cycloheximid (CHX) zeigt. IL-8 wurde hierbei biphasisch freigesetzt, mit einer frühen Freisetzung geringer Mengen dieses Chemokins und später Akkumulation gewaltiger Mengen von bis zu 10 ng/ml. Der Synthese von IL-8 ging die Bildung von TNF und IL-1 voraus (Abb. 14B). Diese sequenzielle Induktion der Zytokinsynthese wurde bereits in Endotoxin-stimulierten PMN beschrieben (104) und auch in weiteren eigenen Arbeiten bestätigt (Anlage 5).

Interessanterweise induzierte TNF, das früh liberiert wurde, vor allem die späte Phase der Freisetzung von IL-8, während IL-1 nicht in diesen Prozess involviert schien, wie Experimente mit neutralisierenden Antikörpern zeigten (Abb. 15A). Auch auf mRNA-Ebene wurde die Induktion von IL-8 und der neutralisierende Effekt von TNF-Antikörpern bestätigt (Abb. 15B). Für die LPS-induzierte Aktivierung der Synthese von IL-8 wurde im Gegensatz dazu in den hier

vorgelegten Untersuchungen ein synergistischer Effekt von TNF und IL-1 detektiert (Abb. 16). Parallel wurden TNF und IL-1 ebenfalls in den Zellüberstand LPS-stimulierter PMN freigesetzt (Abb. 20).

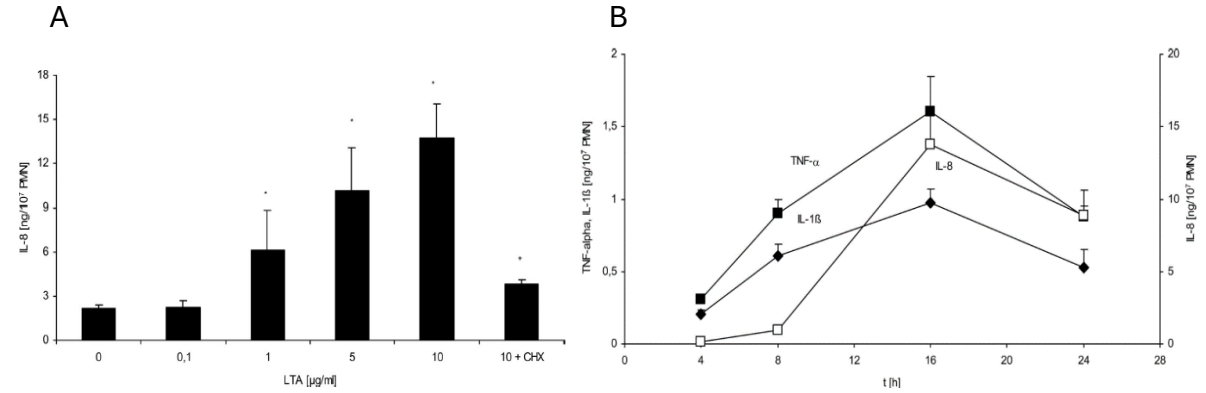

Abbildung 14: Aktivierung der granulozytären Zytokinsynthese durch Stimulation mit LTA (Anlage 4).
*A) Dosisabhängige Aktivierung der IL-8 Synthese in PMN durch LTA nach 16 h. IL-8 in ng/10^7 PMN/ml. CHX: Cycloheximid (1 µg/ml) *p < 0.05*
B) Sequenzielle Induktion der Synthese von TNF, IL-1, und IL-8 durch LTA (10 µg/ml).

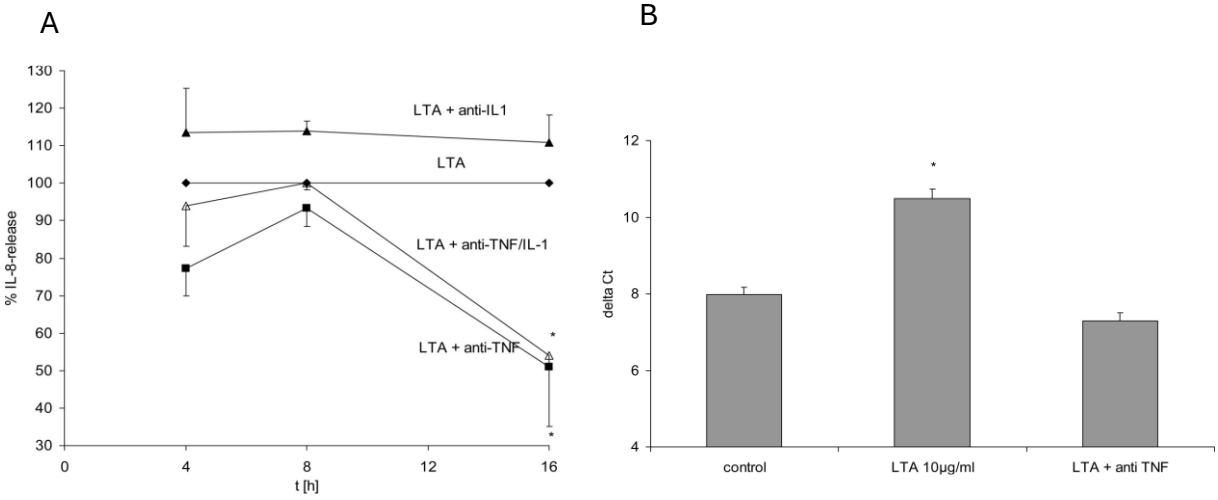

Abbildung 15: Autokrine Rolle von TNF bei der durch LTA induzierten Synthese von IL-8 (Anlage 4).
*A) Stimulation von PMN (10^7/ml) über unterschiedliche Zeiträume mit LTA (10 µg/ml) in Ab- oder Anwesenheit neutralisierender Antikörper gegen TNF und IL-1. Angabe von IL-8 als prozentualer Wert der durch 10 µg/ml LTA induzierten Freisetzung (100 %). Anti-TNF-AK und anti-IL-1-AK: 5 µg/ml. *p < 0.05 vs. LTA.*
*B) Achtstündige Stimulation von PMN (10^7/ml) mit LTA (10 µg/ml) oder Kontrollmedium in Ab- oder Anwesenheit neutralisierender TNF-Antikörper (5 µg/m, LTA + antiTNF) delta Ct: relative IL-8 mRNA-Expression bezogen auf HPRT. *p < 0.05 vs. Kontrolle.*

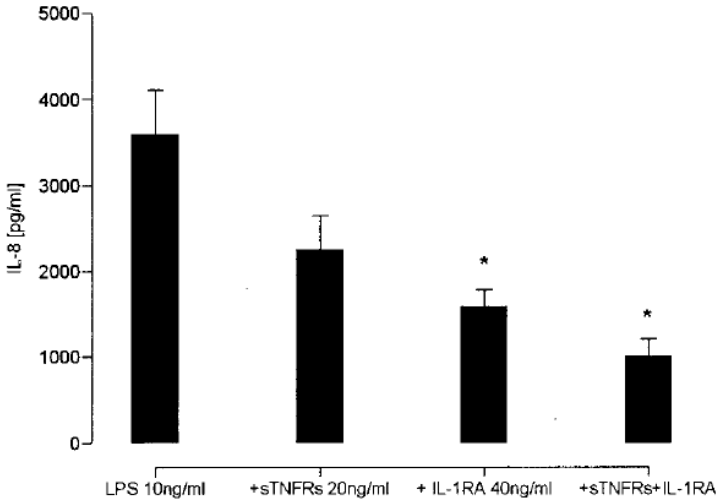

*Abbildung 16: TNF- und IL-1-abhängige Freisetzung von IL-8 aus LPS-stimulierten PMN. Stimulation isolierter PMN (10^7/ml) mit 10 ng/ml LPS über 16 h in An- oder Abwesenheit löslicher TNF-Rezeptoren (sTNFRs) oder IL-1-Rezeptorantagonisten (IL-1RA). *p < 0.05 vs. LPS (Anlage 5).*

Somit scheint die Synthese von IL-8 durch beide Pathogenitätsfaktoren jeweils differenziell reguliert zu sein, wobei die LTA-induzierte Chemokinfreisetzung lediglich von TNF abhängt, während im Falle von LPS sowohl TNF als auch IL-1 über einen autokrinen Mechanismus synergistisch die Chemokinfreisetzung mediieren.

Neben diesen mechanistischen Divergenzen unterschieden sich die durch LPS und LTA induzierte Zytokinsynthese auch quantitativ: während LPS, das in diesen Untersuchungen in einer Konzentration von 10 ng/ml eingesetzt wurde, eine Freisetzung von etwa 3–4 ng/ml IL-8 nach 16-stündiger Inkubation induzierte, waren zum Erreichen einer vergleichbaren Menge an IL-8 etwa 100-fach höhere Konzentrationen von LTA nötig (Abb. 14). Auch in einer weiteren eigenen Arbeit war zur Induktion der Synthese von IL-8 in PMN und Monozyten LPS der weitaus effektivere Stimulus (Anlage 6). Dies steht im Einklang von Untersuchungen von Draing et al., die das niedrigere immunogene Potenzial von LTA versus LPS zur Induktion einer Zytokinfreisetzung beschreiben (105). Obwohl mittlerweile zahlreiche Arbeiten eine Zytokininduktion durch LPS und LTA an PMN zeigen, war gerade das immunogene Potenzial von LTA zum Zeitpunkt der Publikation (Anlage 4) ein relativ neu entdecktes Phänomen und die autokrine Rolle von TNF bei der Freisetzung von IL-8 in diesem Zusammenhang noch nicht beschrieben.

Analog zur Aktivierung durch LTA zeigt sich die LPS-induzierte Freisetzung von IL-8 an neutrophilen Granulozyten und Monozyten abhängig von der Ligation von CD14 (Tabelle 2, Anlage 6). Erhöht sich die Expression von CD14, wie sie hier durch Vorstimulation von Monozyten und Granulozyten mit den in der Granulomatose mit Polyangiitis pathogenetisch relevanten Antikörpern gegen Proteinase-3 (c-ANCA) erzielt wurde (Abb. 17), zeigt sich korrespondierend eine Amplifikation der LPS- und LTA-induzierten Chemokinsynthese (Tabelle 2). Dieser Effekt von c-ANCA war spezifisch für die zelluläre Aktivierung durch LPS und LTA (Anlage 6) und konnte durch Neutralisation von CD14 aufgehoben werden (Tabelle 2).

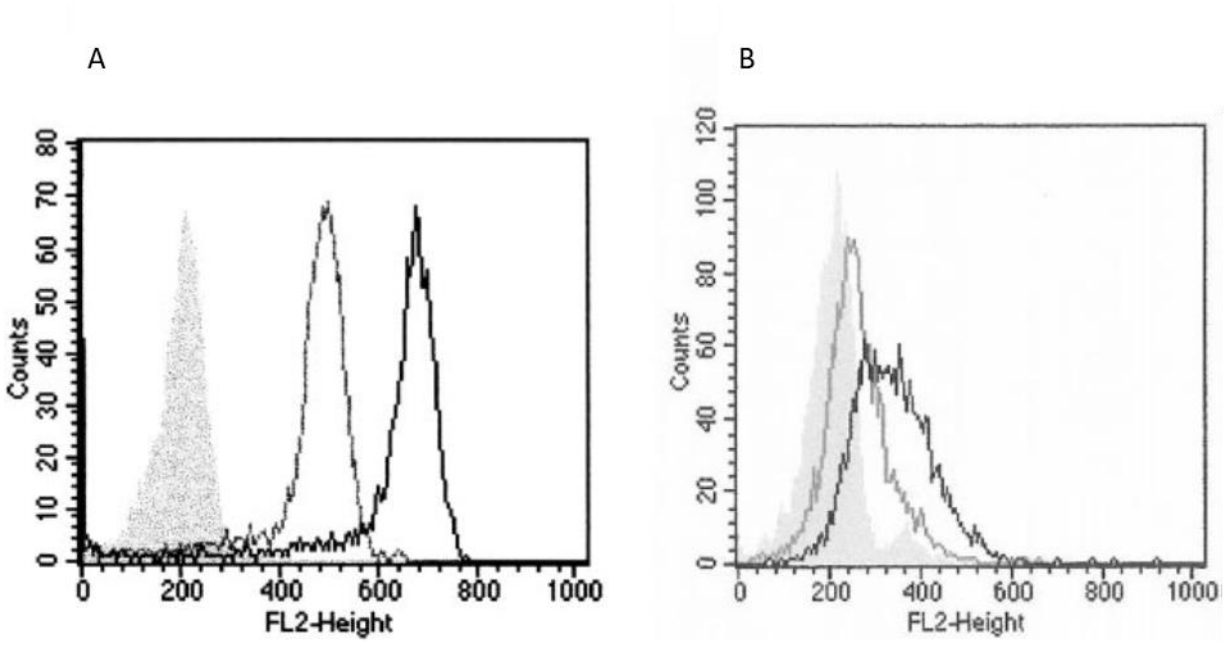

Abbildung 17: Regulation der Membranexpression von CD14 an Monozyten (A) und PMN (B). Stimulation humaner Monozyten (0.5 x 10^6/ml) bzw. PMN (5 x 10^6/ml) mit Kontroll-IgG (1 µg/ml, hellgraue Kurve) oder c-ANCA (murine monoklonale Antikörper gegen Proteinase-3, 1 µg/ml, dunkelgraue Kurve). Gefüllte hellgraue Kurve: Isotyp-Kontrolle. Repräsentative Durchflusszytometrie (Anlage 6).

*Tabelle 2: Effekt von c-ANCA auf die LPS-induzierte Freisetzung von IL-8 und inhibitorische Effekte eines Anti-CD14-Antikörpers (LPS: 10 ng/ml, c-ANCA murine monoklonale Antikörper gegen Proteinase-3 1 µg/ml, anti-CD14 (MY-4) 5 µg/ml). IL-8 in ng/ml. * p < 0.05 vs. LPS (modifiziert nach Anlage 6).*

	Kontrolle	LPS	LPS + anti-CD14
Monozyten			
Kontrolle	7.15 ± 2.25	124.23 ± 3.45	21.43 ± 1.54*
c-ANCA	28.69 ± 1.56	208.32 ± 16.94	28.25 ± 1.25*
PMN			
Kontrolle	0.45 ± 0.12	7.54 ± 0.86	1.88 ± 0.14*
c-ANCA	0.93 ± 0.24	14.2 ± 2.01	4.43 ± 0.41*

Die Expression von CD14 an PMN scheint kein stationäres Geschehen zu sein, sondern einer differenzierten Regulation zu unterliegen. Unter inflammatorischen Bedingungen kann CD14 aus intrazellulären Kompartimenten an die Zelloberfläche transloziert werden (106). Interessanterweise wurde sowohl für eine Stimulation mit Endotoxin (107) als auch mit Lipoteichonsäuren Änderungen des leukozytären Phänotyps mit einer Hochregulation von CD14 beschrieben (108). Diese verstärkte Membranexpression sollte, wie in Anlage 6 beschrieben, in einer beschleunigten Reaktivität mit amplifizierten Zytokinsynthese nach Stimulation mit LPS und LTA resultieren, sodass die bakteriellen Pathogenitätsfaktoren per se die durch sie induzierten inflammatorischen Effekte verstärken können.

CD14, nicht jedoch TLR2 stellt sich ebenfalls als Schlüsselmolekül bei der durch LTA ausgelösten Aktivierung der granulozytären Zytokinsynthese dar, was durch den inhibitorischen Effekt eines CD14-Antikörpers belegt werden konnte (Abb. 18). Auch in

eigenen Untersuchungen in einem experimentellen Modell der septischen Kardiomyopathie ließen sich die kardiodepressiven Effekte von Lipoteichonsäuren, die ebenfalls durch die Freisetzung von TNF mediiert werden, durch Neutralisation von CD14 aufheben (109). Diese Befunde unterstreichen die Bedeutung von CD14 bei der zellulären Aktivierung durch Lipoteichonsäuren.

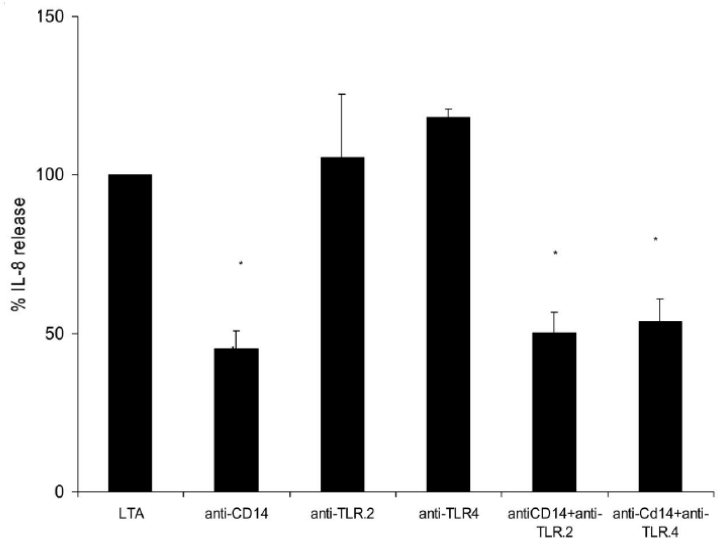

*Abbildung 18: Mechanismen der LTA-induzierten Zytokinfreisetzung. Stimulation isolierter PMN (10^7/ml) mit 10 µg/ml LTA für 16 h in Ab- oder Anwesenheit funktionsblockierender Antikörper gegen CD14 (MY-4, 5 µg/ml), TLR2 (TL2.1, 10 µg/ml), TLR4 (HTA125, 10 µg/ml oder Kombinationen der CD14 und TLR-Antikörper. IL-8 als prozentualer Wert der durch LTA-induzierten Freisetzung (100 %). * p < 0.05 vs. LTA (Anlage 4).*

Weswegen neutralisierende Antikörper gegen TLR2 in unserem Modell in PMN keinen inhibitorischen Effekt auf die LTA-induzierte Zellaktivierung haben, ist abschließend nicht geklärt. Obwohl der CD14-TLR2-Rezeptorkomplex als essenziell für die Aktivierung eukaryonter Zellen durch Lipoteichonsäuren gilt (67, 68), zeichnet die Literatur zur Rolle von TLR2 in der Granulozytenaktivierung durch grampositive Pathogenitätsfaktoren kein einheitliches Bild: Während Draing (105) und Lotz (110) eine klare Abhängigkeit der LTA-induzierten PMN-Aktivierung von TLR2 beschreiben, stellt Ellingsen (111) dies in Frage, da zwar eine Neutralisation von CD14, aber nicht die ebenfalls hier eingesetzten neutralisierenden Antikörper gegen TLR2 einen inhibitorischen Effekt auf die Chemokinsynthese ausüben, was die hier vorliegenden Daten bestätigt.

Die divergierenden Daten können unter anderem auch auf die Isolationsmethode der PMN zurückzuführen sein. In den hier vorgelegten Untersuchungen wurden PMN durch ein steriles Verfahren isoliert, das keinerlei Voraktivierung der PMN erwarten lässt. Kurt-Jones et al. konnten zeigen, dass ruhende PMN, wie sie hier durch sterile Isolation vorliegen, nur sehr geringe Mengen an TLRs exprimieren (112). Die Expression dieser Rezeptoren kann erst durch eine Vorstimulation wie Gabe von LPS oder G-GSF heraufreguliert werden (112), was eine weitere Erklärung für den hier fehlenden inhibitorischen Effekt der hier eingesetzten TLR2-Antikörper bietet.

Zusammenfassend induzieren sowohl LPS als auch LTA über einen CD14-abhängigen Mechanismus die Synthese der proinflammatorischen Zytokine TNF, IL-1 und IL-8 in PMN, wobei die LPS-induzierte Synthese von IL-8 durch die „frühen" Zytokine TNF und IL-1 autokrin mediiert wird. Für die durch LTA stimulierte Freisetzung von IL-8 scheint lediglich TNF notwendig. Zahlreiche Studien belegen eine entscheidende Beteiligung dieser und anderer Zytokine und Wachstumsfaktoren in der Tumorproliferation (21, 24, 113). Da PMN im TME eine dominante Zellpopulation darstellen, könnten Granulozyten-abhängige Zytokine erheblich zur Tumorprogression im Tumormikromilieu beitragen. Die pathogenetische Bedeutung dieser proinflammatorischen Zytokine, insbesondere von IL-1 wird durch die klinischen Effekte des IL-1-Inhibitors Canakinumab auf das inzidentell auftretende Lungenkarzinom eindrucksvoll demonstriert (114).

Priming inflammatorischer granulozytärer Reaktionen durch bakterielle Pathogenitätsfaktoren

Neutrophile Granulozyten existieren prinzipiell in drei Aktivitätszuständen: ruhend, voraktiviert (geprimt) und aktiviert, die man in etwa mit den klassischen Ampelphasen rot – gelb – grün vergleichen kann (115). Priming versetzt den Neutrophilen in einen voraktivierten Zustand, um bei Kontakt mit einem zweiten Stimulus das volle granulozytäre Abwehrpotenzial zu entfalten. Dieser voraktivierte Status geht mit einer Konformitätsänderung der Zellen wie z. B. der Translokation cytosolischer Komponenten der NADPH-Oxidase an die Zellmembran einher als Voraussetzung für die Freisetzung von Sauerstoffradikalen, den sog. Respiratory Burst. Hier werden in erster Linie O_2^- und Hypochlorsäure liberiert. In Modellen des Mamma- und Lungenkarzinoms wurden Neutrophile in einem voraktivierten Zustand detektiert (116, 117). Interessanterweise beschreibt unter anderem eine aktuelle Studie von Rocks et al., dass durch Ozon voraktivierte PMN initiale Schritte der intrapulmonalen Metastasierung von Tumorzellen über die Bildung von NETs verursachen (55).

Dem Phänomen des Primings inflammatorischer granulozytärer Reaktionen könnte also ebenfalls eine protumorigene und pro-proliferative Rolle zukommen. Daher seien an dieser Stelle Untersuchungen zum Priming-Effekt von LPS auf die granulozytäre Sauerstoffradikalsekretion und die Leukotriensynthese dargestellt. In diesen Untersuchungen wurde ein möglicher Effekt von 5-Lipoxygenase-Mediatoren auf das Priming des Respiratory Burst adressiert.

Isolierte PMN wurden nach einer Vorstimulation mit Endotoxin oder Kontrollmedium mit dem bakteriellen Tripeptid N-Formyl-Methionin-Leucin-Phenylalanin (fMLP) aktiviert und die Induktion des Respiratory Burst sowie die Synthese von LTB_4 quantifiziert. Abbildung 19 (Anlage 7) zeigt, dass nach einer Exposition mit LPS die fMLP-induzierte Synthese von LTB_4 sowie die Sauerstoffradikalfreisetzung erheblich gesteigert waren. Auch der Priming-Effekt von LPS war abhängig von einer Ligation des CD14-Rezeptors, im Einklang mit früheren Studien (118) und eigenen Untersuchungen (Anlagen 1, 6). Die gesteigerte Sekretion von Sauerstoffradikalen könnte selbstverständlich in erster Linie zytotoxische Effekte auf benachbarte Tumorzellen haben; jedoch wurden für O_2^- und Hypochlorsäure auch genotoxische Eigenschaften im Sinne einer Protumorigenität postuliert (51).

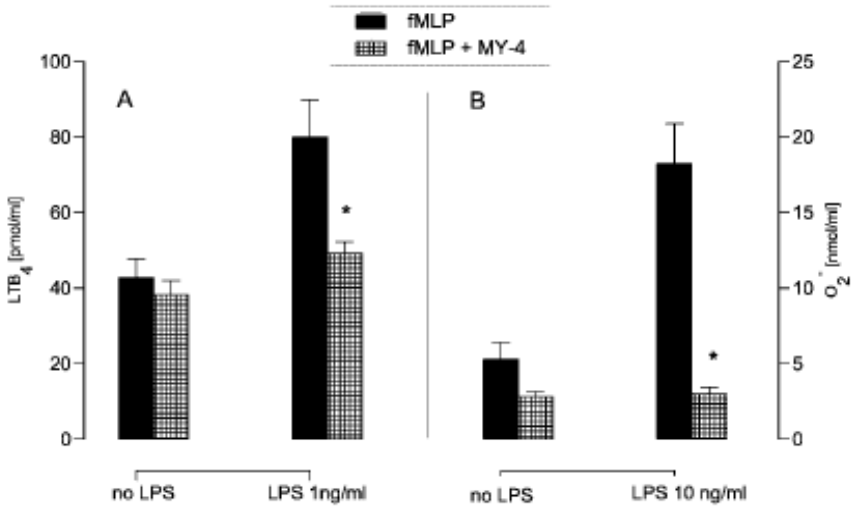

*Abbildung 19: LPS-induziertes Priming der granulozytären Leukotriensynthese (A) und Sauerstoffradikalfreisetzung (B) – Abhängigkeit von CD14. Vor-Inkubation von 10^7 PMN/ml mit 1–10 ng/ml LPS oder Kontrollmedium in Ab- oder Anwesenheit eines CD14-Antikörpers (MY-4, 5 µg/ml). Aktivierung mit fMLP 1 µM; Gabe von AA 10 µM zur Quantifizierung der Leukotriensynthese anhand der LTB_4 Freisetzung pg/ml (A). Messung des Respiratory Burst anhand der O_2-Sekretion in nmol/ml (B). * $p < 0.05$ vs. LPS (Anlage 7).*

Da die Verfügbarkeit freier Arachidonsäure (AA) eine kritische Determinante bei der Leukotriensynthese in PMN darstellt (118), erfolgte die Stimulation der PMN zur Induktion der Leukotriensynthese in Gegenwart freier AA. Unter inflammatorischen Bedingungen wird AA, u. a. auch durch Endotoxin, durch Aktivierung der Phospholipase A_2 von myeloiden, epithelialen und endothelialen Zellen freigesetzt (119, 120). Interessanterweise finden sich auch bis um das Dreifache erhöhte Spiegel freier AA in den Seren von Patienten, die an einem Adenokarzinom der Lunge leiden im Vergleich zu gesunden Kontrollpersonen (121). Auch das TME des Lungenkarzinoms und des Ovarialkarzinoms ist von der Präsenz hoher Spiegel an freier AA geprägt (86, 87). So scheint dieses Substrat der Cyclo- und Lipoxygenasen auch tatsächlich im Tumormikromilieu vorhanden zu sein und könnte zu den protumorigenen Mediatoren PGE_2 und LTB_4 verstoffwechselt werden.

Bemerkenswerterweise war unter Substitution freier AA die Vorstimulation durch LPS für die Aktivierung des Respiratory Burst wesentlich effektiver (Abb. 20). Dies und die Tatsache, dass das Priming des Respiratory Burst durch den spezifischen Inhibitor des 5-Lipoxygenase-aktivierenden Proteins (FLAP) MK-886 in LPS-vorstimulierten PMN auch in Anwesenheit freier AA wieder auf das Niveau der entsprechenden Kontrollen ohne FLAP-Inhibitor reduziert ist (Abb. 20), lässt eine entscheidende Rolle von 5-LO-Metaboliten, in erster Linie LTB_4, bei der Aktivierung des destruktiven granulozytären Potenzials vermuten. Solch autokrine Effekte von LTB_4 wurden für die Aktivierung von PMN in der Literatur (122, 123) und in eigenen Untersuchungen beschrieben (124).

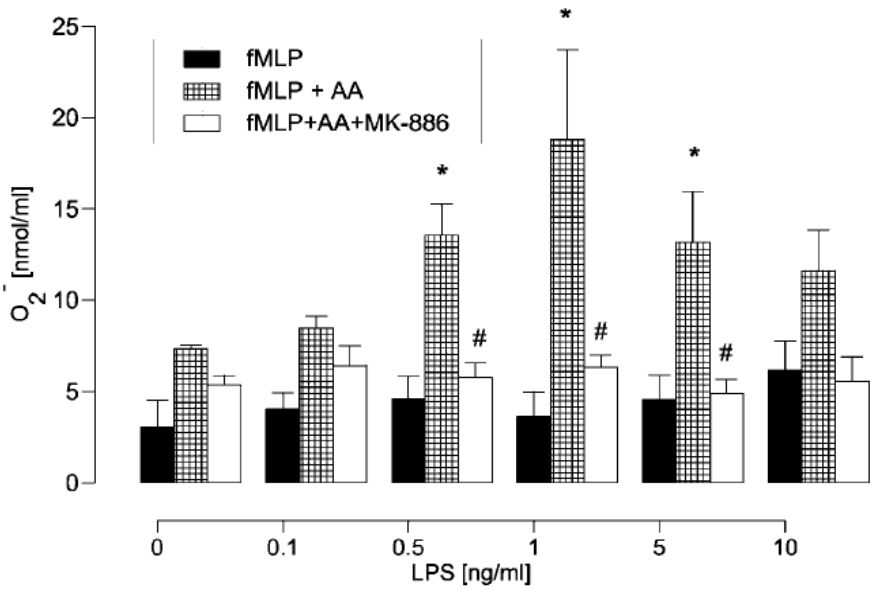

*Abbildung 20: Amplifizierung des LPS-abhängigen Priming des Respiratory Burst durch freie Arachidonsäure (AA) und Inhibition durch den FLAP-Inhibitor MK-886. Vor-Inkubation von 10^7 PMN/ml mit LPS in ansteigenden Konzentrationen oder Mediumkontrolle. Aktivierung mit fMLP 1 µM in Abwesenheit (fMLP) oder Anwesenheit freier AA (fMLP+AA) (AA 10 µM) ohne weitere Intervention oder unter gleichzeitiger Inhibition der 5-LO mit dem FLAP Antagonisten MK 886 (10 µM) (fMLP+AA+MK886). Quantifizierung des Respiratory Burst anhand der Sekretion von O_2^- [nmol/ml]. * $p < 0.05$ vs. Kontrollmedium. # $p < 0.05$ vs. LPS (Anlage 7).*

Charakterisierungen der Eicosanoid-Expression im TME an einem murinen Lungentumormodell zeigen, dass die COX-Metabolite, in erster Linie PGE_2, von epithelialen Tumorzellen gebildet werden, während die Leukotriene exklusiv myeloiden Ursprungs waren (125).

Leukotriene entstehen im Gewebeverbund häufig über einen transzellulären Lipidmediator-Metabolismus, der durch den wechselseitigen Austausch von Substraten und Produkten gekennzeichnet ist, die je nach enzymatischer Ausstattung der Zellen weiter metabolisiert werden können. Bei positiver Rezeptorexpression, wie sie z. B. für A549-Zellen für den LTB_4-Rezeptor Typ 2 (BLT_2) gegeben ist (43), können Tumorzellen durch 5-LO-Metaboliten aktiviert werden. Auch endotheliale Strukturen exprimieren den BLT_2, so wurde z. B. eine LTB_4-vermittelte Freisetzung von VEGF aus humanen Endothelzellen beschrieben (126).

Insgesamt entsteht im Verbund eine Vielfalt von Metaboliten. Es wurde postuliert, dass dieser transzelluläre Lipidmediator-Metabolismus im TME von pathogenetischer Bedeutung ist (32). Die Aktivierung des granulozytären 5-LO-Metabolismus durch LPS könnte zur Tumorprogression im inflammatorischen Tumormikromilieu beitragen. So wurde eindrucksvoll an einem murinen Brustkrebsmodell beschrieben, dass die Antagonisierung von LTB_4 zur Modulation des TME und Reduktion pulmonaler Metastasen führte (127).

Die LPS-induzierte granulozytäre Zytokinsynthese unterliegt einer Autoregulation

Konfrontiert man isolierte PMN mit LPS oder LTA, kommt es zu einer Aktivierung der Zytokinsynthese, wie in den Anlagen 4–6 dargestellt. Da PMN in vielen akuten und chronischen pulmonalen Infektionsherden den prädominanten Zelltyp darstellen, könnte der granulozytären Zytokinsynthese eine prominente Rolle bei der Modellierung des Tumormikromilieus unter infektiösen Bedingungen zukommen. Umgekehrt könnte das inflammatorische Tumormilieu eine entscheidende Determinante für den Verlauf der neutrophilen Inflammationsreaktion darstellen. Während die Aktivierung der granulozytären Zytokinsynthese sehr gut charakterisiert ist, ist über die Apoptose hinaus nur wenig über deren Limitierung bekannt. Physiologische limitierende Mechanismen sollten jedoch existieren, da pulmonale Infektionen wie z. B. die Pneumokokken-Pneumonie klassischerweise zunächst durch eine massive Akkumulation von PMN gekennzeichnet sind, die sich dann jedoch ohne nennenswerte Gewebedestruktion zurückbildet (128). Das granulozytären Mikromilieu könnte hier eine entscheidende Determinante darstellen (129). Bevor sich diese Arbeit den Effekten einer Ko-Kultur von PMN und Tumorzellen auf das inflammatorische Verhalten beider Zelltypen widmet, sollte zunächst die Frage beantwortet werden, ob die Akkumulation von PMN an einem Infektionsherd einer Regulation unterliegt. Möglicherweise könnte die zeitabhängige Akkumulation von *per se* eine kritische Determinante bei der Limitation inflammatorischen Granulozytenverhaltens darstellen. Um diese Fragestellung zu bearbeiten, wurden PMN in ansteigender Zelldichte in einem definierten Volumen mit LPS stimuliert und die Effekte der Zelldichte auf die Zytokinsynthese quantifiziert (Anlage 5).

Tatsächlich zeigt sich hier, dass eine Erhöhung der Zelldichte mit einer Abnahme der Sekretion von IL-8 in den Zellüberstand einhergeht (Abb. 21A). 10×10^6 PMN/ml war hier die Zellkonzentration, die die größten Mengen an IL-8 nach Stimulation mit LPS in den Zellüberstand freisetzte, eine stufenweise Erhöhung der Zelldichte resultierte in einer dichteabhängigen Abnahme der Sekretion dieses Chemokins. Der Reduktion des freigesetzten IL-8 lag nicht etwa eine Sekretionsstörung zugrunde, da sich dieser Effekt auch in den IL-8-Spiegeln aus Zelllysaten von in steigender Zelldichte inkubierten PMN zeigte (Abb. 21B). Zudem wurde der Effekt auch auf transkriptioneller Ebene reproduziert (Anlage 5). Erstaunlicherweise unterlag die Sekretion von IL-1 und TNF keiner Regulation durch die Zelldichte; diese Zytokine wurden kontinuierlich proportional zur Zelldichte freigesetzt (Abb. 21C und 21D). Untersuchungen, in denen 10×10^6 PMN/ml in konditionierten Medien von PMN in hoher Zelldichte inkubiert wurden, zeigten, dass lösliche Faktoren die Inhibition der IL-8-Synthese bedingten (Anlage 5).

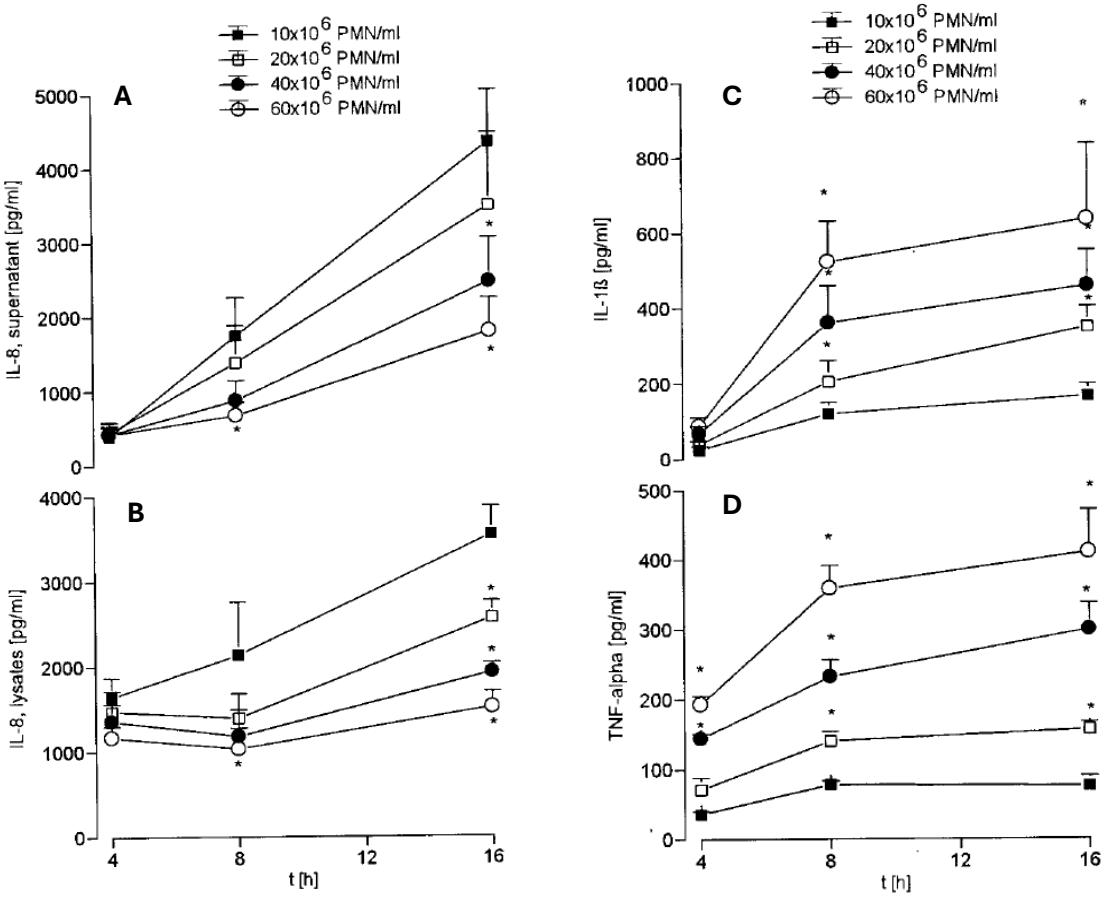

*Abbildung 21: Effekt der Zelldichte auf die Freisetzung in IL-8 in den Zellüberstand (A) und der intrazellulären Spiegel in Zelllysaten (B) sowie der Freisetzung von IL-1 (C) und TNF (D). Aktivierung von PMN in steigender Zelldichte mit 10 ng/ml LPS. Darstellung der Freisetzung von IL-8 (A), IL-1 (C), TNF (D) sowie der intrazellulären Spiegel von IL-8 (B) [pg/ml]. *p < 0.05 vs. 10 x 10^6 PMN/ml (Anlage 5).*

Die Zellüberstände wurden diesbezüglich auf die Freisetzung inhibitorischer Zytokine und Zytokin-Rezeptorantagonisten analysiert. Während die anti-inflammatorischen Zytokine IL-4, IL-10 und IL-13 nicht detektiert werden konnten (Anlage 5), akkumulierten nach einer Erhöhung der Granulozytendichte große Mengen löslicher Rezeptoren für TNF (sTNFRI und sTNFRII) sowie IL-1-Rezeptorantagonisten (IL-1RA) in den Zellüberständen, die sich proportional bis überproportional zur Zelldichte verhielten (Abb. 22). Eine Neutralisierung dieser löslichen Zytokin-Rezeptorantagonisten durch entsprechende Antikörper führte zur Wiederherstellung der IL-8-Synthese bei steigender Zelldichte (Abb. 23). Die Neutralisation von IL-1RA war dabei etwas effektiver als die alleinige Interferenz mit den löslichen TNF-Rezeptoren; vollständig aufgeboben war die Autoregulation der IL-8-Synthese durch simultane Neutralisation aller löslicher Zytokin-Rezeptorantagonisten. In dieser Gruppe zeigt sich eine regelhaft proportional zur Zellzahl ansteigende Sekretion von IL-8 mit Spiegeln von bis zu 12 ng/ml.

Die Involvierung weiterer, in die Autoregulation involvierter löslicher Faktoren, wie Adenosin-Desaminase, IL-4, IL-10, COX- oder 5-LO-Metaboliten, Sauerstoffradikale, Proteasen etc. wurden durch pharmakologische Inhibition ausgeschlossen (Anlage 5). Auch jedweder

unspezifische Effekt, wie etwa ein störender Faktor des Zelldetritus von in hohen Konzentrationen kultivierten PMN wurde durch separate „recovery“-Experimente ausgeschlossen (Anlage 5).

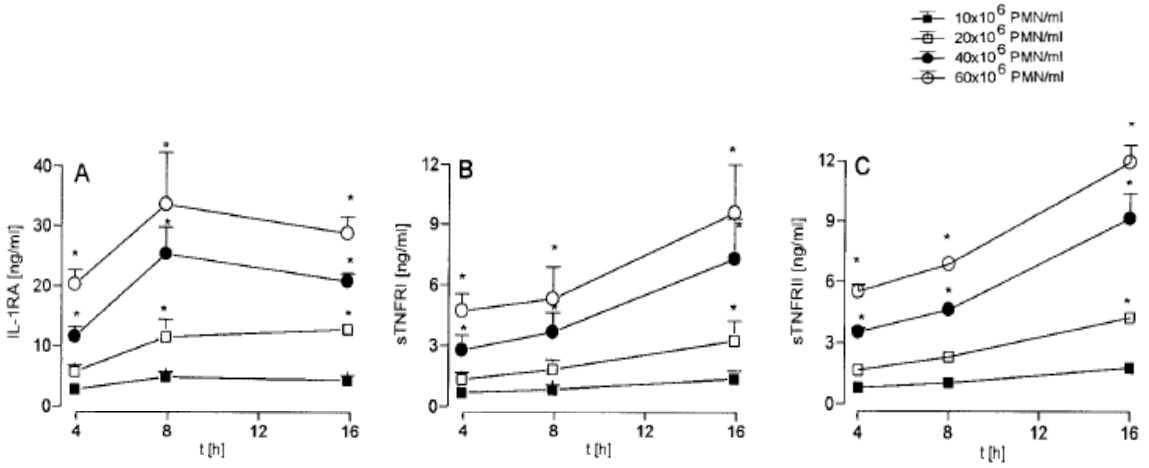

*Abbildung 22: Effekt der Zelldichte auf die LPS-induzierte Sekretion von IL-1 Rezeptorantagonisten (IL-1 RA) (A) und löslichen TNF-Rezeptoren (sTNFRI und sTNFRII) (B und C). Aktivierung von PMN in steigender Zelldichte mit 10 ng/ml LPS für 16 h. Darstellung der Freisetzung löslicher Zytokin-Rezeptorantagonisten in ng/ml. * p < 0.05 vs. 10 x 10^6 PMN/ml (modifiziert nach Anlage 5).*

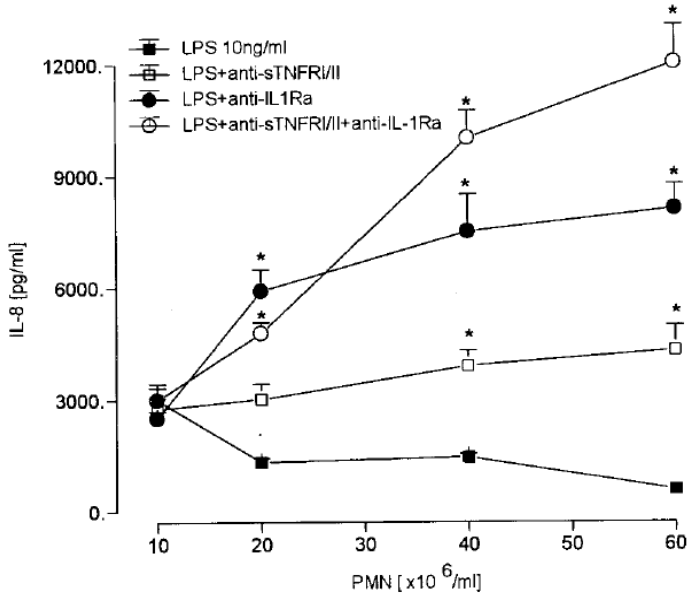

*Abbildung 23: Durch Antagonisierung der löslichen TNF- und IL-1-Rezeptorantagonisten ist die Hemmung der IL-8-Freisetzung bei steigender Zelldichte attenuiert. Aktivierung von PMN in steigernder Zelldichte mit 10 ng/ml LPS für 16 h in Ab- oder Anwesenheit neutralisierender Antikörper gegen lösliche TNF-Rezeptoren (LPS+anti-sTNFRI/II, gegen den IL-1-Rezeptorantagonist (LPS+anti-IL-1RA) oder einer Kombination aller Antikörper (LPS+anti-sTNFRI/II+anti-IL1-RA, 20 µg/ml für alle Antikörper). Darstellung der IL-8-Freisetzung in pg/ml. *p < 0.05 vs. 10 x 10^6 PMN/ml (Anlage 5).*

Dass dem granulozytären Mikromilieu eine Rolle bei der Aktivierung und Beendigung der zellulären Aktivierung zukommt, zeigten Studien, in denen PMN mit humanen Endothelzellen oder Thrombozyten ko-kultiviert wurden. Unter diesen Bedingungen stellte sich unter anderem eine Limitierung der Freisetzung der granulozytären Elastase dar (130, 131). Die hier vorgelegten Untersuchungen demonstrieren erstmals eindrucksvoll, dass die granulozytäre Zelldichte *per se* eine kritische Determinante im Rahmen der Zellaktivierung darstellt. Es existiert eine hochspezifische Autoregulation der granulozytären IL-8-Synthese in Abhängigkeit von der Zelldichte. Lösliche IL-1- und TNF-Rezeptorantagonisten, die nach Stimulation mit Endotoxin proportional zur granulozytären Zelldichte liberiert werden, verhindern die autokrine Aktivierung der IL-8-Synthese. Interessanterweise existiert ein massives Ungleichgewicht zugunsten der löslichen Zytokin-Rezeptorantagonisten im Vergleich zu den natürlichen Agonisten IL-1 und TNF: so wurde der IL-1RA in etwa 80-facher Höhe im Vergleich zu IL-1 freigesetzt; für TNF existierte ein etwa 40-facher Überschuss zugunsten der löslichen Rezeptorantagonisten. Solch ein Überschuss könnte angesichts der weitaus niedrigeren Rezeptoraffinität der löslichen Zytokin-Rezeptorantagonisten gegenüber der physiologischen Liganden IL-1 und TNF nötig sein, um deren physiologische Aktivität zu unterbinden (132, 133).

Die Aktivierung neutrophiler Granulozyten im inflammatorischen Mikromilieu wird seit langem als „zweischneidiges Schwert“ angesehen. Ist sie einerseits essenziell für die Abwehr von Mikroben, so führt eine zu starke oder anhaltende Aktivierung zur Gewebedestruktion, wie sie in akuten oder chronischen inflammatorischen Erkrankungen zu sehen ist (134). Die Existenz der hier beschriebenen granulozytären Autoregulation durch die Zelldichte könnte einen negativen Feedback-Mechanismus darstellen, um einer exzessiven Neutrophilenakkumulation und Aktivierung an einem infektiösen Fokus vorzubeugen. Mittlerweile ist bekannt, dass einer weiteren Gruppe granulozytärer Lipidmediatoren, den sog. Resolvinen, ebenfalls eine regulative Rolle bei Inflammationsprozessen zukommt (135). Ob die hier beschriebene Autoregulation auch im inflammatorischen Tumormikromileu operativ ist, bleibt spekulativ.

Interaktion von NSCLC-Zellen und Granulozyten unter dem Einfluss bakterieller Pathogenitätsfaktoren

Fakt ist, dass auch im Tumormikromilieu Neutrophilen eine ambivalente Rolle zukommt. Einerseits könnten sie, je nach Polarisierungs- und Aktivierungszustand über die Aktivierung ihres gewebedestruierenden und zytotoxischen Potenzials zur Tumorabwehr beitragen (136). Andererseits könnte die Freisetzung inflammatorischer granulozytärer Mediatoren auch eine Tumorpromotion auslösen (136). Die sich anschließenden Experimente, in denen PMN mit humanen NSCLC-Zellen ko-kultiviert wurden, widmen sich dieser Fragestellung.

Neutrophile Granulozyten amplifizieren die Zytokinsynthese humaner NSCLC-Zellen

Hierzu wurden humane NSCLC Zelllinien und PMN in Gegenwart von Endotoxin mono- oder ko-kultiviert. Während LPS in Monokulturen von A549-Zellen zwar eine dosisabhängige, jedoch insgesamt moderate Freisetzung von IL-8 in den Zellüberstand induzierte (Anlage 8), kam es in der Präsenz von PMN zu einer massiven Amplifizierung der Chemokinsynthese auf Mengen bis zu 50 ng/ml, die weit einen etwaigen additiven Effekt des in Monokulturen liberierten IL-8 übertraf (Abb. 24A). Auch die Freisetzung von IL-6, das in den jeweiligen Monokulturen nach Stimulation mit LPS nur marginal vorhanden war, wurde in den Ko-Kulturen induziert (Abb. 24B). Die Amplifizierung der Zytokinsynthese war abhängig von direktem Zellkontakt, da diese aufgehoben war, wenn beide Zelltypen voneinander getrennt in sogenannten „transwells“ (TW) kultiviert wurden (Abb. 24).

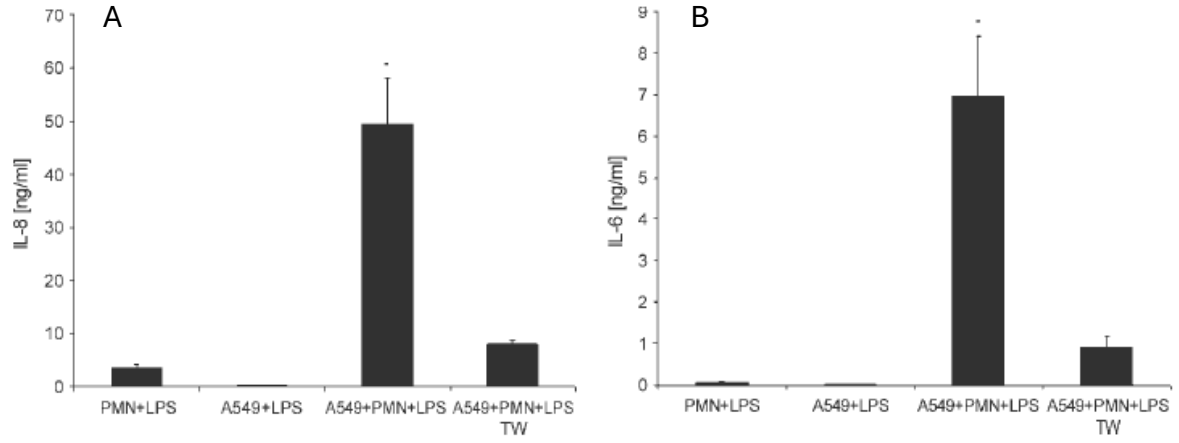

*Abbildung 24: Amplifizierung der IL-8 (A) und IL-6 (B) Freisetzung in Ko-Kulturen von A549-Zellen und PMN. Stimulation jeweils mit 0.1 µg/ml LPS für 24 h. TW: Kultivierung in „transwells“. *p < 0.05 vs. aller anderer Werte (modifiziert nach Anlage 8).*

Der amplifizierende Effekt der Ko-Kulturen auf die Zytokinsynthese konnte an A549-Zellen auch nach Stimulation mit Lipoteichonsäuren beobachtet werden und konnte an der SCLC-Zelllinie H69 reproduziert werden (Abb. 25). Die Reproduktion der Daten für zwei bakterielle Pathogenitätsfaktoren an unterschiedlichen Zelllinien spricht für ein generelles Phänomen.

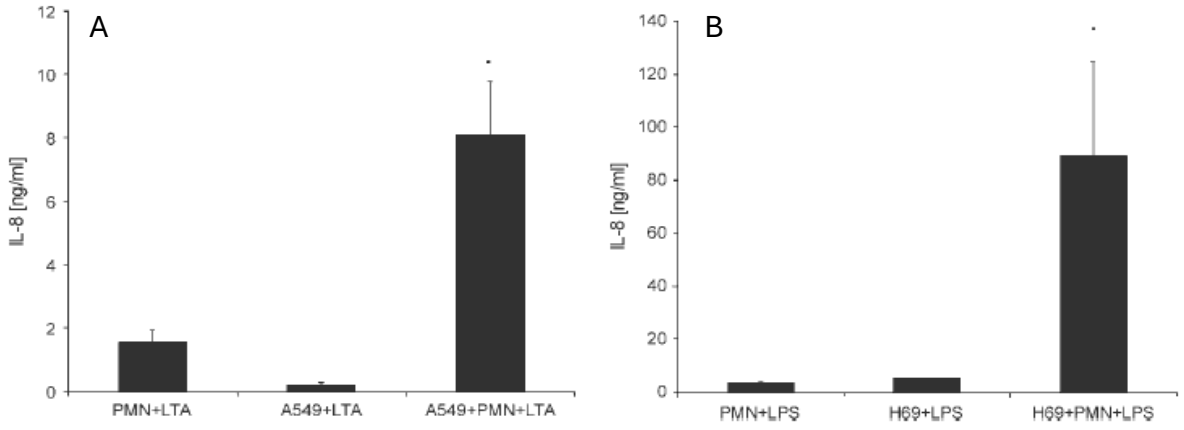

*Abbildung 25: Reproduktion der amplifizierten IL-8-Freisetzung in LTA-stimulierten A549-PMN-Ko-Kulturen (A) und in LPS-stimulierten H69-PMN-Ko-Kulturen (B). LTA 10 µg/ml, LPS 0.1 µg/ml. *p < 0.05 (modifiziert nach Anlage 8).*

Durch Analyse der Zytokininduktion auf transkriptioneller Ebene nach Separierung der beiden Zelltypen konnten A549-Zellen als zelluläre Quelle der amplifizierten IL-8- und IL-6-Synthese identifiziert werden, wohingegen die Transkription von IL-8 in Neutrophilen aus Ko-Kulturen nicht amplifiziert war und generell keine IL-6-Transkription in PMN nachgewiesen wurde (Anlage 8). Die mangelnde Fähigkeit zur IL-6-Synthese in PMN steht im Einklang mit früheren Studien (104, 137).

Da aus eigenen Untersuchungen an PMN (Anlagen 4, 5) und auch aus der Literatur bekannt ist, dass die Synthese von IL-8 in A549-Zellen durch TNF stimuliert wird (138), wurde die Rolle dieses Zytokins bei der Amplifizierung der IL-8-Synthese überprüft. Durch Neutralisation der TNF-Bioaktivität konnte die amplifizierte IL-8-Synthese in den Ko-Kulturen in etwa halbiert werden (Abb. 26). Obwohl A549-Zellen selbst nicht in der Lage sind, TNF zu synthetisieren (139), exprimieren sie den spezifischen Membranrezeptor TNFR1 (140). Eine Konfrontation mit TNF löst in A549-Zellen eine IL-8-Sekretion aus (138). Die zelluläre Quelle von TNF sind im Ko-Kultur-Modell die PMN, deren Kapazität zur TNF-Synthese nach Stimulation mit LPS in diesen Untersuchungen ebenfalls nachgewiesen wurde. So löste hier die Stimulation mit 0.1 µg/ml LPS eine Freisetzung von etwa 150 pg/ml TNF aus (Anlage 8). Auch in weiteren eigenen Arbeiten wurde die Kapazität isolierter PMN zur TNF-Synthese nach Stimulation mit LPS nachgewiesen (Anlage 5). Dass für die Wirkung dieses löslichen Mediators dennoch ein direkter Zellkontakt nötig ist, könnte in der kurzen biologischen Halbwertszeit der Bioaktivität von TNF begründet sein (141). Die Tatsache, dass die Neutralisation von TNF nicht zu einer kompletten Reduktion der IL-8-Synthese im Ko-Kultur-Modell erzielt, spricht dafür, dass

weitere Mediatoren, wie z. B. IL-1 involviert sein könnten, welches neben TNF maßgeblich an der LPS-induzierten Synthese von IL-8 beteiligt ist (Abb. 16).

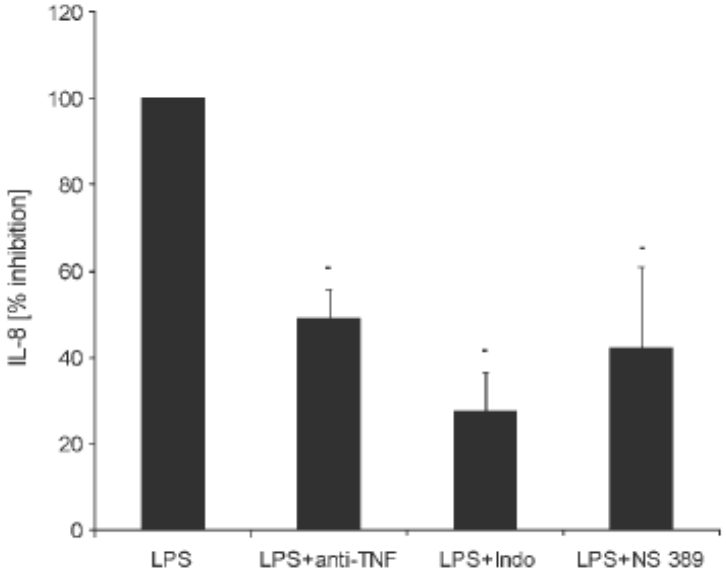

*Abbildung 26: Mechanismen der Amplifikation der IL-8-Synthese in A549-PMN-Ko-Kulturen. Stimulation der Ko-Kulturen mit 0.1 µg/ml LPS in Ab- oder Anwesenheit neutralisierender Antikörper gegen TNF (Anti-TNF: 5 µg/ml), des COX-Inhibitors Indometacin (100 µM) bzw. des COX-2-Inhibitors NS398 (10 µM). IL-8 als prozentuale Freisetzung im Vergleich zur LPS-Kontrolle ohne Inhibitoren (LPS, 100 %). *p < 0.05 vs. LPS (Anlage 8).*

Weitere Mediatoren, die die Amplifizierung der Chemokinsynthese bedingten, waren COX-2-abhängige Lipidmediatoren, was durch den inhibitorischen Effekt des unspezifischen COX-Inhibitors Indometacin und des spezifischen COX-2-Inhibitors NS398 belegt wurde (Abb. 26). Korrespondierend wurde in den Untersuchungen von Pold et al. (142) gezeigt, dass die COX-2-Aktivität in humanen NSCLC-Zelllinien mit der Synthese pro-angiogener CXC-Chemokine, darunter IL-8 korreliert. Eine weitere eigene Arbeit konnte zeigen, dass PGE_2 aus Ko-Kulturen von A549-Zellen und PMN nach Stimulation mit Endotoxin freigesetzt wird (Abb. 29B). Die Entstehung von COX-abhängigen Lipidmediatoren im Ko-Kultur-System könnte auf dem in mehreren Arbeiten (116, 143) dargestellten transzellulären Lipidmediator-Metabolismus beruhen, wobei hier vermutlich PMN als Arachidonsäure-Donorzellen fungieren, da bekannt ist, dass LPS die PLA_2 aktiviert (144). Bei direktem Zell-zu-Zell-Kontakt könnten benachbarte A549-Zellen dieses kurzlebige Substrat internalisieren und unter Stimulation mit LPS weiter zu Prostanoiden wie PGE_2 verstoffwechseln, wie bereits in Anlage 1 unter Substitution freier AA dargestellt wurde.

Neutrophile Granulozyten induzieren eine Proliferation von NSCLC-Zellen in vitro

Schließlich wurde der Effekt neutrophiler Granulozyten auf die Tumorzellproliferation in vitro untersucht. In einem ähnlichen Versuchsansatz wie zuvor beschrieben, wurden NSCLC-Zellen mit PMN in steigender Konzentration (0.5–10 x 10^6 PMN/ml) in An- oder Abwesenheit von

Endotoxin ko-kultiviert (Anlage 9). Sowohl naive als auch LPS-stimulierte PMN induzierten eine Steigerung der Proliferation von A549-Zellen in vitro, die anhand deren MTS-Aktivität quantifiziert wurde (Abb. 27A). Der Effekt der PMN war konzentrationsabhängig, wobei interessanterweise 7,5 x 10^6 PMN den stärksten Effekt auf die NSCLC-Proliferation hatten, was an die bereits beschriebene Autoregulation der granulozytären Aktivität durch die Zelldichte erinnert (Anlage 5). Der pro-proliferative Effekt war auch in Abwesenheit von LPS vorhanden, jedoch war dieser in LPS-stimulierten Ko-Kulturen stärker ausgeprägt, weswegen alle weiteren Versuche unter Stimulation der Ko-Kulturen mit Endotoxin durchgeführt wurden. Dass in der Ko-Kultur tatsächlich die MTS-Aktivität der A549 und nicht etwa die verbleibender PMN gemessen wurde, belegten Kontrollexperimente: Zum einen wurden initial PMN in Monokultur parallel zu den Ko-Kulturen unter identischen Konditionen kultiviert; hier ließ sich keinerlei granulozytäre MTS-Aktivität quantifizieren. Die fehlende Kapazität dieser ausgereiften Zellen in vitro selbst unter inflammatorischen Bedingungen zu proliferieren, wurde bereits in den Untersuchungen von Altznauer und Witko-Sarsat (145, 146) bestätigt. Zum anderen wurde keinerlei Myeloperoxidase-Aktivität nach Entfernen der PMN in den A549-Zellen nachgewiesen (Anlage 9). Im Einklang mit diesen pro-proliferativen Effekten der PMN in vitro existieren Hinweise auf protumorigene Eigenschaften dieser Zellen in vivo: Im Lungenkarzinom wurden erhöhte systemische und lokale Granulozytenspiegel als unabhängiger negativer Prognosefaktor beschrieben (147). Die intratumorale PMN-Dichte korreliert mit weiteren negativen prognostischen Faktoren (148). In murinen Modellen unterschiedlichster Tumorentitäten kam es nach Inhibition der Rekrutierung von PMN zu einer Reduktion des Tumorwachstums, was die hier in vitro beschriebenen pro-proliferativen Effekte neutrophiler Granulozyten bestätigt (149). Korrespondierend zur zuvor beschriebenen Steigerung der Zytokinsynthese war auch der pro-proliferative Effekt der PMN abhängig von direktem Zellkontakt (Abb. 27B).

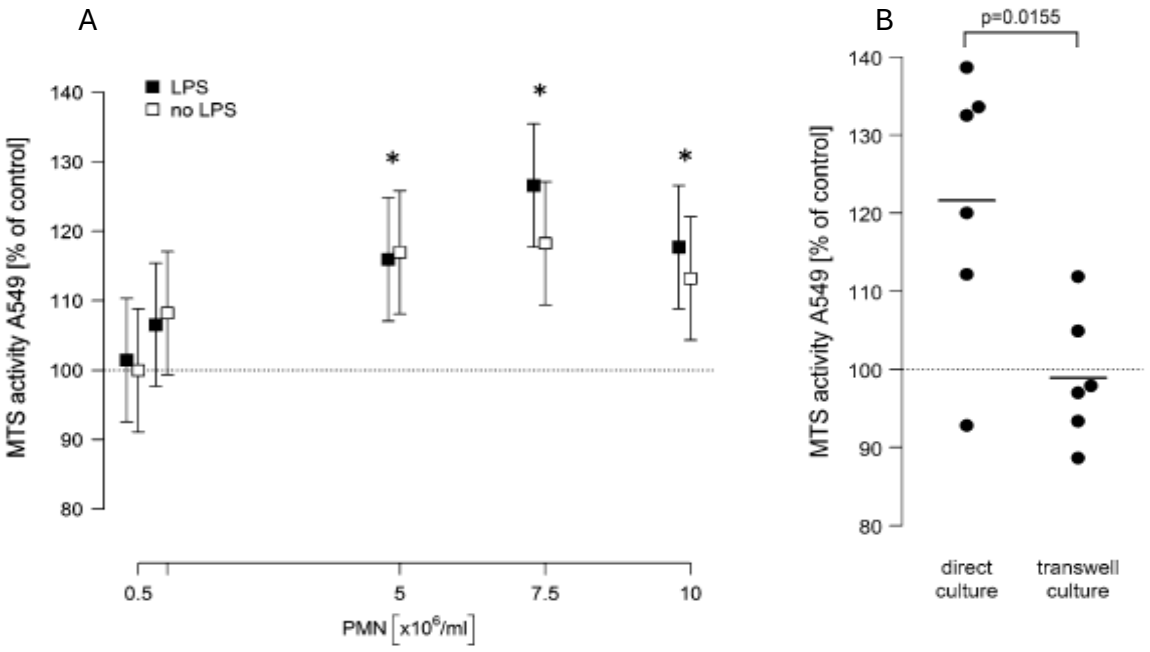

*Abbildung 27: PMN-induzierte Steigerung der Zellproliferation von A549-Zellen. Effekt der Zelldichte der PMN (A) und Abhängigkeit von direktem Zellkontakt (B). Stimulation von Ko-Kulturen von A549-Zellen und PMN in unterschiedlicher Zelldichte in An- oder Abwesenheit von LPS 0.1 µg/ml (A). LPS-stimulierte (0.1 µg/ml) Ko-Kulturen von A549-Zellen und 7,5/10^6 PMN/ml in direkter Ko-Kultur oder in „transwells" (B). Angabe der MTS-Aktivität prozentual zur jeweiligen A549-Monokultur (horizontale Linie entsprechend 100%). *p < 0.05 vs. A549-Monokultur (modifiziert nach Anlage 9).*

Ein pro-proliferativer Effekt durch direkten Zellkontakt könnte z. B. an durch die ß$_2$-Integrin-abhängige Interaktion der PMN mit ICAM-1, welches an der Oberfläche von A549-Zellen exprimiert wird, ausgelöst werden, da die Ligation von ICAM-1 in A549-Zellen eine MAP-Kinase-abhängige intrazelluläre Signaltransduktion auslöst, die ebenfalls mit zellulärer Proliferation assoziiert wurde (150, 151). In PMN induziert die Ligation von ß$_2$-Integrinen die Freisetzung von Sauerstoffradikalen, Proteasen und Lipidmediatoren (152, 153), weswegen die Quantifizierung und pharmakologische Inhibition dieser Mediatoren im Ko-Kultur-Modell erfolgte. Es konnte gezeigt werden, dass die fMLP-induzierte Sekretion der granulozytären Elastase in Anwesenheit von A549-Zellen nahezu verdoppelt war, während die Freisetzung von Sauerstoffradikalen durch die NSCLC-Zellen unbeeinflusst blieb (Abb. 28A und 28B). Interessanterweise war der pro-proliferative Effekt der PMN auf A549-Zellen in Anwesenheit des spezifischen Elastase-Inhibitors AAPVCK, nicht jedoch durch Neutralisation von Sauerstoffradikalen mit Superoxiddismutase (SOD) aufgehoben (Abb. 28C).

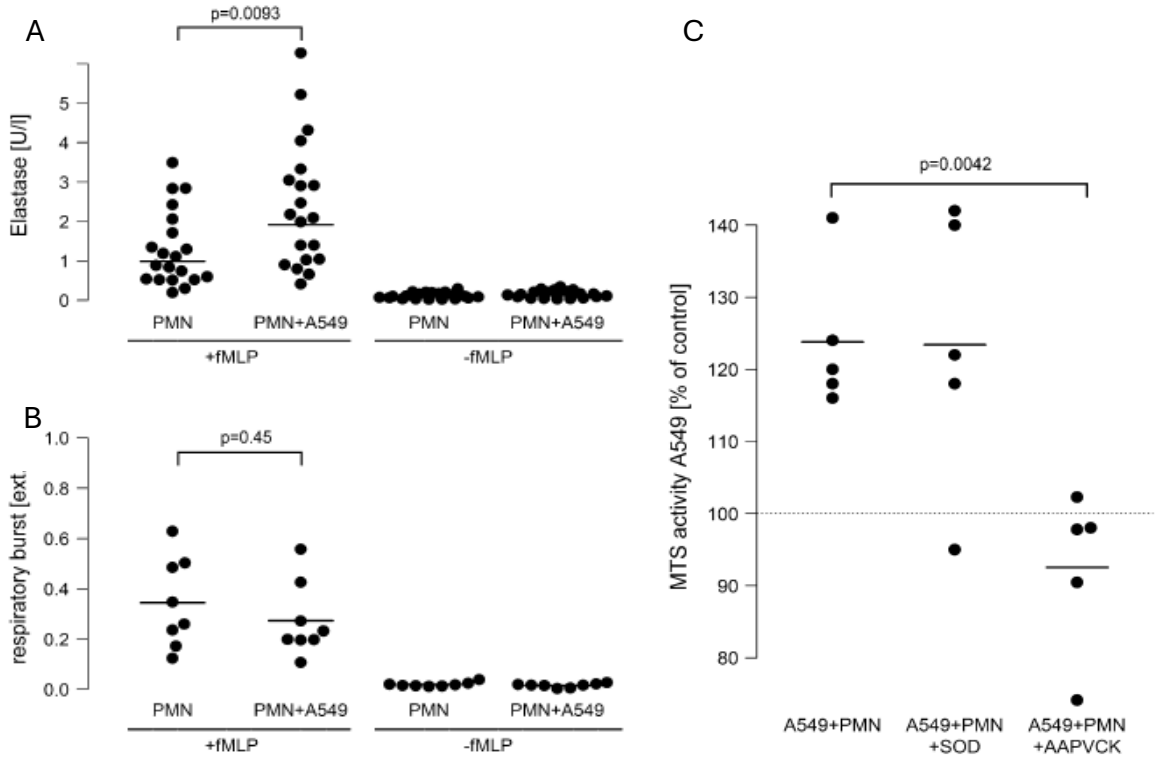

Abbildung 28: Effekt der Ko-Kulturen auf die Degranulierung und den Respiratory Burst und Rolle dieser Mediatoren bei der Proliferation in PMN-NSCLC-Ko-Kulturen.
A/B: Aktivierung LPS-stimulierter Mono- oder Ko-Kulturen von PMN mit 1 µM fMLP oder Mediumkontrolle für 10 min. Elastase-Freisetzung als [U/l]; Respiratory Burst als Reduktion von Cytochrom C [ext. 405 nm]
C) Effekt einer Neutralisierung von Sauerstoffradikalen und einer Elastase-Inhibition auf die Proliferation von A549-Zellen in LPS-stimulierten Ko-Kulturen mit PMN. Angabe der MTS-Aktivität in A549/PM-Ko-Kulturen prozentual zur A549-Monokultur (100 %), horizontale Linie. SOD: Superoxiddismutase (10 µg/ml), AAPVCK (Elastase-Inhibitor): 5 µM, LPS 0.1 µg/ml (modifiziert nach Anlage 9).

Eine protumorigene Rolle der granulozytären Elastase ist ein relativ neu entdecktes Phänomen, waren für diese Protease doch bis dato gewebedestruierende Effekte z. B. im akuten Lungenversagen unter anderem auch aus eigenen Arbeiten bekannt (154, 155). Im Einklang beschrieben Houghton et al. eine Elastase-mediierte Aktivierung tumorintrinsischer Wachstumssignale wie die Initiierung der PDGFR-abhängigen Signaltransduktion (53). Aktuelle Untersuchungen zeigen, dass die pro-proliferativen Eigenschaften dieses Enzyms u. a. auf einer Transaktivierung des EGF-Rezeptorsystems beruhen könnten (156).

Neben der granulozytären Elastase waren auch COX-2-abhängige Metabolite in die amplifizierte Tumorzellproliferation im Ko-Kultur-Modell involviert. Sowohl unspezifische Inhibition der COX mit Indometacin als auch spezifische Hemmung der COX-2-Aktivität mit NS398 hob den im Ko-Kultur-Modell hervorgerufenen pro-proliferativen Effekt komplett auf (Abb. 29A). Aus den Zellüberständen LPS-stimulierter Ko-Kulturen konnten beträchtliche Mengen an PGE_2 quantifiziert werden, während A549-Zellen per se in diesem Modell in Abwesenheit freier AA nur geringe Mengen an PGE_2 freisetzten und PGE_2 aus LPS-stimulierten PMN-Überständen nur marginal detektiert wurde (Abb. 29B). Hier liegt vermutlich der bereits beschriebene transzelluläre Lipidmediator-Metabolismus zugrunde, wobei PMN hier als AA-Donorzellen fungieren. AA wird von den A549-Zellen weiter COX-2-abhängig zu PGE_2 synthetisiert, wie es der inhibitorischen Effekt von NS398 belegt (Abb. 29B).

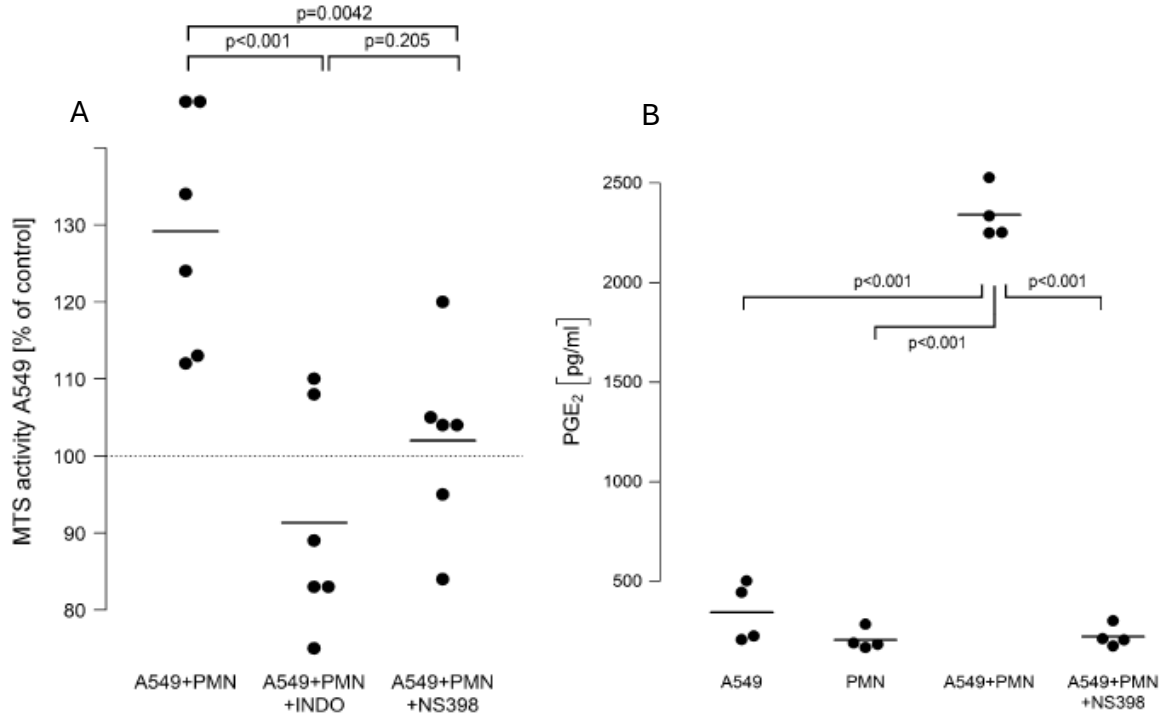

Abbildung 29: Aktivierung des COX-Metabolismus in A549/PMN Ko-Kulturen und Effekt auf die Proliferation. A) Rolle von COX-Mediatoren bei der NSCLC-Proliferation. Stimulation der Ko-Kulturen mit 0.1 µg/ml LPS in Ab- oder Anwesenheit von Indometacin (Indo, 100 µM) oder des spezifischen COX-2-Inhibitors NS398 (10 µM). B) COX-2-abhängige Synthese von PGE_2 in PMN-A549-Ko-Kulturen. Stimulation der Ko-Kulturen mit 0.1 µg/ml LPS in Ab- oder Anwesenheit des spezifischen COX-2-Inhibitors NS398 (10 µM). PGE_2 in pg/ml (modifiziert nach Anlage 9).

Bemerkenswerterweise ist die PMN-induzierte Steigerung der Tumorzellproliferation in den Ko-Kulturen sowohl durch eine Inhibition der Elastase als auch der COX-2 vollständig aufgehoben. Dieses Phänomen könnte in der Interaktion zwischen COX-2-Metaboliten und Elastase begründet sein. Während Inhibitoren der COX die Freisetzung von Elastase in PMN inhibieren (157), kommt es vice versa zu einer Aktivierung der COX-2 durch granulozytäre Elastase in Bronchialepithelzellen (158). Dieser Synergismus könnte im beschriebenen Ko-Kultur-System relevant sein. Da zuvor (Anlage 8) gezeigt wurde, dass in PMN-A549-Ko-Kulturen auch die Synthese von IL-8 über einen COX-2-abhängigen Mechanismus amplifiziert ist, könnte diesem Chemokin ebenfalls eine Rolle bei der PMN-mediierten Proliferationssteigerung im Ko-Kultur-Modell zukommen. In diesem Zusammenhang beschreibt ein Tiermodell eine protumorigene Rolle von PMN im NSCLC, die sich sowohl abhängig von der neutrophilen Elastase und IL-8 darstellt (159).

Zusammenfassend kommt es in Ko-Kulturen von PMN unter dem Einfluss von Endotoxin zu einer Amplifizierung der Zytokinsynthese und zur gesteigerten Tumorzellproliferation. Diese Effekte sind abhängig von direktem Zellkontakt. COX-2-abhängige Mediatoren sind in beide Prozesse involviert, während die granulozytäre Elastase, die in den Ko-Kulturen vermehrt freigesetzt wird, zu einer Steigerung der Tumorzellproliferation beiträgt.

So könnte die wechselseitige Interaktion zwischen neutrophilen Granulozyten und Tumorzellen über eine amplifizierte Synthese pro-proliferativer, chemotaktischer und inflammatorischer Mediatoren zu einer sich selbst unterhaltenden Tumorprogression im TME führen.

Therapeutische Perspektiven

Die Therapie des NSCLC hat sich in den letzten 20 Jahren erheblich gewandelt. Die Tumor-spezifische Systemtherapie, die in den lokal fortgeschrittenen oder metastasierten Stadien indiziert ist, basierte lange auf der Applikation Cisplatin-haltiger „Doubletten“ mit Zytostatika wie Etoposid, Pemetrexed oder Gemcitabin als Kombinationspartner. 2005 wurde mit der Zulassung von Erlotinib als Tyrosinkinase-Inhibitor des EGFR erstmals eine zielgerichtete, „targeted therapy“, zur Systemtherapie des NSCLC zugelassen (160). Die Bedeutung weiterer Targets, wie genetische Alterationen von BRAF und MET oder Translokationen von ALK oder ROS1, wurde identifiziert und spezifische Inhibitoren zur Systemtherapie des NSCLC bei entsprechendem Mutationsstatus zugelassen (161, 162).

Von dieser primär monozentristischen Betrachtung des Tumors im Fokus der therapeutischen Ansätze löste man sich spätestens mit der Zulassung und dem mittlerweile breiten Einsatz der Immuntherapien im NSCLC. Erstes „targeting“ des TME erfolgte bereits 2006 durch die Ergänzung der Systemtherapie des NSCLC um den Angiogenese-Inhibitor Bevacizumab (163).

Durch die Integration der Immuntherapien in die Systemtherapie des NSCLC wurde der therapeutische Fokus primär auf die Interaktion des Tumors mit dem TME gesetzt. Die Immuntherapien basieren auf Inhibition bestimmter Immuncheckpoints wie das auf T-Lymphozyten und dendritischen Zellen exprimierte PD-1, welches mit dem auf Tumorzellen exprimierten Liganden PD-L1 interagiert. Diese Interaktion führt zur Inhibition der T-Zellfunktion, und ermöglicht die sogenannte „immune escape“ der Tumorzelle. Eine Interferenz mit diesem Mechanismus durch Applikation neutralisierender Antikörper gegen PD-1 oder PD-L1 führt zur Re-Aktivierung der spezifischen Immunabwehr gegen Tumorzellen. Die Einführung der Immuntherapien stellte ein völlig neues Therapiekonzept dar, in der erstmals das TME bzw. die Interaktion des Tumors mit dem TME als „target“ in der Systemtherapie im NSCLC berücksichtigt wurde. Mittlerweile sind die Immuntherapien integraler Bestandteil der Systemtherapie des NSCLC in kurativer oder palliativer Intention (164).

Die Berücksichtigung des inflammatorischen Tumormikromilieus und der auch in dieser Arbeit beschriebenen inflammatorischen Vorgänge, die zur Tumorpromotion beitragen, könnte zur Weiterentwicklung der Systemtherapie im NSCLC beitragen.

Noch ist der Einsatz von Coxiben, die selektiv die Cyclooxygenase-2 hemmen, in der Tumortherapie nicht zugelassen. Klinische Studien, in denen Coxibe gleichzeitig zur Systemtherapie bei Patienten mit Lungenkarzinom eingesetzt wurde, zeigten zwar einen klinischen Benefit im Sinne einer verbesserten Lebensqualität, aber keinerlei Unterschiede im Gesamtüberleben (165). Auch in einer großen Phase-3-Studie, in die Patienten mit NSCLC anhand ihrer COX-2-Expression im Gewebe vorselektiert wurden, konnten keine positiven Effekte von Celecoxib auf das Gesamt- oder progressionsfreie Überleben nachgewiesen werden (166). Aus diesen negativen Studienergebnissen wurde gefolgert, dass, obwohl überzeugende Evidenzen für eine pathogenetische Rolle der COX-2 im Lungenkarzinom existieren, der Einsatz der Coxibe im NSCLC vermutlich eher in einem präventiven Setting bei Risikogruppen, insbesondere starken Rauchern, anzustreben sei (167). Ein weiterer möglicher Einsatz wäre in der Kombination mit Immuntherapien denkbar, da insbesondere PGE_2 einen

inhibitorischen Effekt auf T-Lymphozyten ausübt und so die Effekte einer Immuntherapie konterkarieren könnte (168).

Dies könnte vermutlich ebenfalls für Leukotrien-Antagonisten gelten, deren Einsatz in klinischen Studien im NSCLC ebenfalls aktuell nicht primär erfolgversprechend scheint (169). Ein Problem in der fehlenden Wirksamkeit des solitären Einsatzes von COX-2- bzw. 5-LO-Antagonisten könnte im „Shift" des Substrates Arachidonsäure, wie in vitro belegt, zum jeweiligen nicht blockierten Enzym liegen (170). Da sowohl COX-2-abhängige als auch 5-LO-abhängige Eicosanoide protumorigene Eigenschaften haben, würde die Inhibition der COX durch vermehrte Metabolisierung zu 5-LO-Produkten und umgekehrt jeweils konterkariert. Hier wären gegebenenfalls Studien mit kombinierter Inhibition beider Enzyme aussichtsreich. Diesbezüglich zeigten In-vitro-Untersuchungen an humanen NSCLC-Zelllinien eine überadditive Wachstumshemmung bei gleichzeitiger Inhibition beider Mediatorsysteme (171).

Eine weitere denkbare Interventionsmöglichkeit hinsichtlich des Cyclo- und Lipoxygenase-Metabolismus wäre die diätische Supplementierung mit den Omega-3-Fettsäuren Eicosapentaensäure und Docosahexaensäure als kompetitive Inhibitoren des Substrates Arachidonsäure. Die inflammatorische Kapazität der aus der Metabolisierung der Omega-3-Fettsäuren entstehenden Lipidmediatoren ist gegenüber den Omega-6-Mediatoren stark attenuiert. Tatsächlich zeigt eine aktuelle Metaanalyse 57 klinischer Studien, die den Effekt einer Supplementierung von Omega-3-Fettsäuren bei unterschiedlichen Tumorentitäten adressierten, bei einzelnen Tumorentitäten positive Effekte hinsichtlich des Gesamtüberlebens, des progressionsfreien Überlebens, der Lebensqualität und Muskelmasse. Begleitet waren diese klinischen Parameter von einer Reduktion der Serumspiegel zahlreicher proinflammatorischer Mediatoren wie z. B. TNF, IL-1, IL8, VEGF und PGE_2 (172).

Versucht wurde bereits die Integration eines neuen antiinflammatorischen „biologicals" in die Systemtherapie des NSCLC 1 durch den Anti-IL-1 Antikörper Canakinumab in der CANOPY-Studie (173). Canakinumab wurde ursprünglich in der CANTOS-Studie an Patienten mit Arteriosklerose und hohen CRP-Spiegeln als anti-arthrogenes Medikament eingesetzt; eine Post-hoc-Analyse zeigte interessanterweise eine etwaige Halbierung der Inzidenz von Lungenkarzinomen in der mit Canakinumab behandelten Gruppe (174), was auf eine frühe Rolle von IL-1 bei der Entwicklung des Lungenkarzinoms rückschließen lässt. Obwohl die Ergebnisse der CANOPY-Studien bisher keine Vorteile der mit Canakinumab therapierten Gruppen zeigte, werden solch anti-inflammatorische Therapien weiterverfolgt (175).

Die zielgerichtete Antagonisierung von IL-8 ist ein weiteres Beispiel für eine anti-inflammatorische Therapie, die einer klinischen Überprüfung in soliden Tumoren unterzogen wird. Nach erfolgreicher Re-Induktion einer Therapiesensitivität durch den IL-8 Rezeptorantagonisten Reparixin bei Chemotherapie-resistenten Magenkarzinomen (176) wurde aktuell die Wirkung von Reparixin, allein und in Kombination mit Paclitaxel, bei Patientinnen mit Brustkrebs überprüft (177).

Im NSCLC korrelieren hohe IL-8- und auch TNF-Serumspiegel in Patienten mit nicht kleinzelligem Lungenkarzinom mit einer schlechteren Therapieansprache auf Immuntherapie (178). An Tiermodellen des Kolon- und Lungenkarzinoms konnte das Tumorwachstum durch

Deletion des CXCR2 inhibiert werden (156, 179). Vor diesem Hintergrund wurde kürzlich eine klinische Phase-2-Studie initiiert (180), die den Einsatz eines CXCR1/2-Antagonisten beim NSCLC überprüft.

In den von Jamieson (179) beschriebenen Untersuchungen wurde zudem gezeigt, dass die Deletion von CXCR2 mit einer verminderten intratumoralen Granulozytenakkumulation einherging, was auf die entscheidende Rolle von Tumor-assoziierten Neutrophilen (TAN) in der Tumorprogression hindeutet. Das „targeting“ und die „Reedukation“ von TANs und Tumor-assoziierten Makrophagen (TAM) von einem protumorigenen (N2, M2) in einen antitumorigenen (N1, M1) Status könnte ein völlig neues Wirkprinzip bei der Therapie des NSCLC darstellen. Selbstverständlich ist bei einer antigranulozytären Therapie eine schwere Neutropenie zu vermeiden. Eine gezielte, Granulozyten-zentrierte Therapieoption wäre eventuell die Inhibition der Elastase mit Sivelestat, die im Tiermodell inhibierend auf das Wachstum des NSCLC wirkt (181) und bereits klinisch im akuten Lungenversagen Anwendung findet (182). Auch die Inhibition der Rekrutierung von Makrophagen könnte therapeutisch nutzbar sein. Für einen Rezeptorantikörper gegen M-CSF (CSF-1), des wichtigsten Wachstums- und Differenzierungsfaktor für Monozyten und Makrophagen, konnte in unterschiedlichen Tumorentitäten eine therapeutische Wirksamkeit belegt werden (183).

Auch wenn die bisherigen Studien zu anti-inflammatorischen Therapien im NSCLC zum Großteil noch nicht die gewünschten Erfolge gezeigt haben, werden diese weiterverfolgt. Vor dem Hintergrund der komplexen Pathogenese mit Interaktionen zahlreicher inflammatorischer Effektoren bleibt es – in etwa vergleichbar mit den therapeutischen Interventionsstudien im Krankheitsbild der Sepsis – eine Herausforderung, ein einzelned zielgerichtetes inflammatorisches „target“ und auch den Zeitpunkt der Intervention zu identifizieren (184).

All die genannten inflammatorischen Effektoren, wie Lipidmediatoren der Cyclo- und Lipoxygenasen sowie die Zytokine IL-1 und IL-8 werden durch die bakteriellen Pathogenitätsfaktoren LPS und LTA induziert. Zudem kommt es durch LPS und LTA auch zur Aktivierung von PMN, die *per se* zur Tumorpromotion beitragen. Vor dem Hintergrund der in dieser Arbeit dargestellten Rolle bakterieller Pathogenitätsfaktoren in der Tumorprogression des NSCLC und der Tatsache, dass bakterielle Infektionen häufig das Krankheitsgeschehen im Lungenkarzinom aggravieren, muss stets eine konsequente anti-infektive Therapie durchgeführt werden. Deren Benefit auf den Verlauf der Erkrankung konnte überzeugend bestätigt werden (185, 186).

Zusammenfassend ist das Tumorwachstum im inflammatorischen Tumormikromilieu komplex und differenziell reguliert. Bakterielle Pathogenitätsfaktoren besitzen die Kapazität, sowohl durch direkte Aktivierung der Tumorzellproliferation als auch durch Aktivierung protumorigener humoraler und zellulärer Effektoren im Mikromilieu das Tumorwachstum zu beeinflussen. Ein tieferes Verständnis der inflammatorischen Tumorpromotion und -progression könnte zur Entwicklung differenzierter und individualisierter Tumortherapien im Lungenkarzinom beitragen.

III. Zusammenfassende Darstellung

Mittlerweile gilt die Inflammation als ein entscheidender Faktor in der Pathogenese zahlreicher solider Tumoren. Pulmonale bakterielle Infektionen stellen eine häufige Komplikation im Krankheitsverlauf des nicht kleinzelligen Lungenkarzinoms (NSCLC) dar. Die isolierten bakteriellen Pathogenitätsfaktoren Lipopolysaccharide (LPS) gramnegativer Keime und deren grampositive Äquivalente Lipoteichonsäuren (LTA) lösen hierbei pulmonale Inflammationsreaktionen aus. So könnte ein verändertes Tumormikromilieu (TME) entstehen, das u. a. durch die Präsenz proinflammatorischer Mediatoren und die Infiltration neutrophiler Granulozyten (PMN) gekennzeichnet ist. Vor diesem Hintergrund sollte die Frage beantwortet werden, ob LPS und LTA die Tumorprogression im NSCLC direkt oder über die Aktivierung von PMN beeinflussen können.

Die hier vorgelegten Untersuchungen zeigen, dass die bakteriellen Pathogenitätsfaktoren LPS und LTA direkt mit NSCLC-Zellen interagieren und die Tumorproliferation in vitro an unterschiedlichen NSCLC-Zelllinien induzieren (Anlagen 1, 2). Diese Effekte konnten an ex vivo kultiviertem humanen Lungengewebe und in einem Mausmodell reproduziert werden (Anlage 1). Neben diesen unmittelbaren Effekten auf die Tumorzellproliferation induzieren diese Pathogenitätsfaktoren auch eine Strahlenresistenz an NSCLC-Zelllinien (Anlage 3).

Eine Interaktion von bakteriellen Pathogenitätsfaktoren mit Tumorzellen scheint im NSCLC tatsächlich stattzufinden, da Nejman et al. kürzlich LPS und LTA im Tumorgewebe des NSCLC nachweisen konnten (71). In den hier vorgelegten Untersuchungen induzierten LPS und LTA eine quantitativ gleichermaßen ausgeprägte Proliferationsreaktion in NSCLC-Zellen in vitro, die in aktuellen Publikationen reproduziert werden konnte (79, 80). Die pro-proliferativen Effekte von LPS wurden in den hier vorgelegten Arbeiten (Anlage 1) zusätzlich in einem Tiermodell bestätigt. Nach subkutaner Injektion von A549-Zellen entwickelten Mäuse subkutane Tumoren am Injektionsort, wobei das Tumorwachstum nach LPS-Stimulation beschleunigt war und über einen Zeitraum von zwölf Tagen anhielt. Langzeitmodelle, in denen Mäuse repetitiven LPS-Inhalationen unterzogen wurden, bestätigen diese Befunde (97). Ebenso wurden die hier beschriebenen pro-proliferativen Effekte von LTA (Anlage 2) in einem Tiermodell reproduziert (84).

Die Ligation des GPI-verankerten Glykoproteins CD14 initiiert die zelluläre Aktivierung durch LPS und LTA. An NSCLC-Zellen wurde zusätzlich die Rolle des „pattern recognition“-Rezeptors TLR4 für die durch LPS induzierte Tumorzellproliferation herausgearbeitet, während die durch LTA induzierten Effekte abhängig vom TLR2 waren (Anlagen 1, 2). Diese differenzielle Abhängigkeit spricht für die Spezifität der durch die jeweiligen Pathogenitätsfaktoren hervorgerufenen Proliferationsreaktionen. Auch in vivo scheint dem TLR-System eine pathogenetische Relevanz im NSCLC zuzukommen, da die Expression von TLR4 bzw. TLR2 im humanen Adenokarzinomgewebe jeweils mit einer negativen Prognose der Erkrankung vergesellschaftet ist (69, 70). Dazu passend zeigen aktuelle Untersuchungen, dass durch genetischen Knock-out von TLR2 und TLR4 in einem murinen Lungenkarzinommodel, die durch die Inflammation getriggerte Tumorpromotion unterbunden werden kann, was die Relevanz der hier dargestellten Befunde unterstreicht (85).

Ebenfalls scheint der tumoreigene EGF-Rezeptor (EGFR) in die pro-proliferativen Effekte involviert zu sein, was durch die inhibierenden Effekte von Cetuximab, einem EGFR-Antikörper, auf die LPS-induzierte Zellproliferation belegt wurde. Vor dem Hintergrund der Evidenzen, dass der EGFR eventuell einen kritischen Ko-Rezeptor für Endotoxin darstellen könnte (92), lässt sich die Abhängigkeit der LPS-induzierten Tumorzellproliferation vom EGFR erklären. Dazu passend stellte sich die Strahlenresistenz, die in LPS-stimulierten NSCLC-Zellen induziert wurde, EGFR-abhängig dar (Anlage 3).

Nach Bindung von LPS und LTA an die spezifischen TLRs kommt es zu einer Freisetzung proinflammatorischer Mediatoren, wobei aufgrund ihrer Relevanz in vivo der Fokus auf die Cyclooxygenase (COX)-abhängigen Lipidmediatoren und das Chemokin IL-8 gelegt wurde.

Die Stimulation von NSCLC-Zellen und humanem Lungenkarzinomgewebe mit LPS induziert eine Expression des Isoenzyms COX-2 (Anlage 1). Für dieses Enzym wird im Lungenkarzinom eine wesentliche pathogenetische Bedeutung postuliert, da die Expression im NSCLC gesteigert ist (34) und eine Korrelation zwischen Expressionsgrad und Prognose der Erkrankung existiert (35). In Anwesenheit eines COX-2-Inhibitors war der pro-proliferative Effekt von Endotoxin nahezu aufgehoben. Der entscheidende COX-2 Metabolit, der zur LPS-induzierten zellulären Proliferation führt, ist mit hoher Wahrscheinlichkeit PGE_2, das in beträchtlichen Mengen in den Überständen LPS-stimulierter NSCLC-Zellen detektiert werden konnte. PGE_2 stellt im Lungenkarzinomgewebe den quantitativ führenden Metaboliten der COX-2 dar (88) und könnte seine proliferativen Effekte über Aktivierung spezifischer PGE_2-Rezeptoren ausüben, die im TME in vivo exprimiert sind (169). Voraussetzung für eine effektive Induktion der Synthese von PGE_2 war die Präsenz des Cyclooxygenase-Substrates Arachidonsäure (AA). Im Tumormikromilieu des Lungenkarzinoms konnte freie AA nachgewiesen werden (87). Somit scheint die Substratsubstitution nicht artifiziell herbeigeführt, sondern vielmehr einem pathophysiologischen Mechanismus zu entsprechen.

Neben den COX- und 5-LO-Produkten kommt auch den Zytokinen eine pathogenetische Rolle im Lungenkarzinom zu (24–29). IL-8 wurde in NSCLC-Zellen sowohl nach Stimulation mit LPS und LTA dosisabhängig induziert und sezerniert (Anlagen 2, 9). IL-8 scheint entscheidend an der TLR2-mediierten Tumorzellproliferation nach Stimulation mit LTA beteiligt zu sein, da dieser Mediator in den Zellüberständen nachgewiesen werden konnte, die IL-8-Sekretion durch Neutralisation von TLR2 attenuiert war, und eine Inhibition von IL-8 die pro-proliferativen Effekte von LTA aufhob (Anlage 2). Im Einklang hierzu übt IL-8 in verschiedenen Modellen des NSCLC pro-proliferative Effekte in vitro und in vivo aus, die über direkte Aktivierung der spezifischen Chemokinrezeptoren CXCR1 und CXCR2 mediiert werden können (26). Auch eine Kapazität zur Transaktivierung des EGFR wurde für IL-8 postuliert (12). Für die Relevanz dieses Chemokins in vivo spricht zudem die Korrelation des Expressionsgrades von IL-8 mit einer Tumorprogression im Lungenkarzinom (30). Ein möglicher therapeutischer Nutzen einer Antagonisierung von IL-8 wird in einer aktuellen klinischen Studie überprüft (180).

Neutrophile Granulozyten stellen eine wesentliche Komponente des inflammatorischen Tumormikromilieus dar. Ihre Rekrutierung an einen pulmonalen infektiösen Fokus wird durch chemotaktische Substanzen wie IL-8 und Leukotrien (LT)B_4 mediiert. Hierbei stellen die PMN

selbst, einerseits durch ihre enzymatische Ausstattung mit Expression der 5-Lipoxygenase und andererseits über ihre quantitative Prädominanz, eine wesentliche zelluläre Quelle für diese chemotaktischen Substanzen dar. Obwohl es nach Stimulation von PMN mit LPS per se nicht zu einer Leukotriensynthese kommt (114), existiert ein CD14-abhängiger Priming-Effekt durch LPS, der eine Sensibilisierung der granulozytären Aktivierung durch einen zweiten Stimulus hervorruft (Anlage 7). So ist nach Endotoxin-Priming die durch das bakterielle Tripeptid fMLP induzierte Synthese von LTB_4 und auch die Sauerstoffradikalfreisetzung isolierter PMN massiv amplifiziert. Solch autokrine Effekte von LTB_4 wurden unter anderem in eigenen Untersuchungen bestätigt (124).

Darüber hinaus kommt es nach Konfrontation isolierter PMN mit LPS oder LTA zu einer dosisabhängigen Freisetzung von IL-8 in den Zellüberstand (Anlagen 4–6). Diese ist bei beiden Pathogenen durch Ligation von CD14 mediiert (Anlagen 4, 6). Hier scheint auch eine Abhängigkeit der zellulären Aktivierung durch den Expressionsgrad der CD14-Rezeptoren zu bestehen, da gezeigt werden konnte, dass nach einer Heraufregulation von CD14 sowohl die LPS- als auch die LTA-induzierte Zytokinsynthese gesteigert war (Anlage 6). Die Membranexpression von CD14 ist kein stationäres Phänomen, sondern unterliegt einer Regulation durch Mobilisation aus intrazellulären Kompartimenten, die unter anderem durch LPS und LTA in PMN induziert werden kann (107, 108).

In PMN zeigte sich die LPS-induzierte Synthese von IL-8 als abhängig von einer frühen Freisetzung von TNF und IL-1, wobei die durch LTA-induzierte Synthese von IL-8 ausschließlich durch TNF mediiert schien. Neben diesen mechanistischen Divergenzen unterschieden sich die durch LPS und LTA induzierte Zytokinsynthese insofern, als weitaus höhere Konzentrationen von LTA zur Induktion einer quantitativ vergleichbaren Zytokinsynthese notwendig waren. Das niedrigere immunogene Potenzial von LTA versus LPS wurde bereits beschrieben (105). Interessanterweise unterliegt die LPS-induzierte granulozytäre Synthese von IL-8 einer Autoregulation in Abhängigkeit von der Zelldichte, wobei eine Erhöhung der Zelldichte mit einer Abnahme der Sekretion von IL-8 einherging (Anlage 5). Die Inhibition der Chemokinsynthese wird durch überproportional freigesetzte Mengen löslicher TNF-Rezeptoren und des IL-1-Rezeptorantagonisten mediiert. Angesichts der weitaus niedrigeren Rezeptoraffinität der löslichen Zytokin-Rezeptorantagonisten gegenüber den Liganden IL-1 und TNF könnte dieser Überschuss einem physiologischen Mechanismus entsprechen (132, 133). Diese Autoregulation könnte einer exzessiven Granulozytenakkumulation und Aktivierung in einem infektiösen Fokus vorbeugen.

In Interaktion mit NSCLC-Zellen zeigt sich die Synthese von IL-8 keinesfalls limitiert. Im Gegenteil war diese in LPS-stimulierten Ko-Kulturen von PMN und NSCLC-Zellen massiv amplifiziert (Anlage 8). Somit können im inflammatorischen Tumormikromileu unter dem Einfluss bakterieller Pathogenitätsfaktoren große Mengen dieses pathogenetisch relevanten Chemokins entstehen. Der amplifizierende Effekt der Ko-Kulturen auf die Zytokinsynthese konnte an A549-Zellen auch nach Stimulation mit Lipoteichonsäuren beobachtet werden und wurde in der SCLC-Zelllinie H69 reproduziert. Die amplifizierte IL-8-Synthese war, wie schon zuvor in den Monokulturen an PMN beschrieben, partiell durch autokrin sezerniertes TNF bedingt, da die Neutralisation von TNF eine Verringerung der IL-8-Spiegel in den Zellüberständen der Ko-Kulturen bewirkte. Hier könnte das von PMN sezernierte TNF parakrin

die Synthese von IL-8 in A549-Zellen stimulieren, die den TNFRI exprimieren (140). Auch COX-abhängige Mediatoren trugen zur Amplifizierung der Synthese von IL-8 bei. Dazu passend wurde bereits eine Korrelation der COX-2-Aktivität mit der Synthese von IL-8 in humanen NSCLC-Zelllinien beschrieben (142).

Interessanterweise induzierte die Interaktion von PMN und NSCLC-Zellen eine Steigerung der Proliferation der Lungenkarzinomzellen (Anlage 9). Dieser Effekt war ebenfalls durch COX-abhängige Mediatoren der Arachidonsäure vermittelt, da die gesteigerte Proliferation durch Inhibitoren der COX, insbesondere des Isoenzyms COX-2, attenuiert werden konnte. Im Einklang dazu wurde eine massive, COX-2-abhängige Amplifikation der LPS-induzierten Freisetzung von PGE_2 in den Ko-Kulturen im Vergleich zu Monokulturen von NSCLC-Zellen verzeichnet. Diesem Phänomen könnte ein transzellulärer Lipidmediator-Metabolismus, der durch den wechselseitigen Austausch von Substraten und Produkten gekennzeichnet ist, zugrunde liegen (32). Auch die neutrophile Elastase war in die pro-proliferativen Effekte der PMN involviert. In den Überständen von PMN, die in Gegenwart humaner NSCLC-Zellen kultiviert wurden, war die Sekretion von Elastase im Vergleich zu Monokulturen von PMN mehr als verdoppelt. Darüber hinaus attenuierte pharmakologische Inhibition der Elastase die PMN-abhängige Proliferationssteigerung in NSCLC nahezu auf Normalniveau. In-vivo-Studien bestätigen, dass diese Serinprotease ebenfalls die Kapazität zur Induktion eines pro-proliferativen Signaltransduktion unter anderem durch Aktivierung von EGFR besitzt (53, 156).

Zusammenfassend induzieren bakterielle Pathogenitätsfaktoren an diversen Modellen des NSCLC eine Tumorproliferation und Therapieresistenz. Hierbei spielt sowohl die direkte Aktivierung tumoreigener, pro-proliferativer Mechanismen, wie die Stimulation von TLRs und des EGFR, eine Rolle. Des Weiteren ist die Aktivierung von neutrophilen Granulozyten als wichtige zelluläre Komponenten des Tumormikromilieus in Mono- und Ko-Kultur-Systemen in die Tumorzellproliferation involviert. Entscheidende humorale Effektoren, die über auto- und parakrine Mechanismen die Tumorzellproliferation bedingen, stellen inflammatorische Mediatoren dar, wobei in den vorliegenden Untersuchungen Schlüsselrollen insbesondere für COX-2-abhängige Lipidmediatoren, IL-8 sowie die neutrophile Elastase identifiziert wurden. Die inflammatorische Tumorprogression ist ein multifaktorielles, differenziell reguliertes Geschehen, wobei einige der involvierten inflammatorischen Mechanismen Ansätze für innovative Therapieansätze des NSCLC bieten könnten und bereits in klinischer Erprobung sind.

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V. Eigene Publikationen

Originalarbeiten

Erst- und Seniorautorenschaften

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VI. ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit bzw. die mir zuzuordnenden Teile im Rahmen einer kumulativen Habilitationsschrift, selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Ich versichere, dass ich für die nach § 2 (3) der Habilitationsordnung angeführten bereits veröffentlichten Originalarbeiten als Erst- oder Seniorautor fungiere, da ich den größten Teil der Daten selbst erhoben habe, für das Design der Arbeiten verantwortlich bin und die Manuskripte maßgeblich gestaltet habe. Für alle von mir erwähnten Untersuchungen habe ich die in der „Satzung der Justus-Liebig-Universität zur Sicherung guter wissenschaftlicher Praxis“ niedergelegten Grundsätze befolgt. Ich versichere, dass alle an der Finanzierung der Arbeiten beteiligten Geldgeber in den jeweiligen Publikationen genannt worden sind. Ich versichere außerdem, dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Weise einer anderen Prüfungsbehörde vorgelegt wurde oder Gegenstand eines anderen Prüfungsverfahrens war. Mit der Überprüfung meiner Arbeit durch eine Plagiatserkennungssoftware bzw. ein internetbasiertes Softwareprogramm erkläre ich mich einverstanden.

VII. ABKÜRZUNGSVERZEICHNIS

5-LO	5-Lipoxygenase
AA	Arachidonsäure
BLT2	Leukotrien-Rezeptor B2
COX	Cyclooxygenase
CREB	cAMP response element-binding protein
CXCR1/2	Chemokinrezeptor 1/2
E. coli	Escherichia coli
EGFR	Epidermal growth factor receptor
EP	Prostaglandin E_2-Rezeptor
FLAP	5-Lipoxygenase-aktivierendes Protein
fMLP	N-Formyl-Methionin-Leucin-Phenylalanin
GPI	Glycosylphosphatidylinositol
IL	Interleukin
IL-1 RA	Interleukin-1-Rezeptorantagonist
LBP	Lipopolysaccharid-binding protein
LPS	Lipopolysaccharid
LTA	Lipoteichonsäuren
LTB_4	Leukotrien B_4
NET	Neutrophil extracellular trap
NSCLC	Nicht-kleinzelliges Lungenkarzinom
PGE_2	Prostaglandin E_2
PLA_2	Phospholipase A_2
PMN	Polymorphkerniger neutrophiler Granulozyt
S. aureus	Staphylococcus aureus
SCLC	Kleinzelliges Lungenkarzinom
sTNFR I/II	Löslicher TNF-Rezeptor I/II
TLR	Toll-like-Rezeptor
TME	Tumormikromilieu
VEGF	Vascular endothelial growth factor

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Anlage 1

Hattar K, Savai R, Subtil FS, Wilhelm J, Schmall A, Lang DS, Goldmann T, Eul B, Dahlem G, Fink L, Schermuly RT, Banat GA, Sibelius U, Grimminger F, Vollmer E, Seeger W, Grandel U.

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Abstract Lung cancer is frequently complicated by pulmonary infections which may impair prognosis of this disease. Therefore, we investigated the effect of bacterial lipopolysaccharides (LPS) on tumor proliferation in vitro in the non-small cell lung cancer (NSCLC) cell line A549, ex vivo in a tissue culture model using human NSCLC specimens and in vivo in the A549 adenocarcinoma mouse model. LPS induced a time- and dose-dependent increase in proliferation of A549 cells as quantified by MTS activity and cell counting. In parallel, an increased expression of the proliferation marker Ki-67 and cyclooxygenase (COX)-2 was detected both in A549 cells and in ex vivo human NSCLC tissue. Large amounts of COX-2-derived prostaglandin (PG)E_2 were secreted from LPS-stimulated A549 cells. Pharmacological interventions revealed that the proliferative effect of LPS was dependent on CD14 and Toll-like receptor (TLR)4. Moreover, blocking of the epidermal growth factor receptor (EGFR) also decreased LPS-induced proliferation of A549 cells. Inhibition of COX-2 activity in A549 cells severely attenuated both PGE_2 release and proliferation in response to LPS. Synthesis of PGE_2 was also reduced by inhibiting CD14, TLR4 and EGFR in A549 cells. The proliferative effect of LPS on A549 cells could be reproduced in the A549 adenocarcinoma mouse model with enhancement of tumor growth and Ki-67 expression in implanted tumors. In summary, LPS induces proliferation of NSCLC cells in vitro, ex vivo in human NSCLC specimen and in vivo in a mouse model of NSCLC. Pulmonary infection may thus directly induce tumor progression in NSCLC.

Keywords Lung cancer · Infection · Endotoxin · Tumor proliferation · Inflammation

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Introduction

Infections are considered to promote growth of human cancer. Roughly estimated, about 15 % of all malignancies worldwide can be attributed to infectious agents [1]. Although the contribution of different viruses to the development of malignancies has been well recognized, some chronic bacterial infections are also associated with

tumor formation [2]. In this context, the most prominent example is *Helicobacter pylori*, which is an important risk factor for gastric cancer [3, 4].

In lung cancer, one of the leading causes of cancer-related death in the western hemisphere, the association between bacterial infections and cancer development is less obvious. Lung cancer patients frequently suffer from pulmonary infections, and the most common pathogens found in patients with lung cancer are gram-negative bacteria such as *Haemophilus influenzae* and *E. coli* [5, 6]. Although pulmonary infections have been related to a reduction in the median survival of patients with lung cancer [7], it is not clear whether bacterial infections worsen prognosis of lung cancer by actually accelerating tumor growth and metastasis formation. However, it is well established that persistent inflammation can activate cancer growth [8, 9], and in NSCLC, a prominent role for COX-2-derived lipid mediators has been postulated in this context [10, 11]. In vivo, COX-2 protein and mRNA levels are elevated and are associated with a poor prognosis in lung adenocarcinoma [12, 13]. In vitro, overexpression of COX-2 directly increases survival of lung adenocarcinoma cell lines [14]. PGE_2 is the major COX-2-derived metabolite up-regulated in human lung cancer tissue and cell lines [15, 16]. Direct inhibition of apoptosis and an EGFR-associated signaling have been characterized as molecular mechanisms of PGE_2-induced tumor growth [17].

Regarding NSCLC, COX-2 expression and PGE_2 production in epithelial cancer cell lines have been shown to be induced by benzo[a]pyrene, a potent carcinogen contained in cigarette smoke [18]. In bronchial epithelial cells, however, COX-2 is also induced by endogenous and exogenous proinflammatory stimuli such as the bacterial membrane glycolipid LPS [19, 20], suggesting a relevant role for infectious agents in this context. In general, cellular activation by LPS is initiated via the CD14 surface receptor, a GPI-anchored glycoprotein [21] and TLRs, such as TLR4 [22, 23]. However, some LPS types, mainly from non-enterobacteria are recognized by TLR2, presumably due to differences in the lipid A component [24].

In gastric cancer, the expression of different TLRs enables gastric carcinoma cells to interact with *Helicobacter pylori* [25]. This interaction may be followed by the production of tumor-promoting factors such as IL-8. Most importantly, an up-regulation of TLR4 expression was recently demonstrated in human adenocarcinoma of the lung in vivo, and TLR4 expression levels correlated with malignancy [26]. Thus, specific interactions between bacterial pathogens such as LPS and tumor cells may actually occur in NSCLC. However, the consequences of such interactions for tumor cell biology are less clear.

In the current study, we focused on the effects of bacterial endotoxin in vitro on proliferation of A549 cells, a cell line derived from human lung adenocarcinoma, in an ex vivo short-term cultivation model designated short-term stimulation of tissues (STST) using human specimens obtained from patients with NSCLC and in vivo in the subcutaneous A549 adenocarcinoma mouse model. In essence, we found that LPS strongly induces proliferation in these experimental models, which was mediated by COX-2 activation. Furthermore, interference with CD14, TLR4 and EGFR attenuated the proliferative response to LPS. Thus, our data suggest that LPS exposure as a consequence of pulmonary infections could potentially accelerate tumor progression in lung cancer.

Materials and methods

Cell culture

The A549 human lung adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured at 37 °C in a humidified atmosphere (95 % air, 5 % CO2). All cell culture media and supplements were purchased from Gibco (Eggenstein, Germany) unless otherwise indicated. The cells were kept in Dulbecco's modified Eagle's medium (DMEM/F12), supplemented with 10 % FCS, 2 mM L-glutamine, 10^5 U/l penicillin and 100 mg/l streptomycin. Cells were grown to confluence and subcultured every 2–3 days and split at a ratio of 1:10.

Ex vivo cultivation and stimulation of human lung cancer tissues

Three specimens of human NSCLC of adenocarcinoma type were cultured using a novel short-term tissue cultivation model ex vivo as previously reported [27]. Briefly, vital tissue samples were cultured in 2 ml RPMI 1640 supplemented with 10 % FCS at 37 °C and 5 % CO2 for 16 h in the presence or absence of 10 µg/ml of a highly purified LPS from *E. coli* F515 (kindly provided by Prof. Otto Holst, Immunochemistry Group, Research Center Borstel, Germany) [28]. After termination of the cultivation period [27], the specimens were fixed by the HOPE-technique and embedded in paraffin as described elsewhere [29]. Accordingly, the tissue samples were always deparaffinized for subsequent analyses (immunohistochemistry and molecular analysis).

A549 adenocarcinoma mouse model

Tumor growth was assessed by subcutaneous injection of A549 cells (2.5×10^6 cells/200 µl in PBS) into 8-week-old female BALBc/c nu/nu mice. These mice were

purchased from Charles River (Sulzfeld, Germany) and kept under specific pathogen-free conditions. Immediately before subcutaneous injection, A549 cells were treated with highly purified *E.coli* LPS F515 ($n = 6$) or sham incubation with PBS (control, $n = 8$) was performed. Animals were handled in accordance with the European Community recommendations for experimentation. The size of the tumor was measured at days 4, 8 and 12 after implantation by Mitutoyo digital calipers (Mitutoyo Ltd., UK), as previously described [30]. The tumor volume (TV) was calculated by the formula TV (mm^3) = $(L \times W^2)/2$, where L is the longest dimension of the tumor (in mm) and W is the shortest dimension of the tumor (in mm). Each tumor measurement was taken in triplicate by two different investigators (K.H. and R.S.)

MTS assay

The MTS assay (CellTiter 96@ Aqueous One Solution Cell Proliferation Assay, Promega, Mannheim, Germany) quantifies the metabolic activity of cells. This assay is based upon the cleavage of the yellow 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) to purple formazan by metabolic active cells. The production of the colored formazan product is directly proportional to the number of viable cells in culture [31]. Based on these data, the MTS assay is widely used for the assessment of cellular proliferation.

In brief, A549 cells were seeded on 96-well plates (2,500 cells/well) and maintained in culture for 24 h before LPS stimulation. Then, medium was exchanged, and cells were kept in RPMI containing 1 % FCS at a total volume of 200 µl/well. A549 cells were stimulated with different concentrations of LPS (*E. coli* LPS 0111:B4, Sigma, Deisenhofen, Germany or highly purified *E.coli* LPS F515) for various time periods or sham incubation (control) was performed. For Fig. 1a with LPS from *E. coli* 0111:B4, at least five independent experiments were performed, and for Fig. 1b with LPS from *E. coli* F515, at least four independent experiments were performed. In an additional series of experiments in Fig. 2, function-blocking antibodies targeting TLR2 (clone TL2.1, e-Bioscience, San Diego, CA, USA), TLR4 (clone HTA 125, e-Bioscience, San Diego, CA, USA), CD14 (MY-4, Coulter Immunotech, Hamburg, Germany), EGFR (Cetuximab, Merck Serono, Germany) or COX inhibitors (indomethacin, Sigma, Deisenhofen, Germany and NS-398, Calbiochem, La Jolla, CA, USA) were applied simultaneously to LPS. For these inhibitor studies, at least six independent experiments were performed (at least 3 both for LPS 0111:B4 and for LPS F515, respectively).

At the end of the incubation, 20 µl of MTS solution were added to each well and plates were again incubated

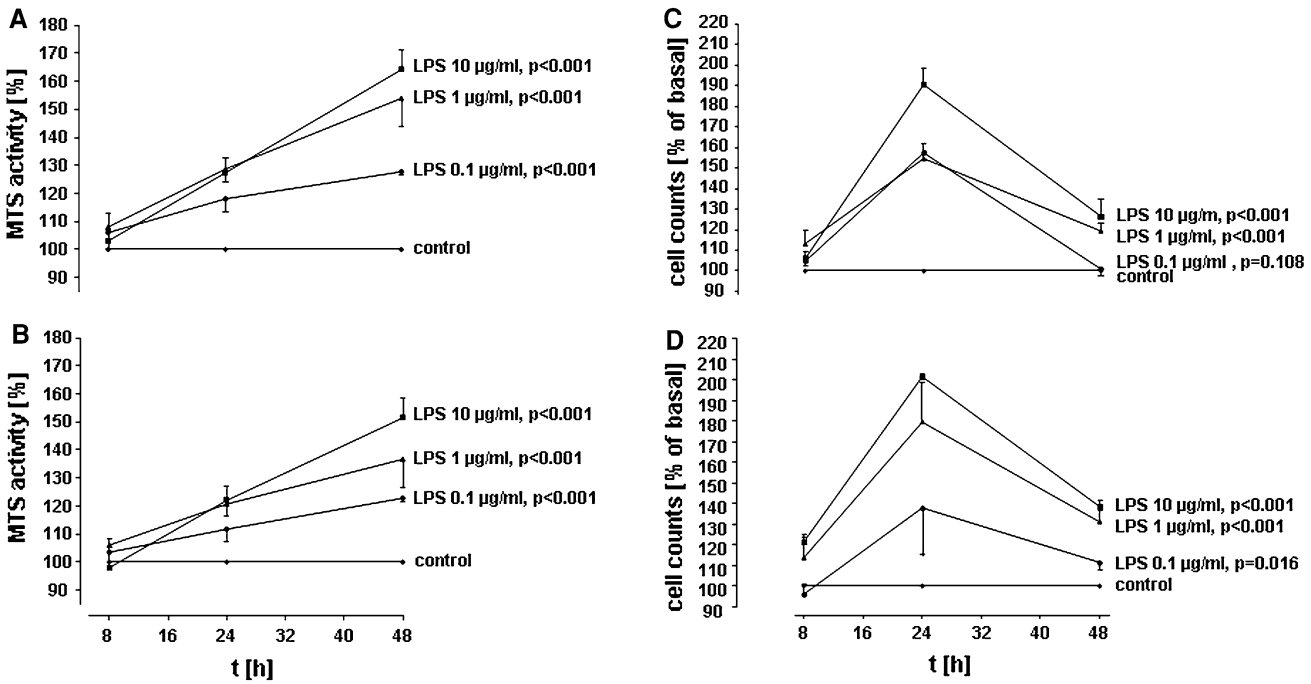

Fig. 1 Time-and dose-dependent induction of A59 proliferation by LPS. A549 cells were incubated with various concentrations of LPS from *E. coli* 0111:B4 (**a/c**) or highly purified LPS from *E. coli* F515 (**b/d**) or sham incubation was performed (*control*). A549 proliferation was assessed by measuring MTS activity (**a/b**) and automatic cell counting (**c/d**). All data are expressed as percentage of unstimulated cells (*control*). Mean ± SEM of at least four independent experiments are given

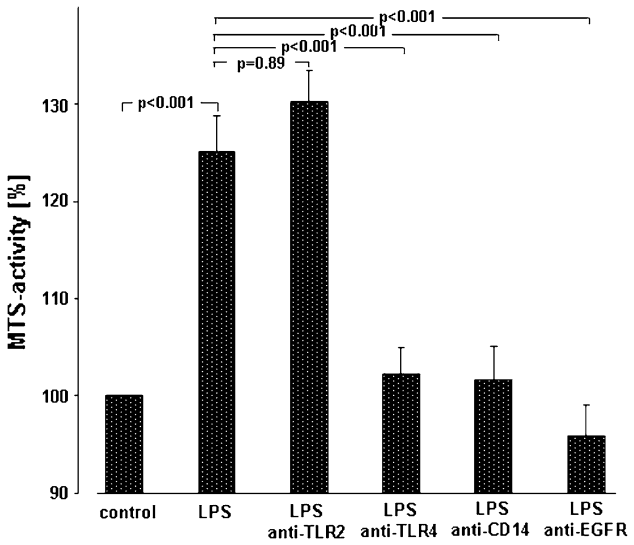

Fig. 2 Mechanisms of LPS-induced A549 proliferation. A549 cells were either sham-incubated (*control*) or exposed to 10 μg/ml of LPS ($n = 3$ for LPS 0111:B4 and $n = 3$ for LPS F515, total $n = 6$) in the absence or presence of neutralizing antibodies targeting TLR2, TLR4, CD14 or EGFR. After 24 h of incubation proliferation was quantified by determining MTS activity. All data are expressed as percentage of unstimulated cells (*control*). Mean ± SEM of six independent experiments are given

for 2.5 h at 37 °C. Absorbance was read at 490 nm, background readings were subtracted from the sample wells, and data were expressed as percentage of controls (sham-incubated cells). All samples were run in triplicate, and all measurements were taken twice after 2.5 h of incubation with the MTS reagent. All data were expressed as percentage increase in MTS activity compared to unstimulated cells (controls), which were set to 100 %.

Assessment of cellular proliferation by cell counting

For cell counting, A549 cells were seeded on 24-well plates (50,000 cells/well) and maintained in culture for 24 h before LPS stimulation. Then, medium was exchanged, and cells were kept in RPMI containing 1 % FCS at a total volume of 500 μl/well. A549 cells were stimulated with different concentrations of LPS (*E. coli* LPS 0111:B4 or *E. coli* LPS F515) or sham-incubated (control). For each LPS type in Fig. 1c, d, at least four independent experiments were performed. At the end of the incubation period, the medium was removed, and cells were washed, detached by treatment with 0.5 % trypsin–EDTA, resuspended in a stop-solution containing 20 % FCS in PBS and finally counted by the cell counter-analyzer system Casy Model TT (Innovatis AG, Reutlingen, Germany). Data were expressed as percentage of controls (sham-incubated cells), which were set to 100 %. All samples were performed in triplicate, and all measurements were taken three times in 200 μl cell casyton suspension each. The orifice tube had an aperture size of 150 μm.

Measurement of PGE_2 and thromboxane A_2

PGE_2 and thromboxane $(Tx)B_2$, the stable hydrolysis product of TxA_2, were quantified in a commercial ELISA system (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions. For these experiments, A549 cells (50,000 cells/well) were seeded on 24-well plates and grown to confluence. Confluent monolayers were washed twice and kept in RPMI containing 1 % FCS at a total volume of 500 μl/well. Then, incubation with different concentrations of LPS (from *E. coli* 0111:B4 or *E. coli* F515) in the absence or presence of the respective antibodies or COX inhibitors for various time periods, or sham incubation (control) was performed. All samples were performed as duplicate. For the time–response curve in Fig. 3c, as well as for the inhibitor studies in Fig. 3a, at least six independent experiments were performed (at least 3 both for LPS 0111:B4 and for LPS F515, respectively). At the end of the incubation period, medium was exchanged, and cells were washed twice and kept in RPMI containing 1 % FCS and further incubated for 8 h with 5 μM arachidonic acid (AA, Sigma, Deisenhofen, Germany). Then, cell supernatants were harvested, cell debris was removed by centrifugation at $13.000 \times g$, and samples were stored at −20 °C until further processing. The measurement of PGE_2 and TxB_2 release was taken by ELISA technique, according to the manufacturer's protocols and is expressed in pg/ml. All samples were performed as duplicate, and each sample was measured twice.

RNA isolation and real-time RT-PCR

For quantification of COX-2 in fig. 3b, Ki-67 and PCNA mRNA, experiments with A549 cells (50,000 cells/well) or short-term stimulation of lung cancer tissues were performed as described above for 16 h. Total RNA was extracted from cells and lung cancer tissues with TRIzol reagent (Invitrogen, Karlsruhe, Germany) or RNeasy minikit (Qiagen, Hilden, Germany), following the manufacturer's protocols. The yield of extracted RNA was determined by Nano Drop (PeqLab, Erlangen, Germany). After digesting residual DNA with DNase (Invitrogen, Karlsruhe, Germany), cDNA was synthesized by RT (Promega, Mannheim, Germany or Applied Biosystems, Darmstadt, Germany). Real-time PCR was performed using 1 μg of cDNA, SYBR Green PCR Master Mix (Invitrogen, Karlsruhe, Germany) and 0.05 M forward/reverse primers; specific primers used for sequence detection were as follows:

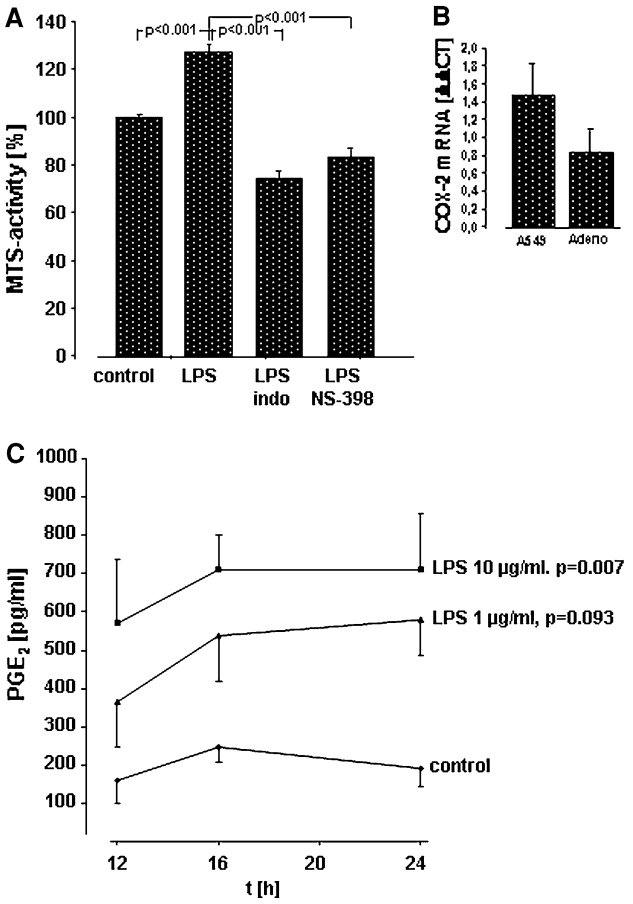

Fig. 3 Activation of COX-2 and release of PGE_2 in A549 cells and human lung cancer tissue in response to LPS. **a** Effect of COX inhibitors on LPS-induced proliferation of A549 cells in vitro. A549 cells were either sham-incubated (*control*) or exposed to 10 µg/ml of LPS ($n = 3$ for LPS 0111:B4 and $n = 3$ for LPS F515, total $n = 6$) in the absence or presence of indomethacin (*indo*) or the COX-2 inhibitor NS-398. After 24 h of incubation proliferation was quantified by determining MTS activity. All data are expressed as percentage of unstimulated cells (*control*). Mean ± SEM of six independent experiments are given. **b** Expression of COX-2 mRNA in response to LPS in A549 cells and human lung cancer tissue. A549 cells and specimen of human adenocarcinoma were either sham-incubated or exposed to 10 µg/ml LPS F515. After 16 h, mRNA was extracted and subjected to quantitative reverse transcriptase polymerase chain reaction. The ΔΔCT values represent relative expression of COX-2 mRNA normalized to the internal reference HPRT mRNA in LPS versus unstimulated A549 cells or lung adenocarcinoma (*Adeno*) tissue. Mean values ± SEM, originating from four independent experiments, each performed in duplicate are given. **c** Release of PGE_2 in A549 cells in response to LPS. A549 cells were either sham-incubated (*control*) or exposed to the given concentrations of LPS (at least $n = 3$ for LPS 0111:B4 and $n = 3$ for LPS F515, total at least $n = 6$) for various time periods. 8 h before the end of the incubation period, and AA was added. PGE_2 release into the cell supernatant is given in pg/ml. Data are expressed as mean ± SEM of at least six independent experiments

for HPRT
5′GGTCCTTTTCACCAGCAAGCT3′8 (forward) and 5′TGACACTGGCAAAACAATGCA3′ (reverse),
for PCNA
5′TTTTCTGTCACCAAATTTGTACCTC3′ (forward) and 5′CTGCATTTAGAGTCAAGACCCTTT3′ (reverse)
for Ki-67
5′AGAAGACAGTACCGCAGATGA3′ (forward) and 5′CGGCTCACTAATTTAACGCTGG3′ (reverse),
for COX-2
5′ATCATAAGCGAGGGCCAGCT3′ (forward 101 bp) and 5′AAGGCGCAGTTTACGCTGTC3′ (reverse 101 bp).

Real-time reactions were carried out on the Stratagene Mix Pro 3000 P (Agilent Technologies Inc) or the Sequence Detection System 7900 HT (Applied Biosystems) with following cycle conditions: denaturation, 95 °C for 10 min, 40 cycles with denaturation at 95 °C for 30 s, annealing at 58–60 °C for 30 s and extension at 72 °C for 30 s. To ensure single-product amplification, a dissociation curve was generated for each gene and the threshold cycle (Ct values) for each gene was determined. The comparative 2−ΔΔCt method was used to analyze mRNA-fold changes between control and LPS, which was calculated as ratio = 2−(ΔCt control−ΔCt LPS). Ct is the cycle threshold and ΔCt (Ct target − Ct reference) is the CT value normalized to the housekeeper gene HPRT obtained for the same cDNA samples [32, 33]. All samples were performed in triplicate, and all measurements were taken three times.

Histological examination of explanted tumors from the A549 adenocarcinoma mouse model

The tumors were frozen in liquid nitrogen and stored at −80 °C. For immunofluorescence, six LPS-treated tumors and eight unstimulated tumors were analyzed. 5 µm whole tumor cross-sections were cut from the central part. Immunofluorescence staining has previously described in detail [34]. Briefly, whole tumor cross-sections were fixed with methanol and acetone (1:1) for 5 min and washed three times with PBS containing 0.1 % BSA and 0.2 % Triton X-100. The unspecific binding sites were blocked with 3 % BSA in PBS for 1 h. For Ki-67, slides were stained with a polyclonal rabbit anti-human nuclear Ki-67 (Abcam Ltd.332, ab833, Cambridge, UK, dilution 1:100 [35]. The secondary antibody, consisting of a goat anti-rabbit IgG (Alexa Fluor 488, Molecular Probes, Eugene, Oregon, USA) was applied at 1:1000. Each section was counterstained for 5 min with 40, 6-diamidino-2-phenylindole (DAPI, Sigma, Deisenhofen, Germany) and mounted with fluorescent mounting medium (Dako, Hamburg, Germany). The cyrosections were also stained with hematoxylin–eosin (H&E) [36].

Microscopic analyses were performed using a fluorescence microscope (Leica DMLA Q550/W, Leica Microsystems, Bensheim, Germany) and Leica Q-Win standard

software for quantification. The whole tumor cross-sections were sequentially scanned. After the scanning procedure, the Ki-67-positive signal of the whole tumor cross-section was detected, and this positive area was measured and related to the whole DAPI-positive area of the same tumor cross-section [37].

Expression of Ki-67 in human lung cancer tissue

Ki-67 was analyzed by immunohistochemistry (IHC) as described earlier [38]. Primary antibody MIB-1 (Dako, Glostrup, Denmark, 333 ng/ml) was used in a final dilution of 1:100. After 30 min at room temperature, visualization was performed by horseradish-peroxidase labeled streptavidin–biotin technique (LSAB2^{TM}, Dako, Denmark) diluted 1:3 and using 3-Amino9-Ethylcarbazole/H_2O_2 as chromogen. Slides were counterstained with Mayer's hemalum and mounted with Kayser's glycerine gelatine. Negative controls were run by omitting the primary antibody.

Statistics

Unlike otherwise indicated, data are given as the relative changes compared to control values and expressed as the mean ± SEM. Raw data were analyzed with R [39]. Linear mixed models were calculated using the package "lme" [40]. Raw data from time series were analyzed using the area-under-the-curve (AUC) approach. AUC values were calculated using the trapezoid rule. Percentages were analyzed using beta regression [41]. Linear mixed models were used for Figs. 1a, b, 2, 3a and 4. Linear models were used for Figs. 1c, d, 3c and 5a. Beta regression was used for Fig. 5b. Residuals of the models were checked for normal distribution, variance homogeneity and influential points. Reported p values are not corrected for multiple testing. Unless otherwise stated, p values below 0.05 keep a family-wise error rate of 5 % (i.e., they would be <0.05 after Bonferroni correction).

Results

Induction of a time- and dose-dependent proliferation of A549 cells by LPS

The A549 monolayers were incubated with different concentrations of LPS (0.1, 1 and 10 µg/ml) for various time periods (8, 24, 48 h). Two different endotoxins were used, either *E. coli* LPS 0111:B4 (Fig. 1a/c) or highly purified *E.coli* LPS F515 (Fig. 1b/d). Both LPS preparations stimulated the proliferation of A549 cells in a time- and dose-dependent manner, as quantified by MTS assay and cell counting when compared to unstimulated controls. The maximal increase in metabolic activity was induced by 10 µg/ml endotoxin stimulation. In response to LPS from *E. coli* 0111:B4, MTS activity was increased to 129 % after 24 h of stimulation and to 157 % after 48 h of incubation (Fig. 1a). Similiar results were obtained for highly purified LPS from *E. coli* F515 (Fig. 1b), with an increase in MTS activity to 122 % after 24 h and to 151 % after

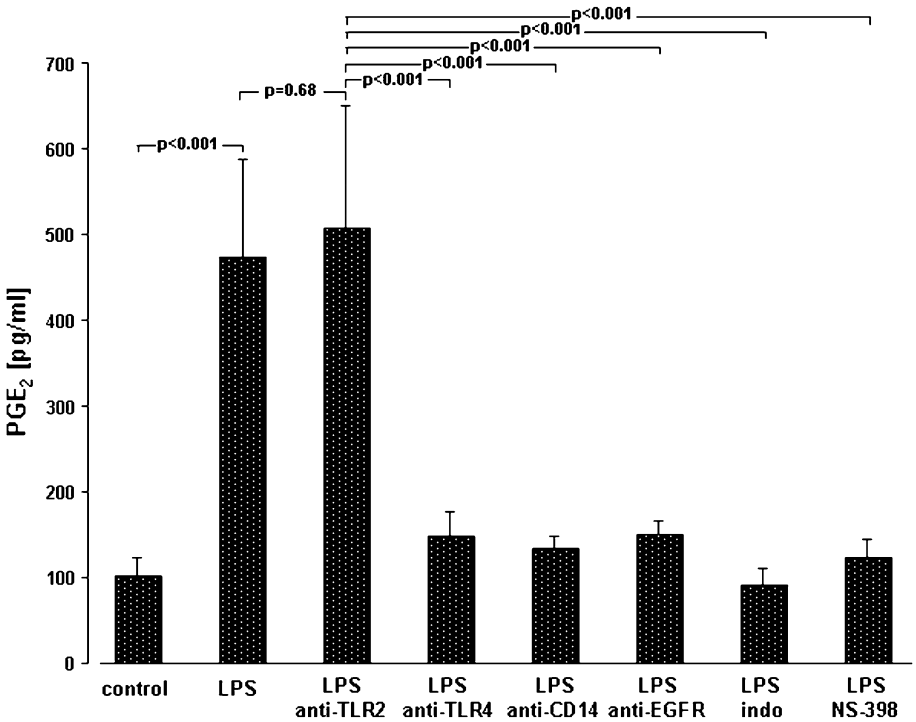

Fig. 4 Mechanisms of LPS-induced PGE_2 synthesis. A549 cells were either sham-incubated (*control*) or exposed to 10 µg/ml of LPS ($n = 3$ for LPS 0111:B4 and $n = 3$ for LPS F515, total $n = 6$) in the absence or presence of neutralizing antibodies targeting TLR2, TLR4, CD14 and EGFR, or the COX inhibitor indomethacin (*indo*) and the specific COX-2 inhibitor NS-398 for 24 h. 8 h before the end of the incubation period, and AA was added. PGE_2 release into the cell supernatant is given in pg/ml. Data are expressed as mean ± SEM of at least six independent experiments

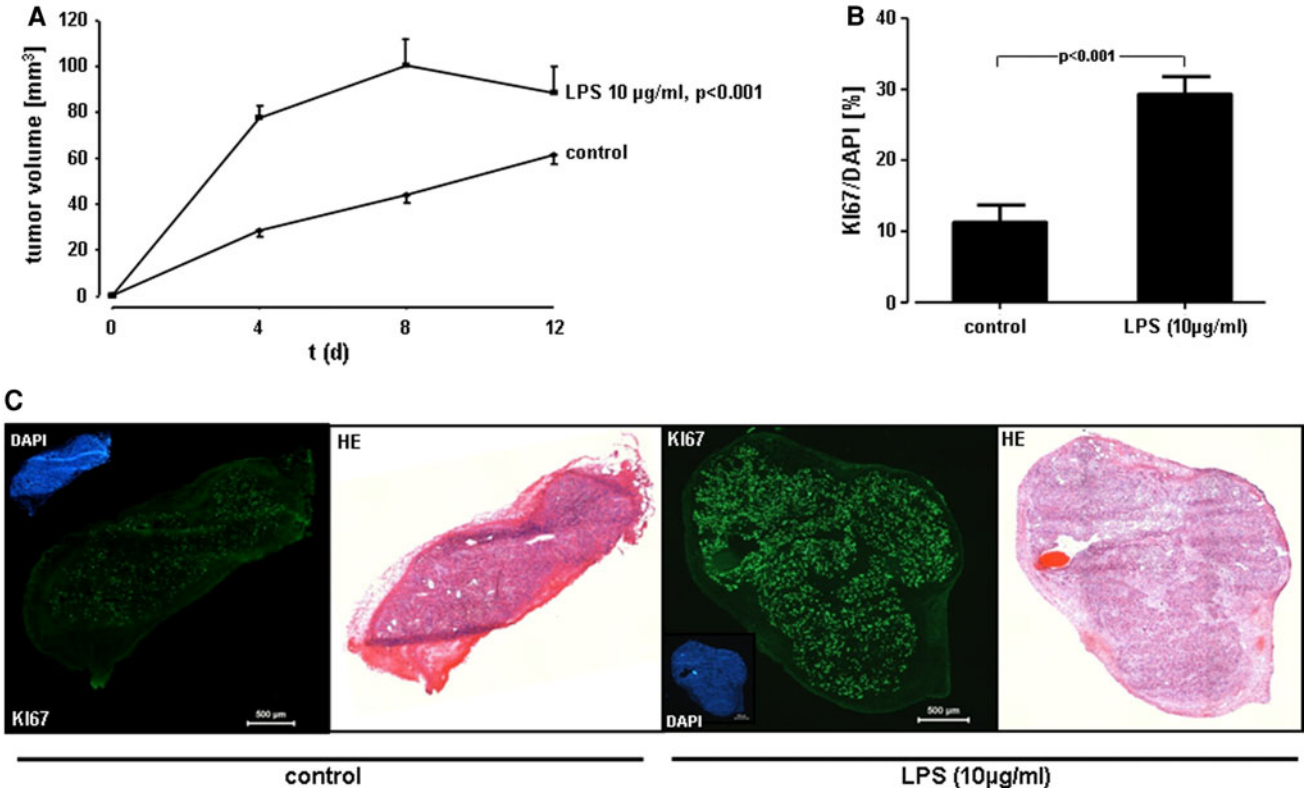

Fig. 5 Proliferative response of A549 cells in vivo. **a** In vivo tumor growth. A549 cells were exposed to 10 µg/ml of LPS F515, or sham incubation was performed. Immediately after treatment, cells were injected subcutaneously into 8-week-old female BALBc nu/nu mice. At indicated time points, the size of the tumor was measured by Mitutoyo digital calipers and is given in mm^3. Data are expressed as mean ± SEM ($n = 8$ for controls and $n = 6$ for LPS). **b** Immunohistofluorescent analysis of Ki-67 in cryosections from A549 tumors. Quantitative analysis of Ki-67 relative to DAPI (%) from the experiments described in (**a**). Data reflect the mean ± SEM ($n = 8$ for controls and $n = 6$ for LPS). **c** Immunofluorescent and H&E staining. Representative images of untreated (*control*) versus LPS-stimulated (10 µg/ml) A549 tumors and of the corresponding DAPI, Ki-67 and the H&E staining, respectively. The *scale bar* corresponds to 500 µm

48 h. Also, cell numbers were maximally increased upon exposure to 10 µg/ml endotoxin. After 24 h, LPS from *E. coli* 0111:B4 elicited an increase in cell numbers to 191 % and to 127 % after 48 h (Fig. 1c), while stimulation with LPS from *E.coli* F515 was equally effective: cell counts were elevated to 201 % after 24 h and to 138 % after 48 h, respectively. Continuous cellular growth was observed during the incubation period (untreated cells were counted as 745 cells after 8 h, 1,060 cells after 24 h and 3,978 cells after 48 h). Moreover, Ki-67 mRNA, as normalized to transcription of the internal standard gene HPRT was up-regulated 3.52 ± 0.52-fold, and PCNA mRNA was up-regulated 5.61 ± 0.88-fold in A549 cells after stimulation with 10 µg/ml LPS ($n = 6$, $n = 3$ for LPS 0111:B4 and $n = 3$ for LPS F515).

Mechanisms of LPS-induced A549 proliferation in vitro

In order to determine the molecular steps in A549 activation by endotoxin, studies with blocking antibodies targeting CD14 (MY-4, 5 µg/ml), TLR2 (TL2.1 10 µg/ml) or TLR4 (HTA125, 10 µg/ml) were performed. As depicted in Fig. 2, LPS increased metabolic activity to 127 % of unstimulated controls in the absence of blocking antibodies. However, this LPS-induced increase in metabolic activity was abolished in the presence of anti-CD14 (102 %) or anti-TLR4 (100 %), whereas blocking of TLR2 activity was not effective (128 %). In addition, targeting EGFR with cetuximab (10 µg/ml) suppressed the LPS-induced proliferation of A549 cells completely (96 %).

Activation of COX-2 and release of PGE_2 in response to LPS

As COX-2-dependent prostanoids play a central role in NSCLC proliferation, the role of COX activation in response to LPS was investigated. In the presence of both the non-specific COX inhibitor indomethacin (100 µM) and the COX-2-specific inhibitor NS-398 (10 µM), the LPS-induced cellular proliferation (127 %) was reduced below control levels (reduction to 74 % for indomethacin

and to 83 % for NS-398 compared with unstimulated controls, Fig. 3a).

In parallel, COX-2 mRNA in A549 cells and in ex vivo stimulated human NSCLC tissue specimens was up-regulated as reflected by the positive ΔΔ CT values for COX-2 mRNA in response to LPS (Fig. 3b). Moreover, a time- and dose-dependent accumulation of PGE_2 was detected in the supernatant of LPS-stimulated A549 cells (Fig. 3c). Again, the higher (10 μg/ml) endotoxin concentration was most effective and elicited an almost fivefold increase in PGE_2 after 24 h. TxB_2 was not released (data not shown). PGE_2 synthesis in response to LPS could be effectively prevented by indomethacin (100 μM) and the specific COX-2 inhibitor NS-398 (10 μM). Both inhibitors diminished LPS-induced PGE_2 release to control levels (Fig. 4). Corresponding to the LPS-activated proliferation of A549 cells, PGE_2 release depended on ligation of TLR4 and CD14. Moreover, targeting EGFR also inhibited PGE_2 release in response to LPS (Fig. 4).

Tumor cell proliferation in response to endotoxin in vivo

In order to investigate the effect of LPS on tumor growth in vivo, LPS-stimulated (10 μg/ml) or unstimulated A549 cells were injected subcutaneously into BALBc nu/nu mice. All mice developed tumors at the site of injection. The size of tumor xenografts was measured over a 12 days period. Already after 4 days, tumor growth was 2.8-fold enhanced upon LPS stimulation. In fact, tumor size was 27 mm^3 in unstimulated tumors (control) and 78 mm^3 in LPS-stimulated tumors (Fig. 5a). These growth effects of LPS were observed up to 12 days after tumor cell transplantation. The increase in tumor size was accompanied by a significant up-regulation of the proliferation marker Ki-67, as assessed by immunofluorescence staining. In LPS-stimulated tumors, 30 % of cells expressed Ki-67 expression, while in unstimulated controls only 12 % of tumor cells were Ki-67 positive (Fig. 5b).

Ki-67 expression in human lung cancer tissue after LPS exposure

Short-term stimulation of NSCLC tissue specimens using the ex vivo STST model [27] with 10 μg/ml LPS for 16 h revealed a twofold up-regulation of the proliferation marker Ki-67 as assessed by immunohistochemistry. Mean values of Ki-67 nuclear staining were 7.5 ± 3.8 % positive cells in untreated NSCLC specimens versus 15.0 ± 5.77 % positive cells in LPS-treated tissue specimens ($n = 3$).

Immunohistochemical staining of Ki-67 in a representative human NSCLC specimen of adenocarcinoma type in the absence or presence of LPS is depicted in Fig. 6.

Discussion

Pulmonary infections are frequently encountered in lung cancer and may worsen prognosis in advanced stages of the disease. While therapy of lung cancer is often hampered by recurrent pulmonary infections, it is still unknown whether lung cancer growth and progression are actually accelerated by bacterial infections of the lung. In the present study, we demonstrated that purified endotoxin, the main pathogenicity factor of gram-negative bacteria, promotes tumor progression in A549 cells in vitro and in a mouse model assessing subcutaneous tumor growth of A549 cells transfected into nude mice. Importantly, the proliferation-enhancing effect of LPS was also evident in ex vivo stimulated intact human tissue specimen obtained from patients with NSCLC, thus giving a strong hint on the clinical significance of the current data.

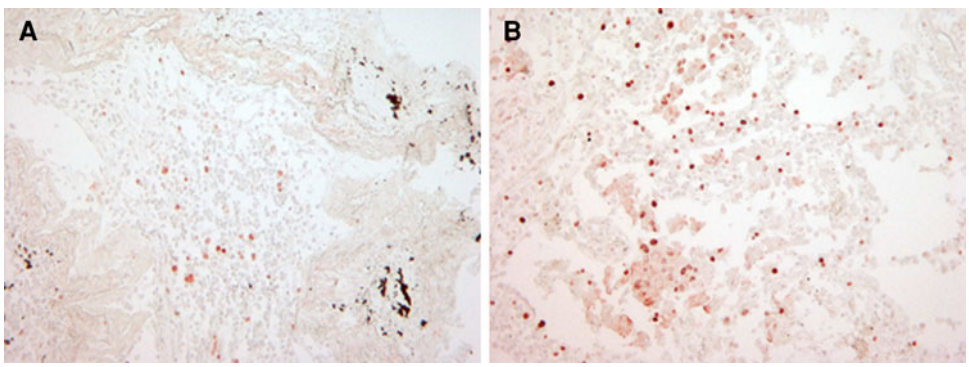

Fig. 6 Expression of Ki-67 in human non-small cell lung cancer tissue in response to LPS. Human lung adenocarcinomas were cultivated in the absence or presence of 10 μg/ml LPS F515 ($n = 3$) for 16 h and subsequently treated with the novel HOPE-fixation technique and paraffin-embedding method. After deparaffinization, Ki-67 protein expression was assessed by IHC. One representative example of both an unstimulated (**a**) and LPS-stimulated (**b**) human NSCLC specimen of adenocarcinoma type is shown (magnification ×200)

Proliferation of tumor cells in response to LPS in A549 cells was induced by the ligation of LPS to CD14 and the pattern recognition receptor TLR4 followed by COX-2-dependent PGE_2 synthesis. Moreover, activation of EGFR was involved in LPS-activated tumor cell proliferation.

Our data suggest that LPS is a relevant and potent stimulator of tumor cell proliferation. In vitro, a clear dose- and time-dependent increase in MTS activity was noted in A549 cells incubated with various concentrations of LPS. The increase in metabolic activity was already noted after 24 h of LPS treatment and reached a maximum after 48 h. The metabolic activity of A549 cells is known to correspond to cellular proliferation [31], which was further validated by automatic cell counting with cell numbers being doubled after 24 h of LPS stimulation and further climbing thereafter, but to a lesser extent than MTS activity. As the best correlation between MTS activity and cell counts was evident after 24 h of stimulation, this time point was chosen for further experiments. In A549 cells, both the widely used LPS from *E. coli* 0111:B4 strain and a highly purified LPS preparation from *E. coli* strain F515 were equally effective, supporting the notion that this effect is not restricted to LPS of a certain bacterial strain or due to any contamination or preparation effect. Moreover, this proliferation- enhancing effect of LPS was not restricted to A549 cells. Intact human NSCLC tissue specimens that were stimulated ex vivo using the STST assay exhibited an increase in Ki-67 expression, a marker of cellular proliferation. The currently used HOPE-fixation technique for NSCLC specimen provides an excellent preservation of proteins and extremely low degradation of nucleic acids [29], thus allowing to illustrate most solidly the intact tumor's response to endotoxin. Additionally, in vivo tumor size was doubled in LPS-challenged tumors in the A549 adenocarcinoma mouse model. These results are in accordance with recent experimental data showing that LPS stimulates tumor growth in human ovarian cancer [42] and animal models of breast cancer [43] and induces apoptosis resistance of NSCLC cells [44].

The potential biologic significance of the proliferation enhancing effects of LPS are supported by the fact that the extent of LPS-induced cellular proliferation observed in our study approached or even exceeded that reported by well known other endo- or exogenous proliferative agents such as IL-8 [45] or benzo[a]pyrene [46]. It is noteworthy that even the single administration of low doses of LPS to A549 cells before subcutaneous implantation in the A549 adenocarcinoma mouse model was sufficient to induce a doubling of tumor size within the 12-day observation period.

As the currently described short-time tumor-promoting effects of endotoxin detected in vitro and in the subcutaneous tumor model might not reflect the physiological situation exactly, the effects of endotoxin and the potential mechanisms (i.e., TLR4, EGFR, COX-2 activation,) need to be validated in orthotopic lung cancer models, for example, in recently described in situ models using bioluminescence [47, 48].

However, our current experimental data clearly suggest that binding of LPS to the LPS receptors CD14 and TLR4 appears to be the initial steps in LPS-induced increase in proliferation. Application of both the blocking anti-CD14 antibody and the anti-TLR4 antibody suppressed the LPS-induced increase in MTS activity in A549-cells, whereas addition of the anti-TLR2 antibody was ineffective. This is of particular interest, as an up-regulation of TLR4 expression has been reported recently in human adenocarcinoma of the lung with TLR4 expression levels correlating with malignancy [26].

In our study, binding of LPS to CD14 and TLR4 was followed by the activation of COX-2 with subsequent PGE_2 release. CD14/TLR4-dependent COX-2 activation may represent a crucial step in mediating tumor proliferation in response to endotoxin, and this conclusion is based on several reasons: first, when CD14 or TLR4 (but not TLR2) were blocked by the respective antibodies, no PGE_2 was released from A549 cells and MTS activity as a marker of cell proliferation remained unchanged; second, when the non-specific COX inhibitor indomethacin was used, neither PGE_2 release nor an increase in MTS activity was noted in LPS-stimulated A549 cells; third, the COX-2-specific inhibitor NS398 was equally effective in inhibiting prostanoid formation and MTS activity as compared to indomethacin; and fourth, marked expression of COX-2 mRNA in both A549 cells and in intact human ex vivo stimulated human lung cancer tissue was observed, following incubation with LPS. Therefore, COX-2 is strongly suggested as the predominant isoform of COX engaged in mediating tumor cell proliferation. Whether COX-2-derived PGE_2 is the key prostanoid mediating LPS-induced NSCLC proliferation cannot directly be derived from our data. However, PGE_2 is the major prostanoid of A549 cells [16], is strongly induced in lung cancer tissue [15] and is known to promote NSCLC growth in vitro [11] and in vivo in a murine model of lung cancer [38].

In addition to CD14-and TLR4-dependent LPS-signaling, activation of the EGFR seems to be a crucial element of the observed tumor cell proliferation. When EGFR was blocked by cetuximab in A549 cells, both the LPS-induced increase in MTS activity and the release of PGE_2 were blunted. This is of special interest, since overexpression of EGFR has been associated with tumor development and poor prognosis in NSCLC [49, 50] and novel inhibitors of this signaling pathway such as cetuximab, erlotinib and gefitinib are of increasing clinical importance [51–54].

Thus, EGFR inhibitors may equally be effective in inhibiting NSCLC growth triggered by infections.

The precise mechanism of LPS-induced EGFR activation cannot be derived from our data. Direct activation of this receptor system and downstream signaling events such as ERK and JNK activation in response to LPS have been described [55, 56]. In addition, we and others have demonstrated that LPS activates IL-8 synthesis in A549 cells [57, 58], and this cytokine is known to transactivate EGFR in NSCLC cell lines as an alternative pathway [59].

In conclusion, this is the first study to demonstrate that LPS effectively induces tumor growth in various experimental models of NSCLC in vitro, ex vivo and in vivo via CD14-, TLR4-, EGFR- and COX-2-signaling. Our results support the hypothesis that pulmonary infections may severely worsen the prognosis of NSCLC by accelerating tumor progression.

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Conflict of interest The authors declare that they have no conflict of interest.

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Anlage 2

Hattar K, Reinert CP, Sibelius U, Gökyildirim MY, Subtil FSB, Wilhelm J, Eul B, Dahlem G, Grimminger F, Seeger W, Grandel U.

Lipoteichoic acids from Staphylococcus aureus stimulate proliferation of human non-small-cell lung cancer cells in vitro.

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ORIGINAL ARTICLE

Lipoteichoic acids from *Staphylococcus aureus* stimulate proliferation of human non-small-cell lung cancer cells in vitro

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Abstract Pulmonary infections are frequent complications in lung cancer and may worsen its outcome and survival. Inflammatory mediators are suspected to promote tumor growth in non-small-cell lung cancer (NSCLC). Hence, bacterial pathogens may affect lung cancer growth by activation of inflammatory signalling. Against this background, we investigated the effect of purified lipoteichoic acids (LTA) of *Staphylococcus aureus* (*S. aureus*) on cellular proliferation and liberation of interleukin (IL)-8 in the NSCLC cell lines A549 and H226. A549 as well as H226 cells constitutively expressed TLR-2 mRNA. Even in low concentrations, LTA induced a prominent increase in cellular proliferation of A549 cells as quantified by automatic cell counting. In parallel, metabolic activity of A549 cells was enhanced. The increase in proliferation was accompanied by an increase in IL-8 mRNA expression and a dose- and time-dependent release of IL-8. Cellular proliferation as well as the release of IL-8 was dependent on specific ligation of TLR-2. Interestingly, targeting IL-8 by neutralizing antibodies completely abolished the LTA-induced proliferation of A549 cells. The pro-proliferative effect of LTA could also be reproduced in the squamous NSCLC cell line H226. In summary, LTA of *S. aureus* induced proliferation of NSCLC cell lines of adeno- and squamous cell carcinoma origin. Ligation of TLR-2 followed by auto- or paracrine signalling by endogenously synthesized IL-8 is centrally involved in LTA-induced tumor cell proliferation. Therefore, pulmonary infections may exert a direct pro-proliferative effect on lung cancer growth.

Keywords Lung cancer · Infection · Lipoteichoic acids · Tumor proliferation · Interleukin-8 · Toll-like-receptor-2

Abbreviations

DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
Lpp	Lipopeptides
LTA	Lipoteichoic acids
MTS	3-(4, 5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NSCLC	Non-small-cell lung cancer
S. aureus	*Staphylococcus aureus*
STR	Short-tandem repeat

Introduction

Lung cancer is the leading cause of cancer-related death in the western hemisphere [1]. In the course of the disease,

Parts of the doctoral thesis of Christian P. Reinert are incorporated in this manuscript.

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patients frequently develop pulmonary infections which have been reported to reduce the median survival substantially [2]. It is not clear, whether this reduction in median survival is merely attributable to the clinical complications of pulmonary infections, or whether bacteria may directly stimulate lung cancer growth.

Although the most common pathogens found in NSCLC are of Gram-negative origin, Gram-positive germs such as *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pneumoniae* account for about 25% of pulmonary infections in lung cancer patients and are the leading cause of septicemia in lung cancer [3]. Cell wall components of bacterial pathogens such as lipopolysaccharides, the so-called "endotoxin" of Gram-negative bacteria and their Gram-positive equivalents, lipoteichoic acids (LTA), peptidoglycanes and lipopeptides (Lpp) [4] are major bacterial pathogenicity factors. After ligation of LPS to the CD14 molecule [5], cellular activation is initiated by binding to toll-like receptors. It is widely accepted that TLR-4 confers responsiveness to LPS [6, 7] while TLR-2 seems to be the key receptor for LTA [8–10]. Once TLR-dependent signalling is initiated, a plethora of proinflammatory mediators such as cytokines and lipid mediators are released by immunocompetent cells [8, 11].

It is well established that persistent inflammation and inflammatory mediators can promote cancer growth [12–14]. In lung cancer, a clear pathogenic role has been attributed to chronic inflammatory diseases such as chronic obstructive pulmonary disease [15]. One early step in the development of lung cancer is the activation of inflammatory cascades resulting in synthesis of growth factors and cytokines such as TGF-ß, IL-1, and IL-8 [15]. Once lung cancer has developed, further tumor progression may be caused by inflammatory mediators [16]. Among these inflammatory mediators IL-8 is of special relevance, because in cultured NSCLC cells and in animal models of NSCLC IL-8 has been shown to promote tumor growth [17, 18]. Moreover, in lung cancer patients, there is a clear correlation between IL-8 expression, tumor angiogenesis and overall survival [19].

Synthesis of IL-8 is induced in response to activation of TLRs in myeloid-derived cells such as macrophages and neutrophils [20, 21]. Interestingly, the expression of TLRs is not restricted to myeloid-derived cells. As TLRs are found in a variety of human cancers of epithelial origin, they could definitively play a role in cancer progression. In gastric cancer, the expression of different TLRs enables gastric carcinoma cells to interact with *Helicobacter pylori* [22], which is followed by the production of tumor-promoting factors such as IL-8 [23] and proliferation of cancer cells [24]. Remarkably, an up-regulation of TLR-4 expression was recently demonstrated in human adenocarcinoma of the lung in vivo and TLR-4 expression levels correlated with malignancy [25]. TLR-2 is equally expressed by NSCLC cells in vitro [26] and TLR-2 mRNA has been detected in the bronchoalveolar fluid of patients with NSCLC [27].

Thus, specific interactions between bacterial pathogens and tumor cells may actually occur in NSCLC. For LPS, enhancement of lung cancer tumor growth has been described in NSCLC cell lines and in xenograft and in orthotopic models of lung cancer [28, 29]. In contrast, the consequences of the interaction between lung cancer cells and LTA are less obvious.

In the current study, we investigated the effect of highly purified LTA from *S. aureus* on proliferation and metabolic activity in human NSCLC cell lines of adeno- and squamous cell carcinoma origin. In essence, we found that LTA is a pro-proliferative stimulus for the tumor cell lines. Cellular activation proceeded via ligation of TLR-2 and endogenously formed IL-8 turned out to be a key mediator in NSCLC proliferation induced by LTA.

Materials and methods

Cell culture and authentication

The human lung adenocarcinoma cell line A549 (ATCC-CCL-185) as well as the human lung squamous carcinoma cell line H226 were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured at 37 °C in a humidified atmosphere (95% air, 5% CO_2). Cells were used up to passage 40. Cells were regularly checked for contamination with mycoplasma by the local department of microbiology by analysis of 16S r DNA followed by amplicon sequencing as previously described [30, 31]. Moreover, both cell lines used were subjected to authentication by the German Collection of Microorganisms and Cell Cultures ("Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH", DSMZ) by short-tandem repeat (STR) DNA profiling [32]. STR profiles of the currently used cell lines showed a full match with the respective reference STR profiles. Thus, the A549 and H226 cells used in the current study were derived from authentic cell cultures. All cell culture media and supplements were from Gibco (Eggenstein, Germany), and cell culture plasticware was from Greiner Bio-One (Frickenhausen, Germany). NSCLC cell lines were grown in Dulbecco's modified Eagle's medium (DMEM/F12), supplemented with 10% FCS, 2 mM L-glutamine, 10^5 U/l penicillin and 100 mg/l streptomycin (culture medium). Cells were grown to confluence and subcultured every 2–3 days at a split ratio of 1:10.

Assessment of cellular proliferation by cell counting

A549 or H226 cells were seeded on 24-well plates (15,000 cells/well for A549 and 30,000 cells/well for H226) and maintained in culture medium for 24 h before stimulation with LTA (*S. aureus*, purified, InvivoGen, San Diego, CA, USA) After removement of culture medium by two washing procedures with RPMI, cells were kept in DMEM/F12 supplemented with 1% FCS at a total volume of 500 µl/well. NSCLC cells were exposed to various concentrations of LTA or sham-incubation was performed (control). In an additional series of experiments in Fig. 4, function-blocking antibodies targeting TLR-2 (clone TL2.1, e-Bioscience, San Diego, CA, USA), TLR-4 (clone HTA 125, e-Bioscience, San Diego, CA, USA), or IL-8 (MAB 208, R&D Systems, Wiesbaden, Germany) were applied simultaneously to LTA. Antibodies targeting TLRs were applied at 0.5 µg/ml, whereas the neutralizing IL-8 antibody was used at 5 µg/ml. At the end of the incubation period from 24 to 72 h, medium was removed, cells were washed twice and subsequently treated with 0.5% trypsin–EDTA. Detached cells were resuspended in a stop-solution (PBS containing 20% FCS) and subsequently counted automatically by the cell counter-analyzer system CasyR Model TT (Innovatis AG, Reutlingen, Germany). Data were expressed as percentage of controls (sham-incubated cells), which were set to 100%. Two technical replicates per sample were run in each independent experiment.

MTS assay

The MTS assay (CellTiter 96@ Aqueous One Solution Cell Proliferation Assay, Promega, Mannheim, Germany) quantifies the metabolic activity of cells. This assay is used to quantify cellular proliferation and is based upon the cleavage of the yellow 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) to purple formazan by metabolic active cells. A549 or H226 cells were seeded on 96-well plates (2500 cells/well for A549 and 5000 cells for H226) 24 h before stimulation with LTA in culture medium. After removement of culture medium and two washing procedures, cells were kept in DMEM/F12 supplemented with 1% FCS at a total volume of 200 µl/well. Both NSCLC cell lines were stimulated with increasing concentrations of LTA for various time periods; alternatively, sham-incubated controls were run for each time period. 2.5 h before the end of the incubation period, 20 µl of MTS solution were added to each well and plates were incubated for another 2.5 h under light protection and continuous shaking. Then, absorbance was read at 490 nm, background readings were subtracted from the sample wells and data were expressed as percentage of controls (sham-incubated cells) which were set to 100%. Five technical replicates per sample were run in each independent experiment.

Measurement of IL-8

IL-8 was quantified from the cell supernatants of LTA-stimulated A549 cells by ELISA technique. For these experiments, A549 cells (15,000 cells/well) were seeded on 24-well plates and grown to confluence. Confluent monolayers were washed twice and kept in DMEM/F12 with 1% FCS at a total volume of 500 µl/well. Then, incubation with different concentrations of LTA or sham-incubation (control) was performed for various time periods. In a separate set of experiments, stimulation with LTA was performed in the absence or presence of antibodies targeting TLR-2 and TLR-4. Two technical replicates per sample were run in each independent experiment. At the end of the incubation period, cell supernatants were harvested, cell debris was removed by centrifugation at 13.000×*g* and samples were stored at −20 °C until further processing. Release of IL-8 was determined in a direct sandwich ELISA, as described previously [33]. To normalize the data, IL-8 was expressed as ΔIL-8, meaning that baseline levels of IL-8 secreted from unstimulated controls were subtracted from those induced by stimulation with LTA.

RNA isolation and real-time RT-PCR

For quantification of IL-8 mRNA, experiments with A549 cells (50,000 cells/well) were performed as described above. Each independent experiment consisted of two technical replicates per sample.

Total RNA was extracted from cells with TRIzol Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocols. Extracted RNA was quantified with Nano Drop (PeqLab, Erlangen, Germany). Residual DNA was digested with DNase (Invitrogen, Karlsruhe, Germany) and cDNA was synthesized by RT (Bio-Rad, München, Germany). Real-time PCR was performed using 1 µg of cDNA, SYBR Green PCR Master Mix (Bio-Rad, München, Germany) and 0.05 M forward/reverse primers; specific primers used for sequence detection were as follows:

for *IL-8*:

5′AGTTTTGCCAAGGAGTGCTAAA3′ (forward) and 5′TGAATTCTCAGCCCTCTTCAAA3′ (reverse).

for *PBGD*:

5′CAGCTTGCTCGCATACAGAC3′ (forward) and 5′GAATCTTGTCCCCTGTGGTG3′ (reverse).

for *TLR2*:

5′AGCCTTGACCTGTCCAACAA3′ (forward) and 5′GGCTTGAACCAGGAAGACGA3′ (reverse).

Real-time-reactions were carried out on the CFX Connect Real-Time PCR Detection System (Bio-Rad, München, Germany) with following cycle conditions: denaturation at 95 °C for 3 min; 40 cycles with denaturation at 95 °C for 10 s, annealing at 59 °C for 10 s and extension at 72 °C for 10 s. To ensure single-product amplification, a dissociation curve was generated for each gene and the threshold cycle (Ct values) for each gene was determined.

Relative mRNA-levels were expressed as ΔCt values which were calculated with the genes Ct values normalized to the housekeeping gene PBGD Ct values. The comparative 2^ΔΔCt method was used to analyze mRNA-fold changes between control and LTA, which was calculated as ratio = 2^(ΔCt control − ΔCt LTA) [28, 34]. Ct is the cycle threshold and ΔCt (Ct gene of interest − Ct housekeeping gene) is the CT value normalized to the housekeeping gene PBGD obtained for the same cDNA samples. The specificity of the primer pair products was tested by agarose (1.5%) gel electrophoresis (Supplementary Fig. 1).

Statistics

Unlike otherwise indicated, data are given as the relative changes compared to control values and expressed as the mean ± SEM. Data analysis was performed in R [35] using the packages "lme4" and "lmerTest" [36]. Data were analyzed with linear mixed models to account for inter-experimental differences. The variable "time" was taken as a factor in a two-factorial model together with "LTA concentration". Data were checked for the agreement with the model assumptions by analysis of the residuals. MTS activity data were inversely transformed before statistical analysis to meet the model assumptions. The diagrams show the means with SEM. The horizontal dashed line indicates the value of the unstimulated controls. For statistical analysis of changes in mRNA expression, student´s *t* test was performed. Groups or conditions with $p < 0.05$ are marked with asterisks.

Results

A549 and H226 cells express TLR-2 mRNA

To confirm that the NSCLC cell lines used express TLR-2 as a prerequisite for specific interaction with LTA, we analyzed TLR-2 mRNA by PCR. PDGD served as housekeeping gene. Unstimulated A549 and H226 cells were clearly positive for TLR-2 mRNA, as depicted in Table 1a. Interestingly, upon treatment with 0.1 µg/ml LTA for 24 h, TLR-2 expression was doubled in A549 cells, whereas H226 cells did not show any up-regulation of TLR-2 mRNA after stimulation with LTA (Table 1b). Specificity of the primer pairs was visualized by agarose (1.5%) gel electrophoresis (Supplementary Fig. 1).

LTA induces proliferation of A549 cells

A549 cells were stimulated with different concentrations of LTA (0.01–1 µg/ml) for various time periods (24, 48, 72 h) or sham-incubation was performed (baseline). The highly purified LTA preparation stimulated the proliferation of A549 cells in a time-dependent manner, as quantified by automatic cell counting (Fig. 1a). LTA-induced increase in proliferation is expressed as percentage of baseline levels, which was set to 100%. Even low concentrations of LTA (0.01 µg/ml) were capable of inducing a significant increase in cellular proliferation. After 24 and 48 h of stimulation, all LTA concentrations induced increases in proliferation by ~25 to 35% of baseline levels. After 72 h of stimulation, increased proliferation was still observed when low (0.01 µg/ml) concentrations of LTA were used, whereas stimulation with 1 µg/ml LTA over this time period was ineffective. The most prominent increase in cellular proliferation was observed after stimulation with 0.1 µg/ml LTA for 48 h (increase by 35.36% ± 1.25% of baseline levels).

Table 1 (a) Basal expression of TLR-2 mRNA in A549 and H226 cells ($n = 4$), (b) regulation of TLR-2 mRNA expression upon stimulation with LTA in A549 and H226 cells, $n = 4$

(a)	A549, Ct	H226, Ct
TLR-2 mRNA	27.69 ± 0.43	27.70 ± 0.39
PBGD mRNA	21.41 ± 0.18	21.94 ± 0.13
(b) TLR-2mRNA	A549, fold-regulation	H226, fold regulation
Control	1	1
LTA 0.1 µg/ml	1.99 ± 0.39*	0,90 ± 0.20

* $p < 0.05$ vs control

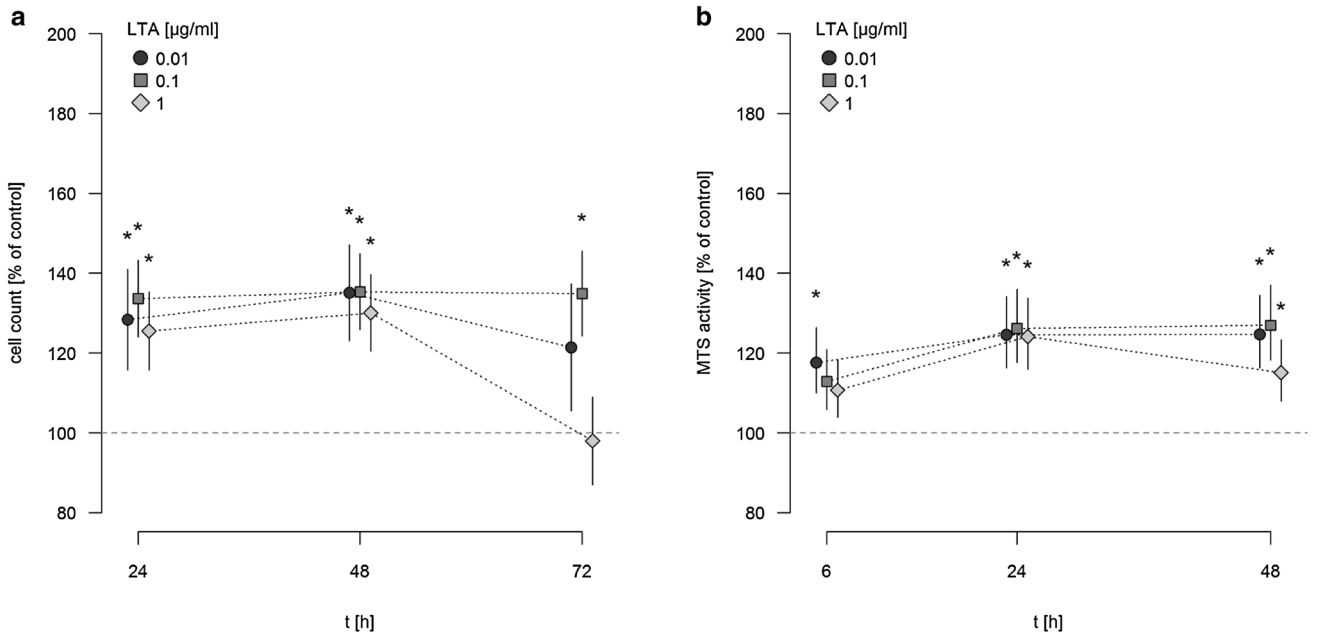

Fig. 1 Time-dependent induction of A549 proliferation and MTS activity by LTA. **a** A549 cells were incubated with various concentrations of LTA from *S. aureus* or sham-incubated (control). A549 proliferation was assessed by automatic cell counting. The *horizontal dotted line* indicates the baseline proliferation of sham-incubated cells, which was set to 100%. All data are expressed as percentage of baseline proliferation. Means ± SEM of at least seven independent experiments are given. *Values marked with an asterisk* differ significantly from controls ($p < 0.05$). **b** A549 cells were incubated with various concentrations of LTA from *S. aureus* or sham-incubated (control). Metabolic activity of A549 cells was quantified by MTS assay. *The horizontal dotted line* indicates the baseline proliferation of sham-incubated cells, which was set to 100%. All data are expressed as percentage of baseline proliferation. Means ± SEM of at least seven independent experiments are given. *Values marked with an asterisk* differ significantly from controls ($p < 0.05$)

LTA failed to induce any pro-proliferative activity of A549 cells when applied at lower concentrations than 0.01 µg/ml: After 24 h, proliferation rates were 105.1 ± 2.7% of baseline upon stimulation with 0.001 µg/ml LTA and 103.4 ± 2.9% after stimulation with 0.0001 µg/ml LTA ($n = 5$). Also after longer incubation periods with 0.001 µg/ml LTA and below, no significant pro-proliferative effect of LTA was observed (e.g. after 48h, proliferation induced by 0.001 µg/ml LTA was 105.5 ± 0.7 and 99.2 ± 1.9% of baseline levels when 0.0001 µg/ml LTA were used, respectively, $n = 5$).

The assessment of metabolic activity, as quantified by MTS assay, showed a comparable pro-proliferative effect of LTA on A549 cells (Fig. 1b). MTS activity was expressed as percentage of baseline levels, which was set to 100%. After 6 h of incubation a significant increase in MTS activity, was noted after stimulation with 0.01 µg/ml LTA. After longer incubation periods of 24 and 48 h, all LTA concentrations increased metabolic activity of A549 cells by ~20 to 25% of baseline levels. Just as observed by automatic cell counting, the most prominent increase in MTS activity of A549 cells was noted after stimulation by 0.1 µg/ml LTA for 48 h (increase by 26.9 ± 1.8% of baseline levels).

LTA induces a time- and dose-dependent release of IL-8 in a TLR-2-dependent manner

When supernatants of LTA-activated A549 cells were analyzed for IL-8 release by ELISA, a dose-dependent release of this chemokine was noted upon stimulation with LTA (Fig. 2). IL-8 release was analyzed after 24, 48, and 72 h, and was expressed as ΔIL-8 versus baseline secretion at indicated time points. Baseline levels of IL-8 liberated from sham-stimulated A549 cells were 53, 98 and 83 pg/ml after 24, 48 and 72 h respectively, and were increased by 117, 129 and 214 pg/ml upon stimulation with 1 µg/ml LTA. In parallel, IL-8 mRNA was upregulated 3.50-fold in response to stimulation with 0.01, 3.54-fold upon stimulation with 0.1 µg/ml, and 11.56-fold in response to 1 µg/ml LTA after 24 h as compared to sham-incubated cells.

When ligation of TLR-2 was blocked by neutralizing antibodies, IL-8 release in response to 1 µg/ml LTA was strongly attenuated, as depicted for a stimulation period for 48 h in Fig. 3. In contrast, neutralizing TLR-4 had no inhibitory effect on LTA-induced release of this chemokine.

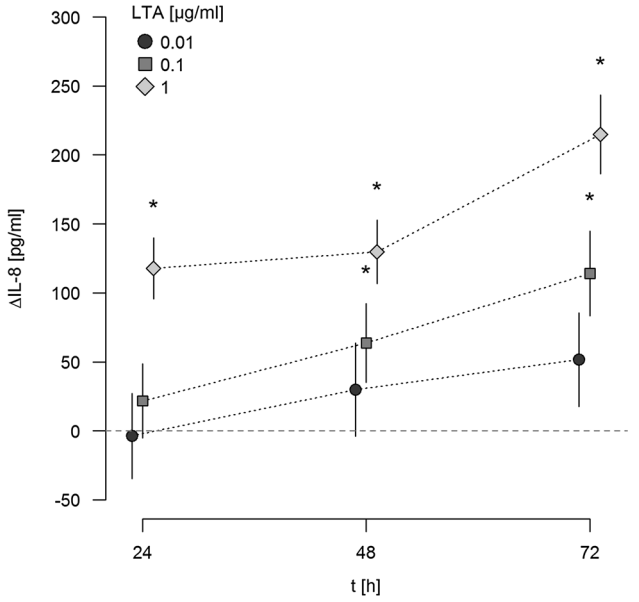

Fig. 2 Release of IL-8 from A549 cells in response to LTA. A549 cells were either sham-incubated (control) or exposed to the given concentrations of LTA. At indicated time points, cell supernatants were collected and analyzed for IL-8 by ELISA technique. IL-8 release is given as ΔIL-8, which is the difference between IL-8 released from LTA-stimulated and sham-incubated cells (indicated by the *horizontal dotted line*). ΔIL-8 is expressed in pg/ml. Means ± SEM of at least four independent experiments are given. *Values marked with an asterisk* differ significantly from controls ($p < 0.05$)

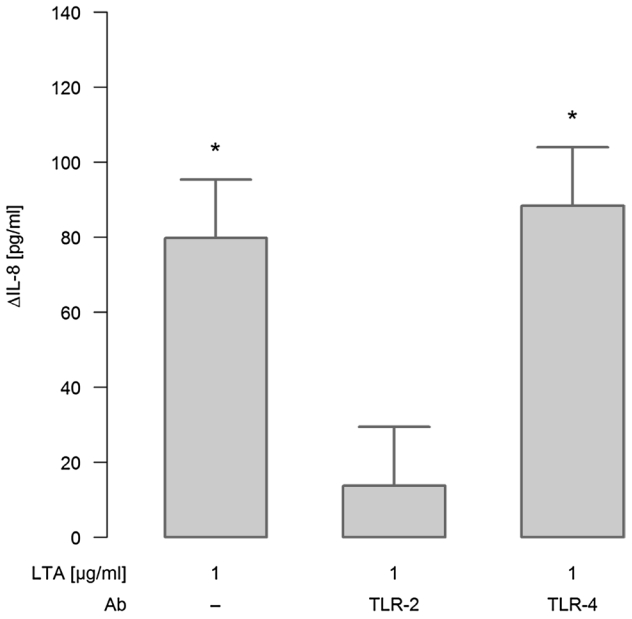

Fig. 3 Effect of TLR-2/-4 antagonists on LTA-induced synthesis of IL-8. A549 cells were either sham-incubated (control) or exposed to 1 µg/ml of LTA in the absence or presence of neutralizing antibodies targeting TLR-2 or TLR-4 for 48 h. Release of IL-8 into the cell supernatants was analyzed by ELISA technique and is given as ΔIL-8 (pg/ml), which was calculated by subtracting IL-8 values of sham-incubated cells from LTA-stimulated cells. Data are expressed as means ± SEM of at least six independent experiments. *Values marked with an asterisk* differ significantly from controls ($p < 0.05$)

Mechanisms of LTA-induced A549 cell proliferation in vitro

In order to determine the mechanism of LTA-stimulated A549 cellular proliferation, TLR-2, TLR-4 and IL-8 were blocked by antibodies under different experimental conditions. Concretely, stimulation with 0.1 and 1 µg/ml LTA was performed in the absence or presence of these neutralizing antibodies for 24 and 48 h, and cellular proliferation was assessed by automatic cell counting. Stimulation of cellular proliferation induced by 0.1 and 1 µg/ml LTA for 24h was inhibited by interference with TLR-2, whereas inhibition of TLR-4 had no effect (Fig. 4a/c). This inhibitory effect was still reproducible when stimulation with LTA (0.1 and 1 µg/ml) was performed for 48 h (Fig. 4b/d). Most interestingly, neutralization of endogenously produced IL-8 abolished LTA-induced proliferation of A549 cells under all experimental conditions (Fig. 4 a–d). Exogenous IL-8 (2 ng/ml) induced a pro-proliferative response of 126.8 ± 3.7% ($n = 6$) as compared to baseline levels (100%), which was not blocked by the presence of anti-TLR2-antibodies (increase in proliferation to 125.7 ± 3.8%, $n = 6$), nor did the TLR-2 antibodies affect baseline proliferation (baseline proliferation in the presence of TLR-2 antibodies was 100.7 ± 2.2%, $n = 6$).

The pro-proliferative effects of LTA can be reproduced for the NSCLC squamous carcinoma cell line H226

Finally, we tried to reproduce the pro-proliferative effect of LTA in a NSCLC cell line of squamous cell origin. For this purpose, H226 monolayers were stimulated with increasing concentrations of highly purified LTA (0.01, 0.1, and 1 µg/ml) for various time periods (24, 48, 72 h). As assessed by automatic cell counting, LTA induced proliferation of H226 cells approximately to the same extent as previously observed in A549 cells. A strong proliferative response in H226 cells was elicited even by the low LTA-concentration of 0.01 µg/ml. Similar to A549 cells, lower concentrations of LTA did not exert any pro-proliferative effect (data not given). The pro-proliferative effect of LTA was observed over the whole incubation period of 72 h. In parallel, MTS activity was enhanced upon stimulation with LTA, with an early response after 6 h of stimulation and an ongoing increase of MTS activity until 48 h (Fig. 5a/b).

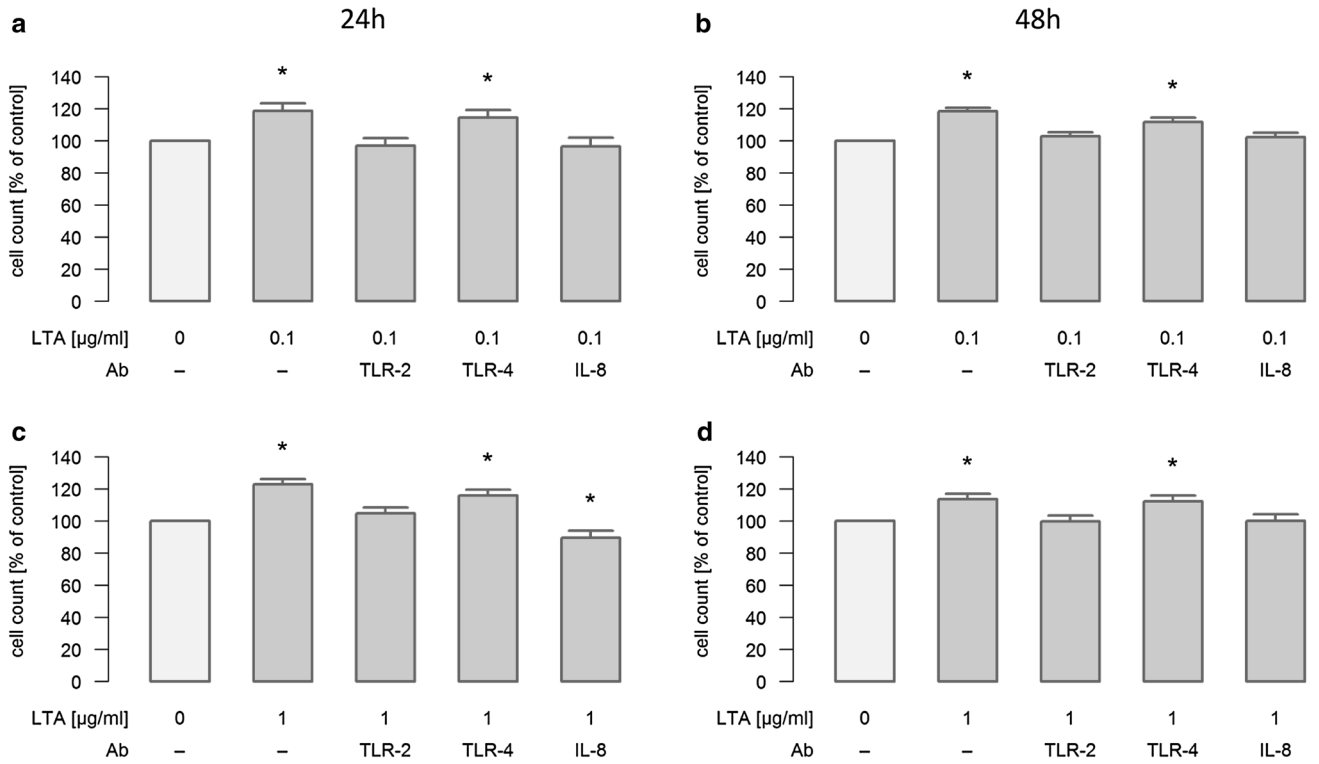

Fig. 4 Mechanisms of LTA-induced proliferation of A549 cells. A549 cells were either sham-incubated (control) or exposed to 0.1 (**a/b**) or 1 µg/ml (**c/d**) of LTA for 24 or 48 h in the absence or presence of neutralizing antibodies targeting TLR-2, TLR-4 or IL-8. A549 proliferation was assessed by automatic cell counting. All data are expressed as percentage of baseline proliferation of sham-incubated cells, which was set to 100%. Means ± SEM of at least four independent experiments are given. *Values marked with an asterisk* differ significantly from controls ($p < 0.05$)

Discussion

Pulmonary bacterial infections are frequently found in advanced stages of lung cancer and may contribute to the progression of this disease [2, 3]. In this context, bacterial pathogenicity factors may play a decisive role by stimulating cancer cell growth. While a strong pro-proliferative effect has been described for LPS, the endotoxin of Gram-negative bacteria, in lung, liver, ovarian, gastric and breast cancer [28, 29, 37–40], less is known about the pathogenicity factors of Gram-positive germs in this context.

In the present study, we observed that highly purified LTA from *S. aureus* induces proliferation in the lung adenocarcinoma cell line A549. The increase in cellular proliferation assessed by automatic cell counting was paralleled by an enhanced MTS activity. The increase in metabolic activity was clearly related to the increase in cell numbers thus confirming the observation that turnover of MTS is directly proportional to the numbers of viable cells in culture [41]. Notably, in both assays, LTA induced an increase in proliferation by ~30%, thus approaching or even exceeding the biological activity of other well-known endo- or exogenous proliferative agents such as IL-8 [17] or benzo[a]pyrene [42]. The pro-proliferative effect of LTA was not restricted to A549 cells but could be reproduced in a NSCLC line of squamous cell origin, suggesting a pathogenic role of LTA in lung cancer progression in general.

The pro-proliferative effects were clearly caused by LTA and not by contaminating LPS. First, we used highly purified LTA prepared according to the method of Morath et al. [43]. Second, compared to LPS much lower concentrations of LTA (0.01 µg/ml) were sufficient to stimulate cellular proliferation of A549 cells [28, 29]. Third, inhibition of TLR-4 by an antibody that effectively inhibited LPS-induced cellular responses [44, 45], did not affect the LTA-induced proliferation of the NSCLC cell line. And fourth, the LPS-induced pro-proliferative response in A549 cells displayed different kinetics compared to LPS, with a maximum response at 24 h and a rapid decline thereafter [28].

However, we cannot completely rule out that contamination with staphylococcal Lpp may have contributed, at least in part, to the biological activity of the LTA preparation used here. Despite broad evidence of the immunostimulatory potency of LTA [reviewed in 46], Lpp display some immune activation functions by ligation of TLR-2 [26], and some recent reports suggested that the immunostimulatory

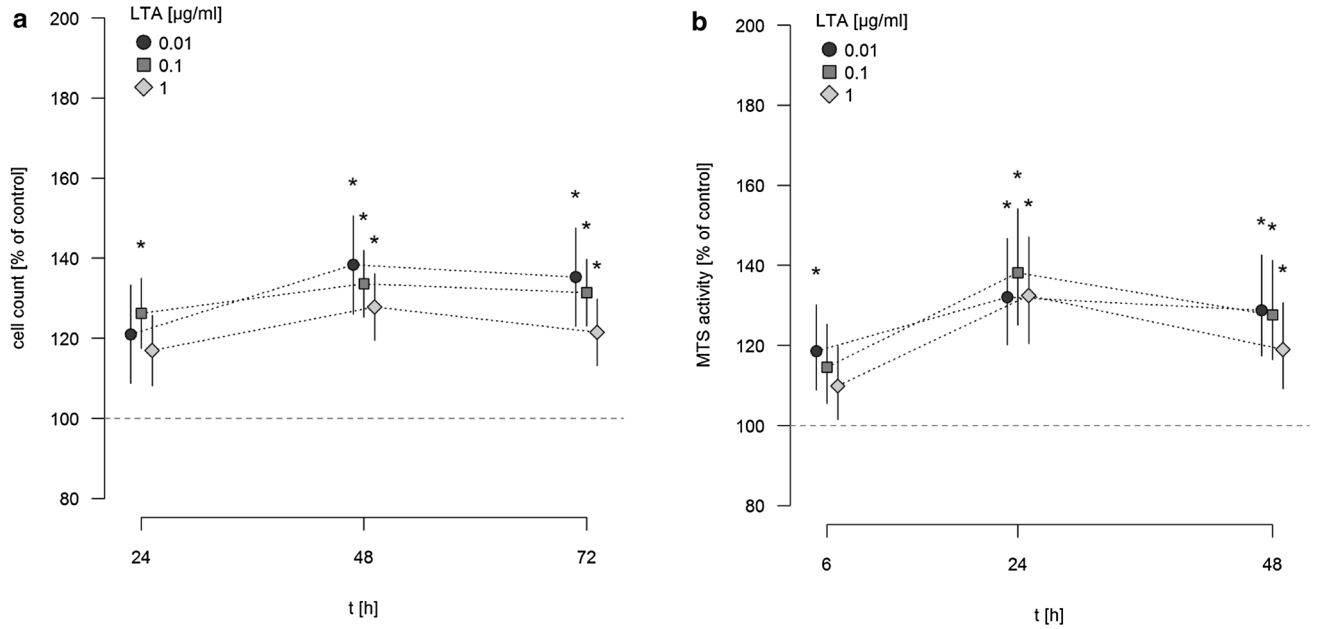

Fig. 5 Time-dependent increase in H226 proliferation and MTS activity by LTA. H226 cells were incubated with various concentrations of LTA from *S. aureus* or sham-incubation was performed (control). H226 proliferation was assessed by automatic cell counting (**a**) and metabolic activity was quantified by MTS assay (**b**). The *horizontal dotted line* indicates the baseline proliferation (**a**) and MTS activity (**b**) of sham-incubated cells which was set to 100%. All data are expressed as percentage of baseline proliferation. Means ± SEM of at least four independent experiments are given. *Values marked with an asterisk* differ significantly from controls ($p < 0.05$)

effects of LTA were, at least in part, mediated by contaminating Lpp [47, 48]. However, synthetic LTA has been demonstrated to activate cytokine synthesis [49] and in a recent report performed in full blood, it has also been shown that binding of LTA to TLR-2 is a prerequisite to elicit cytokine synthesis [50]. Thus, we believe that LTA is a potent activator of cellular inflammatory reactions.

It is noteworthy that, in contrast to LPS-induced stimulation [28, 29], LTA-mediated cellular proliferation displayed no clear dose-dependency. LTA-concentrations from 0.01 to 1 µg/ml induced proliferative responses. This observation is reminiscent of the "all or nothing" principle in human biology, which means that a cellular reaction is initiated as soon as a certain "threshold" concentration of receptor saturation is reached [51]. Therefore, one could speculate that TLR-2 receptors are "saturated" by 0.01 µg/ml LTA, as lower concentrations of LTA failed to induce any significant pro-proliferative response. The absence of a clear dose-dependency in LTA-induced cellular proliferation corresponds well to the studies of Rezania et al., in which increased metabolic activity of human prostate cancer cell lines over a wide range of LTA-concentrations displayed no dose-dependency [10].

When higher concentrations of LTA (1 µg/ml) were used, cellular proliferation could not be further enhanced and was even reduced to baseline levels after the longest incubation period. This may be explained to the previously reported pro-apoptotic effects of LTA in high concentrations which may counteract the pro-proliferative effects of LTA at least in higher concentrations [52].

In our experimental setup LTA induced proliferation of A549 cells by a sequence of TLR-2 ligation, and subsequent activation of IL-8 synthesis. As a prerequisite for specific interaction with LTA, constitutive expression of TLR-2 mRNA was proven in both cell lines by real-time RT-PCR. Interestingly, LTA even induced up-regulation of TLR-2 mRNA in A549 cells. The relevance of TLR-2-expression was clearly demonstrated by the use of function blocking antibodies to TLR-2 and TLR-4. While LTA-induced release of IL-8 was abrogated in the presence of a TLR-2-antibody, blocking of TLR-4 did not affect IL-8 secretion in response to LTA. The specificity of the TLR-2 antibody was proven by the fact that it did not affect baseline or IL-8 induced proliferation of A549 cells. The TLR-2-dependency of LTA-induced proliferation is in line with previous studies which demonstrated that LTA-induced cellular responses proceed via TLR-2 and further down-stream signaling involving pathway-specific TRAFs, activation of NF-κB and subsequent cytokine synthesis [8, 9, 53]. Supporting our findings, it has recently been demonstrated that TLR-2 activation is also operative in human gastric and breast cancer [54].

Clearly, biological activity of IL-8 was a prerequisite for A549 cell proliferation in our study. When IL-8 was

inhibited by a neutralizing antibody proliferation of A549 cells in response to LTA was completely abolished. The observation that in the low concentration of LTA (0.01 µg/ml) no significant amounts of IL-8 were detected, does not stand against this mechanism, as there was still a trend towards elevated IL-8 levels. Moreover, IL-8 mRNA was elevated nearly to the same extent whether 0.01 or 0.1 µg/ml LTA were used. We assume that IL-8 is liberated from A549 cells and activates adjacent A549 cells in an auto- or paracrine way without necessarily reaching significant concentrations in the cell supernatants under all experimental conditions.

Auto- and/or paracrine activation of A549 proliferation by IL-8 correspond well to the in-vitro-studies of Luppi et al., which demonstrated that exogenously added IL-8 stimulates proliferation in the NSCLC cell lines [55]. In line with these results, we demonstrated that addition of exogenous IL-8 stimulated A549 proliferation. Although we did not address the exact signaling events in our study, one mechanism of autocrine cell activation may be direct stimulation of the CXCR receptor type 1, which has been shown to be the decisive receptor subtype mediating proliferative responses upon stimulation with IL-8 in A549 cells [56]. Alternatively, IL-8 may activate NSCLC growth by trans-activation of EGFR, one of the key "drivers" of NSCLC, especially when activating mutations are found [57]. The capacity of IL-8 to transactivate EGFR has not only been shown in A549 cells [55], but also in gastric epithelial and endothelial cells [58, 59]. Besides its direct effect of tumor cell activation, IL-8 is a potent pro-angiogenic factor in NSCLC [18, 19], which may be of further relevance in lung cancer patients.

In conclusion, this is the first study which demonstrates that purified LTA of *S. aureus* effectively induces growth of NSCLC cell lines of adeno- and squamous cell carcinoma origin. These effects are mediated by ligation of TLR-2, and IL-8 was identified as a decisive endogenous mediator activating tumor cell growth. Thus, infections with Gram-positive bacteria may directly contribute to tumor growth in lung cancer.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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Anlage 3

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Research Paper

Targeting CREB-binding protein overrides LPS induced radio-resistance in non-small cell lung cancer cell lines

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ABSTRACT

Non-small cell lung cancer (NSCLC) has a very poor prognosis even when treated with the best therapies available today often including radiation. NSCLC is frequently complicated by pulmonary infections which appear to impair prognosis as well as therapy, whereby the underlying mechanisms are still not known. It was investigated here, whether the bacterial lipopolysaccharides (LPS) might alter the tumor cell radiosensitivity. LPS were found to induce a radioresistance but solely in cells with an active TLR-4 pathway. Proteome profiling array revealed that LPS combined with irradiation resulted in a strong phosphorylation of cAMP response element-binding protein (CREB). Inhibition of CREB binding protein (CBP) by the specific inhibitor ICG-001 not only abrogated the LPS-induced radioresistance but even led to an increase in radiosensitivity. The sensitization caused by ICG-001 could be attributed to a reduction of DNA double-strand break (DSB) repair.

It is shown that in NSCLC cells LPS leads to a CREB dependent radioresistance which is, however, reversible through CBP inhibition by the specific inhibitor ICG-001. These findings indicate that the combined treatment with radiation and CBP inhibition may improve survival of NSCLC patients suffering from pulmonary infections.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death in both men and women in the western hemisphere [1]. With a fraction of 80 to 85% non-small cell lung cancer (NSCLC) is the most prevalent type of lung cancer [2].

NSCLC is generally treated by a complex combination of surgery, radiotherapy and chemotherapy. But, despite the tremendous progress made in all three disciplines the overall survival of NSCLC patients is still very low with a five-year survival rate below 20% [3]. The outcome of these patients is getting even from bad to worse, when NSCLC is associated with a pulmonary infection, which is unfortunately the case in about 70% of lung cancer patients [4]. When no pulmonary infections were present the overall survival of lung cancer patients as determined 28 months after treatment was still

above 30%, in contrast to only 10% when patients were suffering from infections [5].

The most common pathogens found in patients with lung cancer are gram-negative bacteria such as *Haemophilus influenza* and *Escherichia coli* [4, 6]. Lipopolysaccharides (LPS), the so called "endotoxin" of gram-negative bacteria components of cell wall are the major bacterial pathogenicity factors [7, 8].

It was previously shown by us [9] that LPS are able to stimulate tumor growth both *in vitro* as well as *in vivo*. Such a pro-proliferative effect of LPS was also reported for lung, liver, ovarian, gastric as well as breast cancer [9–14]. These data might in part explain the negative impact of pulmonary infections on the outcome of NSCLC patients. However, LPS may also worsen the prognosis of these patients via a reduction of the treatment response to radiation or chemotherapy. But so far data are lacking on any interaction between LPS and radio- or chemoresistance.

Cancer cells respond to irradiation in a heterogeneous manner depending on their intrinsic properties (DNA repair capability) or extrinsic environment (hypoxia, e.g.) [15, 16]. The main irradiation induced damage are DNA double strand breaks (DNA-DSBs), which when either mis- or non-repaired may result in a decreased clonogenicity of tumor cells and thereby affecting tumor cell survival [16–18].

It is well known, that after ligation of LPS to the CD14 molecule [19], cellular activation is initiated by binding of this complex to the toll-like receptor 4 (TLR-4) [20–23]. Through this complex LPS are able to activate various MAPK pathways such as ERK, JNK, p38 and the IKK pathway. The MAPK pathways directly or indirectly phosphorylate and activate various transcription factors, including Elk-1, c-Jun, c-Fos, ATF-1, ATF-2, SRF, and CREB. Some of these pathways are already known to have a strong effect on cell proliferation, survival, inflammation, immune regulation as well as DNA repair also including repair of DNA-DSBs [24, 25]. The LPS induced pathways via TLR-4 were also found strongly to depend on a crosstalk to EGFR [26], which is clearly known to be involved in DSB repair [27, 28]. These data suggest that LPS may affect DSB repair either via one of these transcription factors mentioned above or indirectly via EGFR.

Therefore we tested the hypothesis, in how far LPS may affect the cellular response to radiotherapy by reducing its effect on cell survival due to a depressed repair of DNA-DSBs.

RESULTS

No effect of LPS on colony forming ability

The effect of the LPS induced pathway via TLR-4 is known strongly to depend on a crosstalk to EGFR [26]. Therefore, this study was performed with three cell lines clearly differing in these parameters. Two cell lines (A549, H1975) with high expression of TLR-4 were selected with the first being EGFR wild-type and the second carrying an EGFR driver mutation; while the third cell line (H520) shows a very low expression of TLR-4 and is EGFR-deficient (Table 1). In a previous work of our group, it was shown that LPS are able to stimulate proliferation of A549 cells [9]. It is now shown here that this treatment does, however, not result in an increased plating efficiency and respectively colony forming ability, neither for A549 nor the other two cell lines (Figure 1).

LPS induce radioresistance in H1975 and A549, but not in H520 cells

Next, we investigated the effect of LPS on cellular radiosensitivity. Cells were incubated with different concentrations of LPS for 16 h before exposed to X-ray doses up to 8 Gy followed by further incubation for colony growth. Interestingly, LPS were found to induce a radioresistance in H1975 and A549 cells but not in H520 cells (Figure 2E and 2F). For the first two cell lines this radioresistance was already apparent at low doses and clearly increased for higher radiation doses (Figure 2A and 2C). For H1975 and A549 cells a significant increase in radioresistance was obtained at 6 Gy for the concentration of 10 μg/ml LPS, respectively (Figure 2B and 2D).

Strong up-regulation of CREB dependent pathway after combined treatment with LPS and irradiation

To understand the underlying mechanism of the LPS-induced radioresistance further experiments were carried out with H1975 cells. In a first step, H1975 cells were incubated with 10 μg/ml LPS followed by irradiation (6 Gy) as described above and 24 h after treatment a proteome profiling array was performed for 43 multiple human kinases using the Human Phospho-Kinase Antibody Array Kit (Figure 3A). After single treatment with either LPS (10 μg/ml) or irradiation (6 Gy) for many kinases a change in phosphorylation was seen (Supplementary Figure 1A and 1B). However, after the combined treatment a more than additive up-regulation was solely detected for few of them, namely the phosphorylated form of cAMP response element-binding protein (CREB), the lymphocyte-specific protein tyrosine kinase (Lck), the tyrosine-protein kinase Fyn (Fyn) and the tyrosine-protein kinase Fgr (Fgr) (Figure 3A and 3B). For all other kinases the combined treatment did not result in an additive increase but was mostly identical to the effect of irradiation or LPS treatment alone (Figure 3A and 3B, Supplementary Figure 1A and 1B).

Because Lck, Fyn, Fgr are all members of the SRC family and upstream of CREB we verified the enhanced phosphorylation of CREB by western blotting (Figure 3C). In line with the expression data only a moderate increase (1.2) was seen for pCREB phosphorylated at S133 when treated by irradiation or LPS alone (Figure 3D). In

Table 1: Basal expression of *TLR-4*- and *EGFR*-mRNA in H1975, A549 and H520 cells

	H1975 (–ΔCt value)	A549 (–ΔCt value)	H520 (–ΔCt value)
TLR-4 mRNA	–7.69 ± 0.23	–7.25 ± 0.25	–10.20 ± 0.57
EGFR mRNA	2.63 ± 0.20	0.60 ± 0.12	–8.20 ± 0.58

Data are presented as mean ± SEM (n = 3).

contrast, again a significant increase (1.7) was observed for pCREB, when H1975 cells were exposed to the combined treatment.

Inhibition of CREB binding protein (CBP) decreases the LPS-induced radioresistance

To clarify if the increased phosphorylation of CREB (S133) detected after treatment with LPS and irradiation was causally involved in the LPS-induced radioresistance in H1975, we used the specific CBP inhibitor (CBPi) ICG-001 [29, 30]. We also measured the impact of EGFR using the EGFR kinase inhibitor (EGFRi) AG1478 [31]. The effect of CBPi on the phosphorylation of CREB was successfully confirmed by Co-IP (Figure 4A). Both inhibitors, CBPi and EGFRi, either alone or combined with LPS were found to have no effect on cellular survival (Figure 4B). Interestingly, when CBPi was added to the combined treatment of irradiation and LPS, besides the clear abrogation of the LPS-induced radioresistance

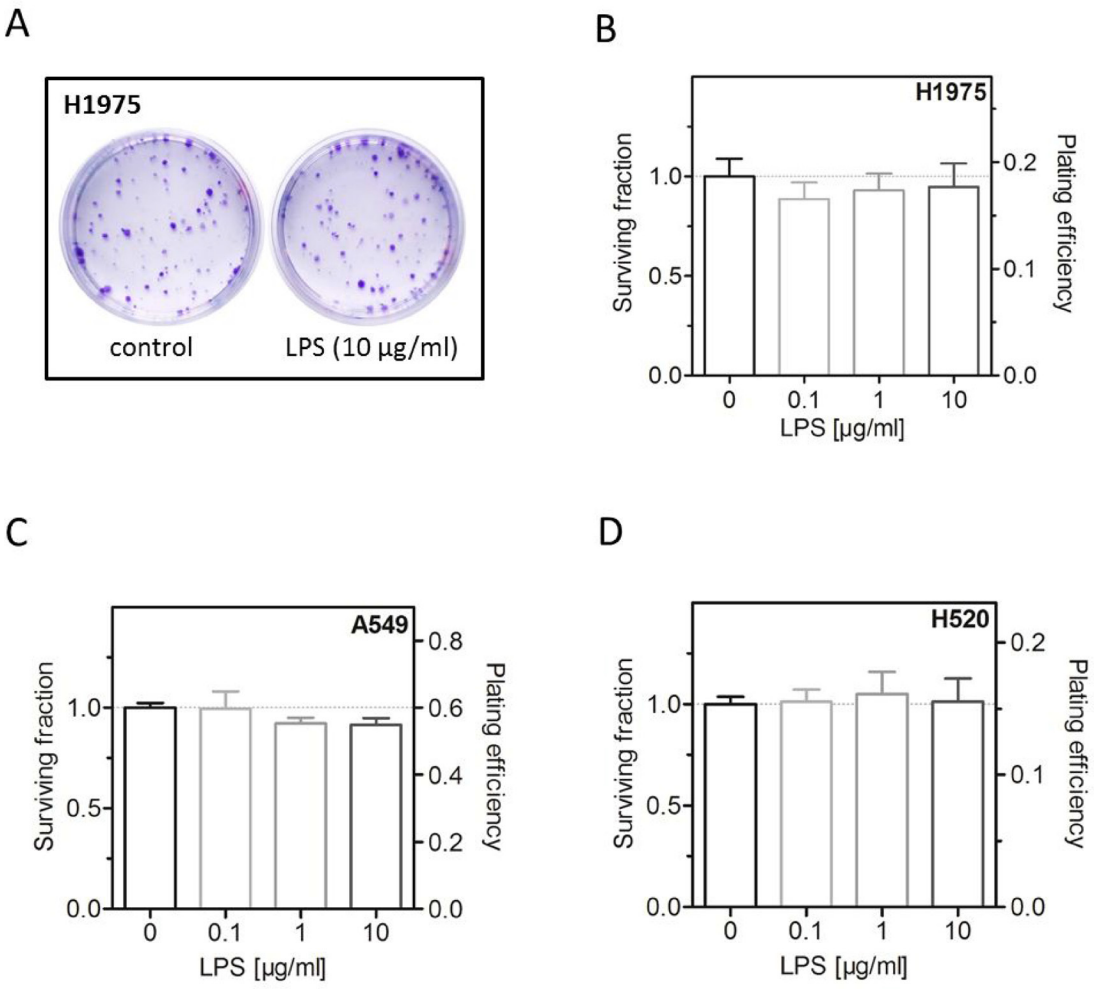

Figure 1: LPS have no effect on colony forming ability. (**A**) Representative images of colonies formed of LPS- (10 µg/ml) or sham-treated (control) H1975 cells. (**B**) Survival fraction and plating efficiency of LPS- (0.1, 1, 10 µg/ml) or sham-treated (control) H1975 cells. (**C**) Survival fraction and plating efficiency of LPS- (0.1, 1, 10 µg/ml) or sham-treated (control) A549 cells. (**D**) Survival fraction and plating efficiency of LPS- (0.1, 1, 10 µg/ml) or sham-treated (control) H520 cells. Data are presented by mean ± SEM, $n \geq 3$.

also a significant further increase in radiosensitivity was observed (Figure 4C and 4D). In contrast, when EGFRi was added to the combination of LPS and irradiation only an abrogation of the LPS-induced radioresistance was seen (Figure 4C and 4D). Both, CBPi and EGFRi combined with irradiation alone, were found to have no effect on radiosensitivity as shown for an X-ray dose of 6 Gy (Figure 4D). These data demonstrate that pCREB appears to have a prominent role in the LPS-induced radioresistance and that this effect only partially depends on EGFR.

Inhibition of CBP impairs DNA double-strand break in cells treated by LPS

We finally asked whether the changes in radiosensitivity seen here, when irradiation was combined with LPS alone or together with CBP inhibitor, may also result from an altered DSB repair capacity. DSB repair was assessed by counting the number of co-localized γH2AX/53BP1 foci 24 h after irradiation, which is known to be an excellent read-out for an altered DSB repair [32–34] (Figure 5A). For unirradiated cells (control, LPS, CBPi, LPS+CBPi) on average one residual γH2AX/53BP1 foci was counted per nucleus (Figure 5B). Upon treatment with 4 Gy this number increased to about four residual γH2AX/53BP1 foci per nucleus. There was a slight, but statistically significant reduction when irradiation was combined with LPS, while no change was seen when irradiation was combined with the CBPi alone. However, when CBPi was added to the combination of LPS and irradiation a significant increase was seen to about five residual γH2AX/53BP1 foci per nucleus (Figure 5B). This variation in residual γH2AX/53BP1 foci was found to correlate with the differences in surviving fraction seen after the respective treatments (Figure 5C). These data strongly indicate that the modulation of the cellular radiosensitivity caused by LPS alone or in combination with CBPi appear to result from its affected DSB repair.

DISCUSSION

The purpose of the current study was to investigate the effect of the bacterial pathogenicity factor LPS on the response to radiotherapy in several NSCLC cell lines. The clinical background is given by the observation that pulmonary bacterial infections worsen prognosis of lung cancer patients [5]. However, it remains unclear, if this impairment of prognosis is a simple epiphenomenon of infections, or if bacterial pathogens are causally involved in the development of therapy resistance in NSCLC. In this context, the response to radiotherapy is of particular relevance, as it is the backbone of both curative and palliative therapeutic settings in NSCLC [35, 36].

LPS are the major pathogenic factors of gram-negative bacteria, which are the mostly seen in lung cancer patients [4, 6–8]. LPS are known to activate TLR-4 dependent pathways, which also require the protein kinase activity of the epidermal growth factor receptor (EGFR) [26, 37]. Therefore, three NSCLC cell lines were selected for this study strongly differing in the LPS response with H1975 (EGFR driver mutation) and A549 (EGFR wildtype) both showing a TLR-4 expression and H520 (EGFR-deficient) with a low TLR-4 expression (Table 1).

It was previously shown by us that LPS effectively stimulate tumor growth in various experimental models of NSCLC *in vitro*, *ex vivo* and *in vivo* using the A549 cell line [9]. It is now observed that LPS per se have no effect on the colony forming capacity of H1975, H520 and A549 (Figure 1). These results illustrate a new aspect of LPS to modulate the biology of NSCLC as LPS in fact induce proliferation [9, 38–40] but have no effect on the clonogenicity of tumor cells. Clonogenicity of tumor cells is an important factor for tumor development, progression and recurrence after treatment. It is known that the radiosensitivity of tumor cells *in vitro* well correlates to the tumor´s response to radiotherapy *in vivo* [41]. Thus, the existence of a correlation of stemness *in vivo* and clonogenicity *in vitro* was suggested pointing out the relevance of intrinsic radiosensitivity of cancer stem cells for radiosensitivity of tumors of different histologies [42–45].

Interestingly, we revealed for the first time that LPS are able to induce a significant radioresistance in NSCLC cell lines showing an expression of the LPS binding receptor TLR-4 (Table 1 and Figure 2). This result reveals that the effect of LPS on radiation response also depends on the formation of an active LPS/TLR-4 complex and gives a proof that the observed effect was specifically mediated by LPS. Furthermore, it is known, that in NSCLC a high expression of TLR-4 was found and the level of TLR-4 correlated with the malignancy of these tumors [46]. Thus, the currently shown interaction of LPS with TLR-4 positive NSCLC cells resulting in resistance to radiotherapy may well be operative *in vivo* and may explain the impaired prognosis of TLR-4 positive patients [5].

The LPS/TLR-4 complex is able to stimulate numerous pathways [24]. To investigate which LPS/TLR-4-dependent pathways were responsible for the observed radioresistance we performed a proteome profiling array. It was found that the LPS and irradiation induced a more than additive up-regulation of the cAMP response element-binding protein (CREB) pathway including phospho-Lck, -Fyn, -Fgr, which are all members of the SRC family, finally resulting in the enhanced phosphorylation of CREB, when H1975 cells were treated with both LPS and irradiation (Figure 3). The functional relevance of CREB in mediating LPS-induced radioresistance was proven by the efficacy of CBPi, restoring radiosensitivity in LPS-treated cells.

CREB is a member of the CREB/ATF family of transcription factors that play a key role in the nuclear responses to a variety of external signals that lead to cell growth and proliferation, differentiation, apoptosis and

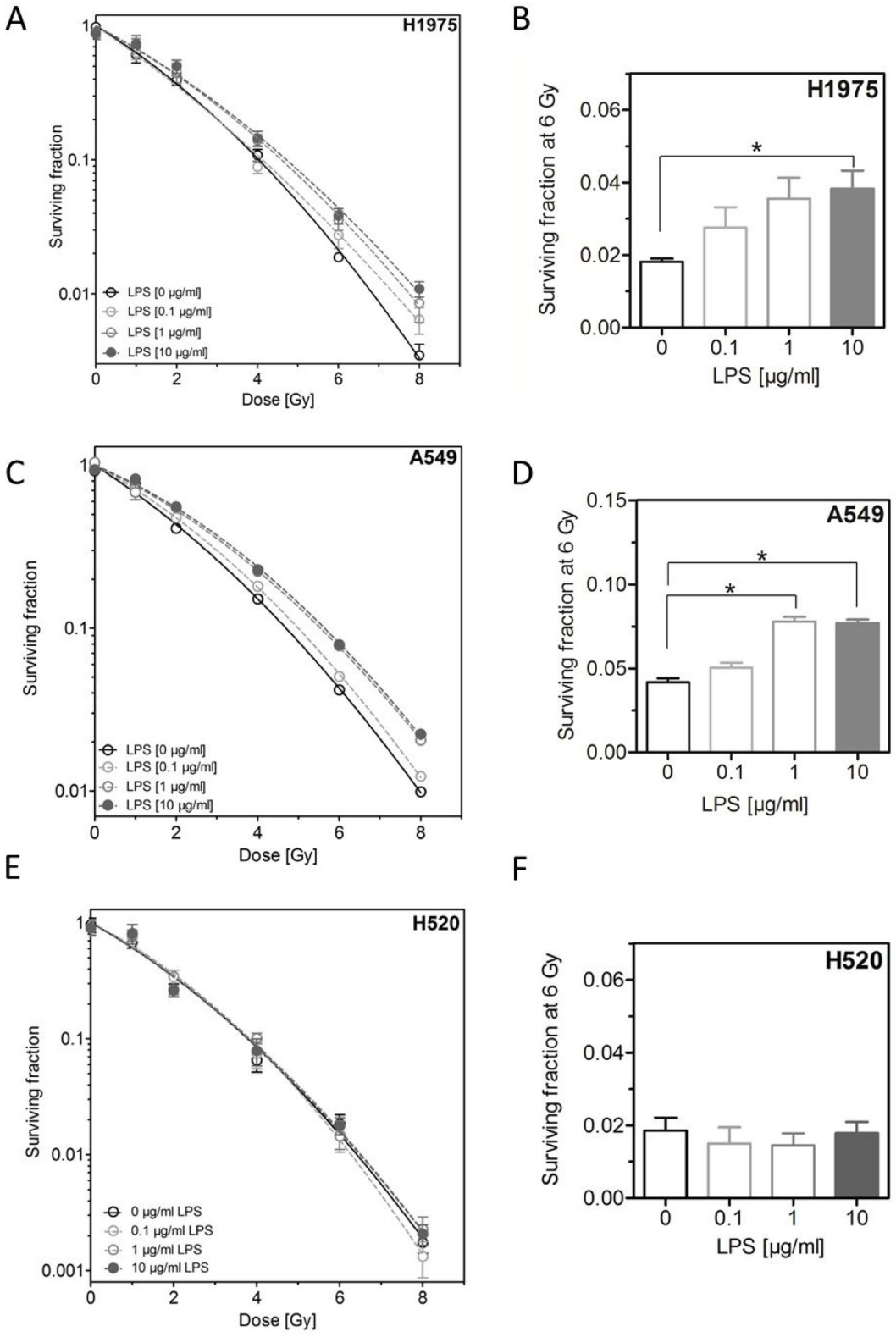

Figure 2: LPS induce radioresistance in H1975 and A549, but not in H520 cells. (**A**) Survival fractions of LPS- (0.1, 1, 10 µg/ml) or sham-treated (control) H1975 cells after irradiation with 0–8 Gy. (**B**) Survival fractions at 6 Gy of LPS- (0.1, 1, 10 µg/ml) or sham-treated (control) H1975 cells. (**C**) Survival fractions of LPS- (0.1, 1, 10 µg/ml) or sham-treated (control) A549 cells after irradiation with 0–8 Gy. (**D**) Survival fractions at 6 Gy of LPS- (0.1, 1, 10 µg/ml) or sham-treated (control) A549 cells. (**E**) Survival fractions of LPS- (0.1, 1, 10 µg/ml) or sham-treated (control) H520 cells after irradiation with 0–8 Gy. (**F**) Survival fractions at 6 Gy of LPS- (0.1, 1, 10 µg/ml) or sham-treated (control) H520 cells. Data are presented by mean ± SEM, $n \geq 3$; $^{*}p < 0.05$ for comparison versus control, as determined by ANOVA following by Bonferroni's Multiple Comparison Test.

survival [47–49]. CREB is phosphorylated at serine/threonine residues depending upon the stimuli from extracellular components and several upstream kinases [50]. Activated/phosphorylated CREB recruits its transcription co-activator, CREB-binding protein (CBP) to a cAMP response element (CRE) region of target genes [51]. This recruitment of CBP is a critical step for the transcriptional activation of CREB [52]. Several previous studies showed that CREB is highly upregulated and hyperphosphorylated in non-small cell lung cancer (NSCLC) tumor specimen and that this upregulation is significantly associated with poor survival rates [53–55]. Against this background, the currently observed *in vitro*-observations may be of major relevance *in vivo* in NSCLC. Besides these facts, there are already several reports, which showed that LPS induce pCREB for example in human monocytes, normal lung tissue, and in the A549 cell line [24, 56–58], indicating a close interaction between LPS and the CREB pathway. Our results revealed that LPS per se induce the phosphorylation of CREB in H1975 cells about the factor 1.2 (Figure 3D).

After irradiation a similar increase of phosphorylated CREB about the factor 1.2 was measured (Figure 3D). In line with our results there are several reports, which showed that irradiation activate the CREB pathway in different types of cancer (hematological, gastrointestinal, lung, prostate) [49]. These results may be explained by the fact that pCREB is already highly upregulated in this cell line like Aggarwal *et al*. assumed [55] and therefore LPS *per se* and irradiation *per se* produced only a slight increase the phosphorylation of CREB in H1975 cells.

However, the phosphorylation levels of CREB were significantly increased upon combined treatment with LPS and irradiation (Figure 3). The relevance of this considerably enhanced level of pCREB for the LPS-induced radioresistance was demonstrated by using the specific inhibitor ICG-001 of the CREB-binding protein (CBP) [29, 30]. Interestingly, inhibition of CBP not only resulted in a complete abrogation of the LPS-induced radioresistance but even caused to a further reduction in radiosensitivity (Figure 4). The specificity of the currently used inhibitor ICG-001 was proven by Co-Immunoprecipitation, clearly showing a strong reduction in the phosphorylation level of CREB when ICG-001 (CBPi) is present (Figure 4A).

It was demonstrated that the changes in cell survival are well correlated with the respective differences in the numbers of residual DNA-DSBs, as recorded after the identical treatments using the γH2AX/53BP1 foci technique (Figure 5). These data demonstrate that LPS modulate cellular radiosensitivity via the CREB pathway due its effect on DSB repair capacity.

There are already several reports indicating that CREB is involved in DSB repair [49]. It was shown for CHO cells that a down-regulation of CREB in a dominant-negative mutant results in a depressed DSB repair, which will then also lead to an enhanced cellular radiosensitivity [59]. The reduction in DSB repair was shown to result from a down-regulated non-homologous End-joining (NHEJ) [60], one of the two major DSB repair pathways acting in mammalian cells, because inhibition of CREB was found to result in an impaired acetylation of Ku70, which is a key component of NHEJ [61]. Overall, our current data suggest that the radioresistance observed upon LPS treatment, might be due to an increased NHEJ activity caused by the elevated amount of phosphorylated CREB induced by the combined treatment (Figure 3). And, vice versa, the abrogation of this radioresistance with even a radiosensitization when CBPi is added to this combination is considered to result from a strong reduction of NHEJ down to a level, which is below that of untreated cells.

In contrast to pCREB no additive increase in pEGFR was seen, when LPS was combined with irradiation (Figure 3A and 3B). Accordingly, when EGFR was inhibited during the combined treatment with LPS and irradiation a clear abrogation of the LPS-induced radioresistance was achieved, but with no further radiosensitization below the irradiated control as observed under CBPi (Figure 4). These findings are in line with the concept that EGFR is needed, but not directly involved in the LPS-TLR-4 pathway, as described by De *et al*. [26]. They have shown that the LPS induced pathway via TLR-4 strongly depends on a crosstalk to EGFR by pLyn. Maybe another member of the SRC family like Lck, Fyn, Fgr are in particular after irradiation mediating in the crosstalk of EGFR and TLR-4, because pLyn was not increased after LPS and irradiation (Figure 3A and 3B). Further analyses are necessary to prove this proposed mechanism in detail.

The observation made here, that LPS are able to induce radioresistance in TLR-4 positive NSCLC cell lines, which, however, can be abrogated by the inhibition of CBP with even a radiosensitization, is maybe of interesting clinical relevance. NSCLC is often associated with pulmonary infection, where circulating LPS are present [4]. Targeting pCREB in combination with radiotherapy may be a novel option for a more effective and specific therapy of these cancer patients, who are, so far, characterized by an extremely poor outcome.

In conclusion, it is shown here that LPS have no impact on the clonogenicity of tumor cells, but induce a radioresistance in TLR-4 and EGFR expressing NSCLC cells. However, this resistance can be abrogated by the inhibition of CBP, which even results in a radiosensitization. These data indicate that the combination of CBPi and radiotherapy might be a promising new strategy to improve the outcome of NSCLC patients, which suffer from pulmonary infections.

MATERIALS AND METHODS

Cell culture

Experiments were performed with the human NSCLC adenocarcinoma cell lines H1975 (CRL-5908)

which has a L858R/T790M double mutations in EGFR, A549 (CCL-185) which are EGFR wild type and the human squamous cell carcinoma cell line H520 (HTB-182) which is EGFR-negative obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in RPMI 1640 (E15-840; PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal bovine calf serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) and 105 U/l penicillin, and 100 mg/l streptomycin (Pan - Biotech GmbH, Aidenbach, Germany), at 37° C in humidified atmosphere containing 5% CO_2 in air. Authentication of all used cell lines was performed by short tandem repeat analysis at the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany).

Treatment with LPS

For the LPS treatment the cells were seeded on cell culture flasks (Falcon, Corning, Inc., Corning, NY, USA) and grown for 24 h. The cells were stimulated with 0.1, 1 or 10 µg/ml highly purified LPS 0111:B4 from *E. coli* (Sigma-Aldrich, St. Louis, MO, USA) for 16 h before irradiation and after irradiation during the experimental setup with sham-treated cells used as control. Trypan

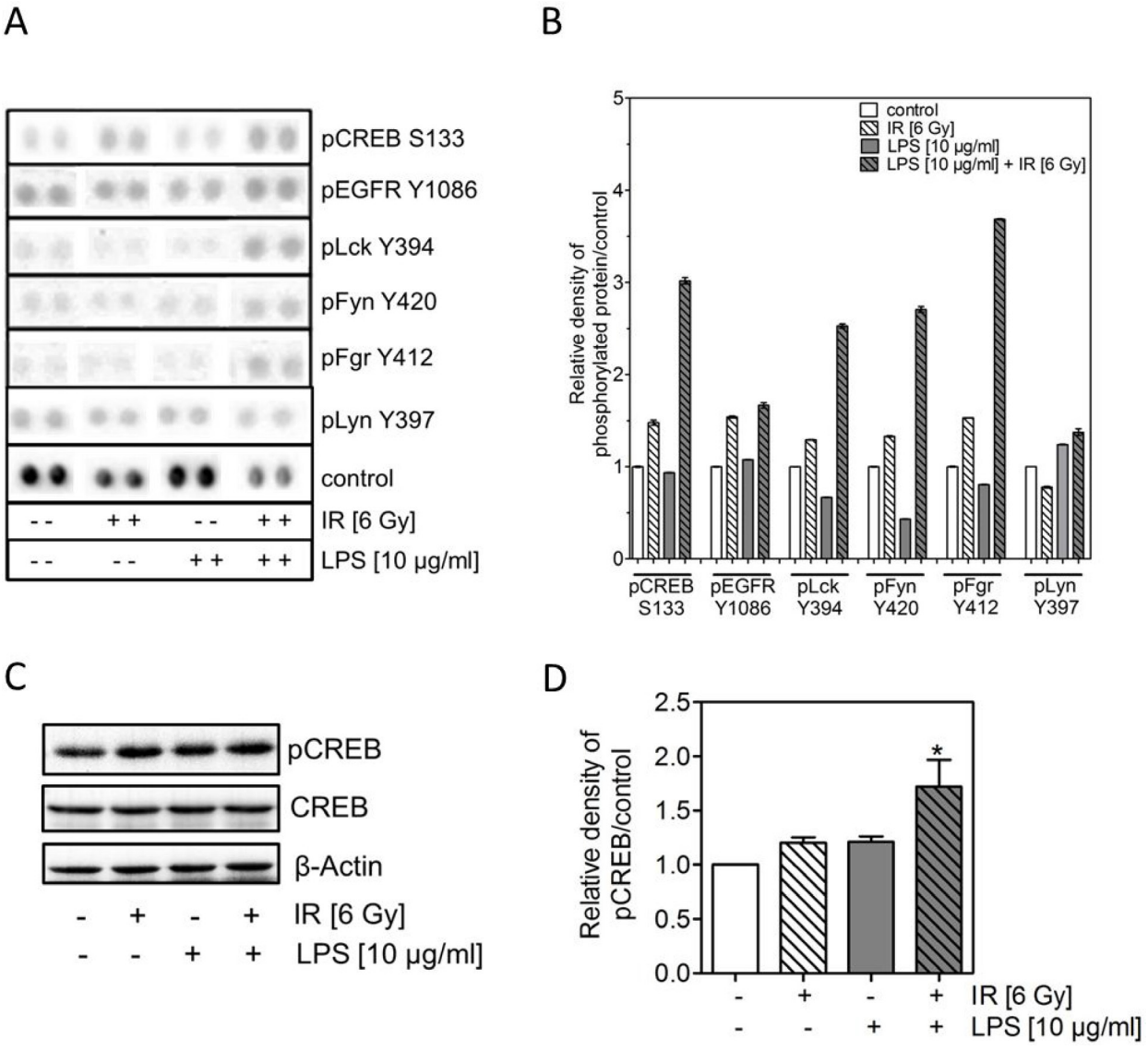

Figure 3: LPS combined with irradiation induces an up-regulation of pCREB. (**A**) Representative images of signals phosphorylated forms from a proteome profiling array using the Human Phospho-Kinase Antibody Array Kit of H1975 cells 24 h after treatment with and without 10 µg/ml LPS and irradiation with 0 or 6 Gy. (**B**) Quantitative analyses of results of A) are presented as mean ± SEM. (**C**) Representative western blots of pCREB, CREB in LPS- (10 µg/ml) treated and/or irradiated (6 Gy) H1975 cells after 24 h; ß-actin was used as loading control. (**D**) Densitometric analyses of pCREB bands after normalization to ß-actin of H1975 cells after LPS and/or irradiation, as indicated (±). Western blot data are presented as mean ± SEM, $n \geq 3$, $^{*}p < 0.05$ for comparison versus control, IR, LPS as determined by ANOVA following by Bonferroni's Multiple Comparison Test.

blue (Sigma-Aldrich, St. Louis, MO, USA) staining was used for obviate any toxicity of used LPS doses in our experimental setups.

Treatment with the CBP-inhibitor ICG-001 and the EGFR-inhibitor AG1478

The CBP-inhibitor ICG-001 (Merck Millipore, Darmstadt, Germany) and the EGFR-inhibitor AG1478 (Merck Millipore, Darmstadt, Germany) were prepared and diluted according to manufacturer instructions. Cells were incubated with 2 µM ICG-001 or 1 µM AG1478 1 h before irradiation and after irradiation during the experimental setup. The control group was treated with corresponding DMSO (Dimethylsulfoxid, Sigma-Aldrich, St. Louis, MO, USA) dilution.

X-irradiation

For X-irradiation, a 6-MeV X-ray beam generated by a clinical linear accelerator was used. The maximum dose rate was 4 Gy/min. X-irradiation was delivered at room temperature and applied doses ranged from 0 to 8 Gy. Cell culture flasks were arranged between 15 mm water-equivalent plates to generate doses maximum in the cell layer.

Colony formation assay

For colony formation assay cells were seeded in 6 cm culture dishes after irradiation (0–8 Gy), the cell number was determining with respect to the plating efficiency and dose in order to obtain 100 colonies. After incubation for 10–14 days, the cells were fixed, stained with 0.1% crystal

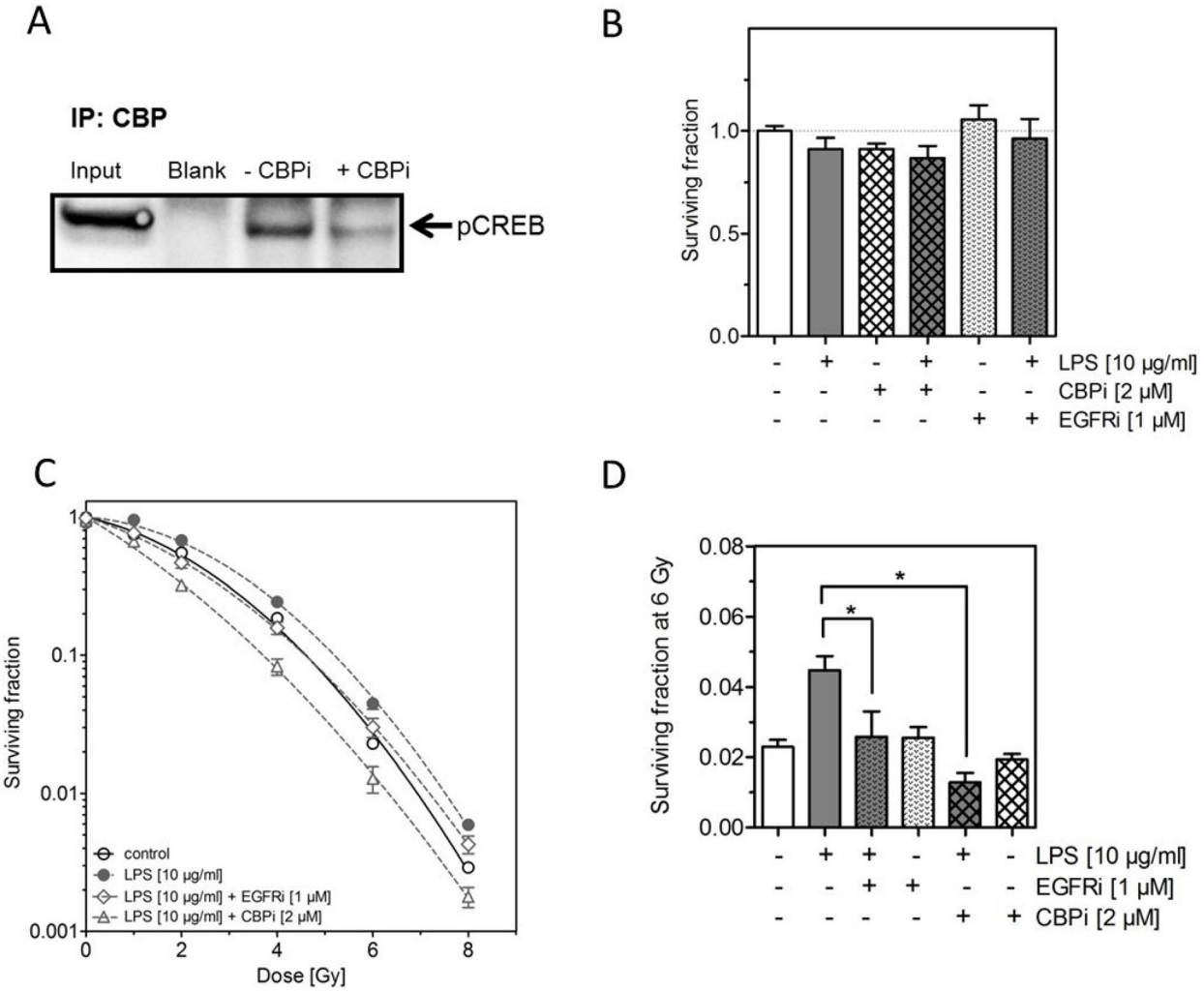

Figure 4: Inhibition of CREB binding protein (CBP) abrogates the LPS-induced radioresistance. (**A**) Representative image of IP against CBP followed by western blot of pCREB in H1975 cells 24 h after treatment with LPS (10 µg/ml) and 6 Gy with or without ICG-001 (CBPi) (2 µM), as indicated (±). (**B**) Survival fractions of LPS- (10 µg/ml) or sham-treated (control) H1975 cells with or without CBPi or EGFRi, as indicated (±). (**C**) Survival fractions of LPS- (10 µg/ml) or sham-treated (control) H1975 cells after irradiation with 0–8 Gy with or without CBPi (triangle) or EGFRi (diamond). (**D**) Survival fractions at 6 Gy of LPS- (10 µg/ml) or sham-treated (control) H1975 cells with or without CBPi or EGFRi, as indicated (±). Data are presented as mean ± SEM, $n \geq 3$, $^{*}p < 0.05$ for comparison versus LPS at 6 Gy as determined by ANOVA following by Bonferroni's Multiple Comparison Test.

violet (Sigma-Aldrich, St. Louis, MO, USA) and colonies >50 cells were counted. Surviving fractions (SF) were calculated as published previously [62, 63].

Proteome profiling

Proteome profiling arrays were performed using Human Phospho-Kinase Antibody Array Kit (#ARY003B, R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions. Cells were grown in T25 culture flasks, rinsed with PBS, and lysed. Protein concentration was estimated with BCA Protein Assay Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 2000 µg of each cell lysate was added to pre-blocked antibody array membranes for incubation. Membranes were treated with detection antibody cocktail followed by streptavidin-HRP, and signal was detected using the chemiluminescence method as instructed. The Array was analyzed by BioDocAnalyze Software (Analytik Jena AG, Jena, Germany). The relative expression levels are shown normalized to the control group ($n = 2$).

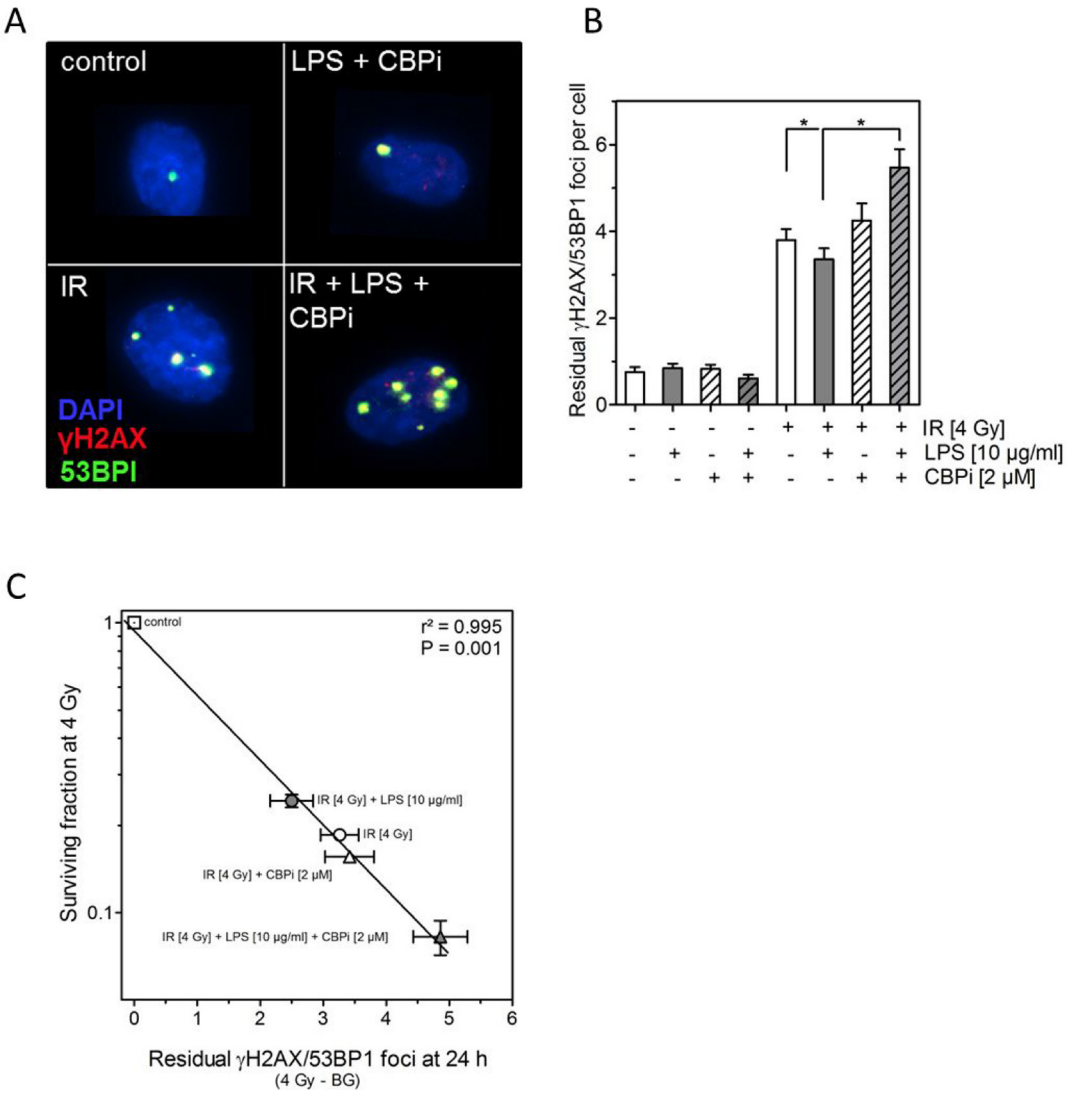

Figure 5: Inhibition of CBP (CBPi) impairs DNA double-strand break in cells treated by LPS. (**A**) Representative images of residual γH2AX/BP53 foci in LPS- (10 µg/ml) or sham-treated (control) H1975 cells detected 24 h after irradiation (IR) with 4 Gy and with or without CBPi (2 µM). Magnification, objective 60×. (**B**) Quantification of the number of residual γH2AX/BP53 foci measured 24 h after irradiation (IR) with 4 Gy and with or without CBPi (2 µM) in LPS (10 µg/ml) or sham-treated (control) H1975 cells, as indicated (±). Quantification was performed by counting at least 200 nuclei per sample. Data are presented as mean ± SEM, $n \geq 3$, $^{*}p < 0.05$ for comparison versus IR and IR + LPS as determined by ANOVA following by Bonferroni's Multiple Comparison Test. (**C**) Association between the survival fractions at 4 Gy and the number of residual γH2AX/BP53 foci detected 24 h after irradiation with 4 Gy (the number of foci in the corresponding unirradiated cells was subtracted as background) of LPS- (10 µg/ml) or sham-treated (control) and with or without CBPi (2 µM) treated H1975 cells. Data were analyzed by linear regression analysis.

Western blot

Cells were rinsed with PBS prior to adding buffer 6 (R&D Systems, Minneapolis, MN, USA) for protein isolation. Protein concentration was determined with the BCA assay (Pierce-ThermoFisher Scientific, Rockford, IL, USA). After SDS-PAGE and transfer of proteins onto a polyvinyl difluoride (PVDF) membrane; nonspecific sites were saturated with 5% milk. Incubation was performed overnight (4° C) with the following primary antibodies: anti-phospho-CREB phospho S311 (ab32096, diluted 1:500, Abcam, Cambridge, UK), anti-CREB (ab32515, diluted 1:500, Abcam, Cambridge, UK), and anti-beta-Actin rabbit mAb (#4970, diluted 1:2000, Cell Signaling Technology, Cambridge, UK). Immunodetection was performed by incubation 1 h with peroxidase-conjugated secondary antibody goat anti-rabbit IgG (#7074, diluted 1:2000, Cell Signaling Technology, Cambridge, UK), with an ECL system (Thermo Fisher Scientific Inc., Waltham, MA, USA). The signals were quantified by densitometric scanning (Bio Rad ChemiDoc XRS+, Bio-Rad Laboratories, Inc., Hercules, USA).

Co-Immunoprecipitation

For the Co-Immunoprecipitation the cells were washed with PBS. The cells were lysed by incubating 20 min with Co-IP buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10% Glycerol, 1% Triton-100). After pre-cleaning an equal amount of proteins (>500 µg) were incubated with an antibody against Anti-KAT3A/CBP antibody (ab2832, 10 µl, Abcam, Cambridge, UK) or Rabbit IgG, polyclonal (ab27478, 10 µl, Abcam, Cambridge, UK) for the isotype control over night. After the incubation with this antibody 50 µl protein A/G Plus-agarose beads (Santa Cruz Biotechnology, Dallas, TX, USA) were added and incubated overnight a 4° C. After, the beads were washed 5 times with Co-IP buffer. The samples were resuspended in 2× Laemmli buffer with 10% beta-Mercaptoethanol and 20 mM DTT and boiled for 10 min. Proteins were analyzed by SDS-PAGE and Western Blot. For the Western Blot the incubation was performed overnight (4° C) with the primary antibody anti-phospho-CREB (phospho S311) (ab32096, diluted 1:500, Abcam, Cambridge, UK). Immunodetection was performed by incubation 1 h with peroxidase-conjugated secondary antibody goat anti-rabbit IgG (#7074, diluted 1:2000, Cell Signaling Technology, Cambridge, UK), with an ECL system (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Immunofluorescent microscopy and quantification of γH2AX/53BP-1 foci

Cells were grown on glass cover slips for 24 h. After incubation with LPS for 16 h cells were irradiated with a dose of 4 Gy. For analysis of γH2AX/53BP1 foci, cells were fixed and stained 24 h after irradiation with 4% paraformaldehyd (Carl Roth GmbH + Co. KG, Karlsruhe, Germany)/PBS (Sigma-Aldrich, St. Louis, MO, USA) for 10 min and stored at 4° C. Fixed cells were permeabilized with 0.2% Triton X-100 (SERVA Electrophoresis GmbH, Heidelberg, Germany), 1% BSA for 10 min and blocked in 3% BSA for 1 h. Primary antibody incubation was done for 1 h at room temperature using the following antibodies: Anti-phospho-Histone γH2A.X (Ser139) close JBW301/ mouse (1:500, Merck Millipore, Billerica, MA, USA), 53BP1 anti-rabbit (1:500, Bio-Techne-Novus Biologicals Minneapolis, MN, USA). After washing three times with 0.5% Tween 20/PBS for 10 min, the cells were incubated for 1 h with secondary anti-mouse Alexa-fluor 594 (1:800, Invitrogen, Carlsbad, CA, USA) and anti-rabbit Alexa-fluor 488 (1:1200, Invitrogen, Carlsbad, CA, USA). Cells were again washed three times and mounted in ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA, USA) including DAPI for staining of nuclei. Immunofluorescence was analyzed using the IX81 microscope (objective: 60x, Olympus, Shinjuku, Tokio, Präfektur Tokio, Japan) and Xcellence Software (Olympus, Shinjuku, Tokio, Präfektur Tokio, Japan). For analysis z-stacked images were taken from each sample and foci counted manually. The number of foci in irradiated samples was calculated by background subtraction from non-irradiated controls. These experiments were performed at least two times in duplicates and at least 200 nuclei were counted.

RNA isolation and real-time RT-PCR

For quantification of *TLR-4* and *EGFR* mRNA, total RNA was extracted with RNeasy Mini Kit (QIAGEN N.V., Hilden, Germany) according to the manufacturer's protocols. Extracted RNA was quantified with Nano Drop (PeqLab, Erlangen, Germany). The cDNA was synthesized by RT (Bio-Rad, München, Germany). Real-time PCR was performed using 1 µg of cDNA, SYBR Green PCR Master Mix (Bio-Rad, München, Germany) and 0.05 M forward/reverse primers; specific primers used for sequence detection were as follows:

TLR-4: 5′ccagcattccaatttgaaacaaatg3′ (forward) and 5′gagaggtccaggaaggtcaagtttc3′ (reverse).

EGFR: 5´gtgaaaacaccgcagcatgt3′ (forward) and 5′cccgtagctccagacatcac3′ (reverse)

PBGD: 5′cagcttgctcgcatacagac3′ (forward) and 5′gaatcttgtcccctgtggtg3′ (reverse).

Real-time-reactions were performed as described before [64]. The mRNA expressions were expressed as –ΔCt value (Ct value gene of interest – Ct value gene of reference gene [PBGD]).

Calculation and statistical analyses

If not otherwise indicated, results are presented as mean values ± standard errors of the mean (SEM) for at least 3 independent experiments. The level of significance was evaluated by one-way ANOVA, followed by Bonferroni's Multiple Comparison Test. Differences at

p-values of < 0.05 were considered statistically significant and are indicated in the figures by an asterisk.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Anlage 4

Hattar K, Grandel U, Moeller A, Fink L, Iglhaut J, Hartung T, Morath S, Seeger W, Grimminger F, Sibelius U.

Lipoteichoic acid (LTA) from Staphylococcus aureus stimulates human neutrophil cytokine release by a CD14-dependent, Toll-like-receptor-independent mechanism: Autocrine role of tumor necrosis factor-[alpha] in mediating LTA-induced IL-8 generation.

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Lipoteichoic acid (LTA) from *Staphylococcus aureus* stimulates human neutrophil cytokine release by a CD14-dependent, Toll-like-receptor-independent mechanism: Autocrine role of tumor necrosis factor-α in mediating LTA-induced interleukin-8 generation

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Objective: **In sepsis, Gram-positive and Gram-negative bacteria provoke similar inflammatory processes. Whereas lipopolysaccharides (LPSs) are acknowledged as the principal immunostimulatory components of Gram-negative bacteria, the effect of the Gram-positive cell wall component lipoteichoic acid (LTA) is less well characterized. In the present study, we investigated the effect of highly purified LTA from *Staphylococcus aureus* on cytokine generation by isolated human neutrophils.**

Subjects: **Isolated human neutrophils from healthy volunteers.**

Interventions: **Incubation of neutrophils with purified LTA from *S. aureus* in the absence or presence of interleukin (IL)-10, anti-CD14, or anti-Toll-like-receptor antibodies.**

Measurements: **Measurement of tumor necrosis factor (TNF)-α, IL-1β, and IL-8 by enzyme-linked immunosorbent assay. Analysis of IL-8 mRNA by reverse transcriptase polymerase chain reaction.**

Conclusions: **The LTA challenge provoked a dramatic release of cytokines, with an early appearance of TNF-α and IL-1β and a delayed liberation of IL-8. The first phase of IL-8 production was induced directly by LTA, whereas the second phase was endogenously mediated by TNF-α, as it was largely abrogated by neutralizing anti-TNF-α antibodies. In contrast, IL1-β was not involved in LTA-induced IL-8 generation. Interestingly, the late phase of IL-8 generation could also be attenuated by exogenous IL-10, probably as a consequence of its downregulatory effects on TNF-α generation. When investigating the mechanism of LTA-induced cellular activation, activity-neutralizing antibodies demonstrated that CD14 was involved in LTA-mediated neutrophil cytokine generation. Using antibodies that neutralize the activity of Toll-like receptor 2 (TLR2) or 4 (TLR4), we also show that CD14-dependent, LTA-induced neutrophil activation did not proceed via TLR2- or TLR4-mediated pathways. In conclusion, LTA is a potent activator of human neutrophil cytokine generation, with the synthesis of the chemokine IL-8 being largely dependent on TNF-α generation in an autocrine fashion. This LTA-induced effect was inhibited by IL-10, dependent on CD14, and independent of TLR 2 or 4. (Crit Care Med 2006; 34:835–841)**

Key Words: sepsis; neutrophils; lipoteichoic acid; cytokines; CD14 antigen; Toll-like receptors; interleukin-10

In sepsis and septic organ failure, Gram-positive and Gram-negative bacteria elicit similar immune responses. Contact of bacterial products with effector cells results in excessive release of proinflammatory cytokines, with tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 being centrally involved in the pathogenesis of sepsis (1, 2). The first-line effector cells being recruited and activated upon microbial invasion are polymorphonuclear leukocytes (PMNs) (3). Although traditionally defined as terminal effector cells with little capacity for protein synthesis, neutrophils have recently been recognized as capable of synthesizing large amounts of proinflammatory cytokines (4, 5). Because neutrophils clearly predominate over other cell types in acute inflammation, neutrophil-derived cytokines may be centrally involved in the pathogenetic sequela of sepsis and septic shock (6).

The membrane glycolipid lipopolysaccharide (LPS) was characterized as the main mediator of immune responses to Gram-negative bacteria. By interacting with CD14, a glycosylphosphatidylinositol (GPI)-anchored glycoprotein (7, 8), and Toll-like receptors (TLRs) (9–12), LPS mediates a plethora of cellular responses, such as activation of the transcription factor NF-κB and induction of cytokine synthesis.

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In contrast to LPS, the mechanisms involved in cell activation by Gram-positive bacteria are less well characterized. The main wall components of Gram-positive bacteria are lipoteichoic acid (LTA) and peptidoglycan. LTA is found in the cell membrane of virtually all Gram-positive bacteria and is liberated into the circulation after antibiotic treatment (13, 14). *In vivo*, LTA has been shown to contribute greatly to the onset of sepsis and septic shock (15, 16). Although activation of inflammatory cell functions, e.g., cytokine generation, has been shown *in vitro* (17–19), the molecular mechanisms of LTA-induced cell activation have not been characterized in detail. However, binding of LTA to different pattern-recognition receptors such as CD14, TLR2, and TLR4 seems to be essentially involved (20–23). Early studies with commercially available LTA were adversely affected by substantial LPS contamination (24). Recently, S. Morath has established a novel purification procedure for LTA of *Staphylococcus aureus*, which yields an LPS-free preparation (25). Both this highly purified LTA and synthetic LTA induce cytokine generation in whole-blood systems and mononuclear cells (26, 27). Although LTA has been shown to readily bind to human neutrophils (28), few studies have explored the effect of LTA on neutrophil physiology. In one recent study, purified LTA delayed neutrophil apoptosis and induced activation of degranulation and IL-8 synthesis (21), whereas other studies indicated that leukocyte activation by LTA was restricted to mononuclear cells (27, 29).

Given the important role of neutrophil-derived cytokines in the pathogenesis of sepsis (3, 6), we analyzed the effect of highly purified LTA from *S. aureus*, a clinically relevant pathogen in sepsis (30), on human neutrophil cytokine responses.

MATERIALS AND METHODS

Materials

Ficoll-Paque was purchased from Pharmacia (Uppsala, Sweden), fetal calf serum from Greiner (Frickenhausen, Germany), and all other media and supplements from GIBCO (Eggenstein, Germany), unless otherwise indicated. LPS (E. coli, 0111:B4) and cycloheximide were purchased from Sigma (Deisenhofen, Germany). All antibodies used for cytokine enzyme-linked immunosorbent assays (ELISAs) and recombinant cytokines were purchased from R & D Systems (Wiesbaden, Germany). IL-6 was analyzed by a commercial ELISA kit from R & D Systems (Wiesbaden, Germany). Neutralizing antibodies targeting TNF-α and IL-1β as well as recombinant human IL-1 receptor antagonist (IL-1RA) were also from R & D Systems. Function-blocking antibodies targeting TLR2 (clone TL2.1) and TLR4 (clone HTA 125) were from e-Bioscience (San Diego, CA). The lactate dehydrogenase (LDH) kit was from Boehringer Ingelheim (Ingelheim, Germany). Horseradish peroxidase–conjugated streptavidin and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Zymed Laboratories (San Francisco, CA). The anti-CD14 antibody MY-4 was from Coulter Immunotech (Hamburg, Germany), and the anti-CD18 antibody clone MHM23 was purchased from Dako (Hamburg, Germany). RLT buffer and columns were from the RNeasy Mini Kit (QIAGEN, Hilden, Germany), as were the QIAShredder columns. Cell culture plasticware was purchased from Falcon (Mannheim, Germany). The Platinum SYBR Green qPCR SuperMix UDG was purchased from Invitrogen (Karsruhe, Germany).

Isolation of Human Neutrophils

Neutrophils were isolated from venous blood of healthy donors by centrifugation over a Ficoll-Paque gradient as previously described (31). Purified cells were finally resuspended in RPMI medium containing 1% fetal calf serum at 10^7 PMNs/mL. Cell purity was >97%, as quantified by repeated flow cytometry after various incubation periods. Cell viability was >96%, as assessed by trypan blue dye-exclusion and determination of spontaneous LDH release.

Cell Culture and Stimulation

In the standard protocol, neutrophils were plated in 24-well low-cluster tissue culture plates at 10^7/mL (500 μL/well) and incubated at 37°C in a 5% CO_2-humidified atmosphere under continuous agitation. Neutrophils were incubated with LTA in various concentrations or 100 ng/mL LPS or sham-stimulated (control). For each time point, a separate neutrophil sample was run. After 4, 8, 16, and 24 hrs of incubation, cells and cell supernatants were separated by centrifugation at 13,000*g*, and cell supernatants were harvested and stored at −80°C. During the incubation, monitoring of LDH release and repeated cell counting were performed, yielding no relevant cell lysis after 4 and 8 hrs. After >16 hrs of incubation, cell counts were diminished to 52 ± 5% of initial values, accompanied by an LDH release of 48 ± 6%. Because of these variations, cytokine secretion was expressed as ng/10^7 PMNs.

Cytokine ELISA

Release of TNF-α, IL-1β, and IL-8 was determined by ELISAs as previously described (32), whereas IL-6 was determined in a commercial ELISA system.

Quantitation of IL-8 mRNA

mRNA Extraction. Aliquots of 2×10^7 cells were transferred into 1.5-mL reaction tubes. After centrifugation at 300*g*, the supernatant was discharged and the pellet was lysed in 600 μL buffer RLT containing 6 μL β-mercaptoethanol. After homogenization with QIAShredder columns, RNA extraction was performed according to the protocol. A DNase digestion of 20 mins at 30°C was included. RNA was eluted with 30 μL of RNase-free water.

cDNA Synthesis and Real-Time PCR. For cDNA synthesis, reagents and incubation steps were applied as described previously (31).

Relative mRNA Quantitation. Relative mRNA quantitation was performed by the Sequence Detection System 7700 (Applied Biosystems) and real-time PCR.

On the basis of the following equation (where T_o is the initial number of target gene mRNA copies; R_o is the initial number of standard gene mRNA copies; E is efficiency of amplification; CT,T is the threshold cycle of the target gene; CT,R is the threshold cycle of the standard gene; and K is the constant),

$$\frac{T_o}{R_o} = K \times (1 + E)^{(CT,R-CT,T)} = K \times (1 + E)^{\Delta CT} \quad [1]$$

we used comparative quantitation (ΔC_T) normalizing IL-8 to an unregulated internal standard gene HPRT (31, 33).

Purification of Lipoteichoic Acid

LTA was isolated from *S. aureus* as previously described in detail (25).

Statistics

For statistical comparison, one-way analysis of variance (ANOVA) was performed, followed by Tukey's honestly significant difference test when appropriate. A level of $p < .05$ was considered to be significant.

RESULTS

LTA Induces a Dose-Dependent Activation of Neutrophil IL-8 Generation. Isolated neutrophils (10^7/mL) were incubated with highly purified LTA from *S. aureus* at various concentrations (01–10 μg/mL). Within 16 hrs of incubation, IL-8 was liberated from LTA-stimulated cells in a dose-dependent manner, with

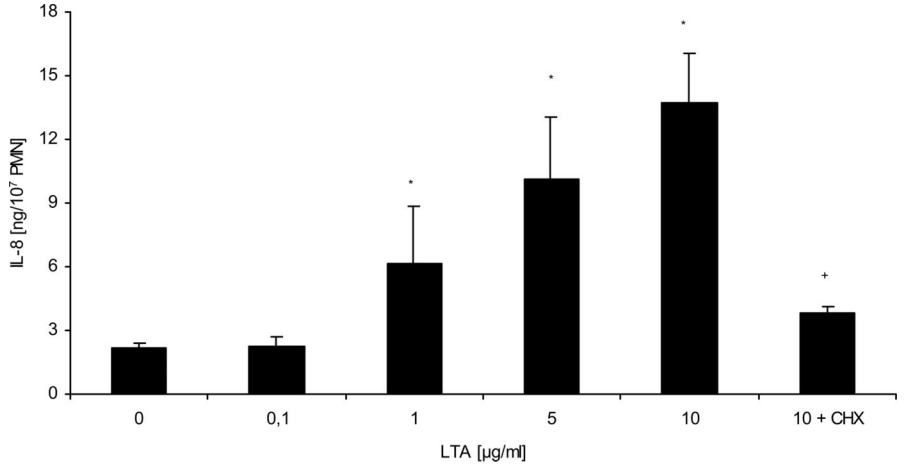

Figure 1. Dose-dependent activation of neutrophil interleukin (*IL*)-8 generation by lipoteichoic acid (*LTA*). Isolated neutrophils (10^7/mL) were challenged with purified LTA from *Staphylococcus aureus* at various concentrations (0.1–10 μg/mL), or sham incubation (*0*) was performed. The protein synthesis inhibitor cycloheximide (*CHX*, 1 μg/mL) was given simultaneously with 10 μg/mL LTA. After 16 hrs of incubation, cell supernatants were harvested, and release of IL-8 was quantified by enzyme-linked immunosorbent assay. Mean ± SEM values of at least four independent experiments each are shown. Values marked (*) differ significantly from sham-incubated cells, and values marked (+) differ significantly from corresponding values without CHX.

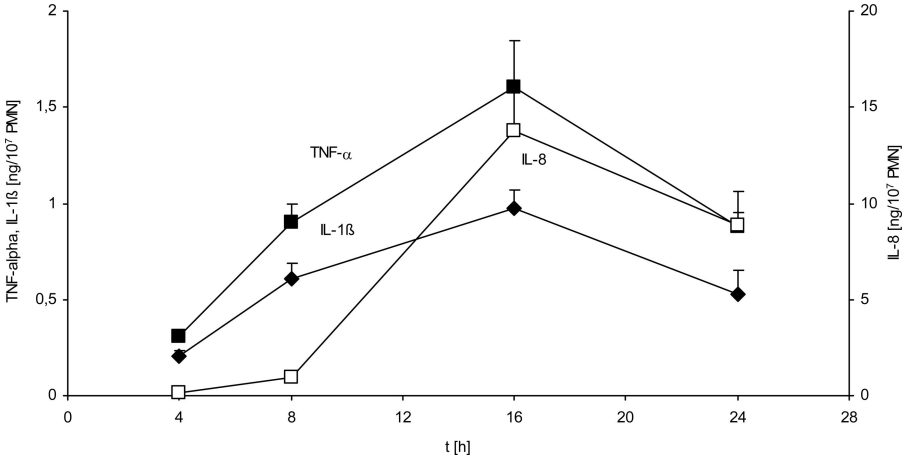

Figure 2. Time course of cytokine release in response to lipoteichoic acid (*LTA*). Isolated neutrophils (10^7/mL) were challenged with 10 μg/mL LTA or sham incubation was performed. At indicated time points, cell supernatants were harvested, and release of tumor necrosis factor (*TNF*)-α, interleukin (*IL*)-1β, and IL-8 was quantified by enzyme-linked immunosorbent assay. Mean ± SEM values of at least five independent experiments each are shown. Note the different scales for TNF-α and IL-1β vs. IL-8.

10 μg/mL LTA being most effective (Fig. 1), eliciting an about 6.5-fold increase in IL-8 (as compared with sham incubation) for 16 hrs. Cytokine secretion was dependent on *de novo* protein synthesis, as it was largely abrogated by cycloheximide (1 μg/mL). Higher concentrations of LTA (20–100 μg/mL) did not elicit any further enhancement of cytokine generation. For this reason, all subsequent studies were performed with 10 μg/mL LTA. In comparison with LPS-induced neutrophil activation, optimal concentrations of endotoxin (100 ng/mL) induced comparable amounts of IL-8 (19.16 ± 2.3 ng/10^7 PMNs) after a 16-hr incubation.

LTA Induces a Time-Dependent, Sequential Liberation of TNF-α, IL-1β, and IL-8. Isolated neutrophils were treated with LTA (10 μg/mL) or were sham-incubated for various time periods. Upon LTA challenge, a dramatic time-dependent activation of cytokine release into the cell supernatants was noted. The IL-8 release was preceded by liberation of low levels of TNF-α and IL-1β, which accumulated rapidly after stimulation, whereas the liberation of IL-8 was more prolonged and peaked after 16 hrs of incubation (Fig. 2). Cell supernatants were analyzed for IL-6, but this cytokine could not be detected in LTA-stimulated PMN supernatants (data not shown).

Autocrine Role of TNF-α in Mediating LTA-Induced Neutrophil IL-8 Release and mRNA Induction. On the basis of these kinetics, a role for TNF-α and IL-1β in LTA-induced IL-8 generation by neutrophils was proposed. Therefore, function-blocking antibodies targeting TNF-α and IL-1β, each at 5 μg/mL, were coapplied with 10 μg/mL LTA. Over the 4-hr and 8-hr incubation periods, these cytokine antagonists did not significantly influence the liberation of IL-8 induced by LTA. However, the accumulation of IL-8 after 16 hrs of stimulation with LTA was significantly reduced by the anti-TNF antibody (to 50.9 ± 15.8%, n = 4), whereas neutralizing IL-1β was not effective and a combination of both inhibitors did not increase the inhibitory effect of the anti-TNF-α antibody *per se* (Fig. 3).

When the anti-TNF-α antibodies were applied 8 hrs after stimulation with LTA, they were still fully effective (inhibition of LTA-induced IL-8 synthesis to 55.83 ± 5.9%, n = 4).

Well in line with these kinetics, when analyzing IL-8 mRNA by reverse transcriptase polymerase chain reaction (RT-PCR) we found a maximal induction after 8 hrs of LTA incubation in pilot studies (data not given). At this time point, LTA-induced IL-8 mRNA synthesis was fully blocked by neutralization of TNF-α, as depicted in Figure 4.

IL-10 Inhibits LTA-Induced IL-8 Release by Interfering with TNF-α Synthesis. To explore the effect of the anti-inflammatory cytokine IL-10 on neutrophil cytokine synthesis in response to LTA, neutrophils were stimulated with 10 μg/mL LTA for 4, 8, or 16 hrs, while IL-10 (10 ng/mL) was coapplied with LTA. Similar to the inhibitory capacity of the neutralizing TNF antibody, IL-10 blocked the late phase of LTA-mediated neutrophil IL-8 synthesis, whereas the early phase (i.e., 8 hrs after stimulation with LTA) of IL-8 synthesis was insensitive to the anti-inflammatory cytokine (Fig. 5, *top*).

When cell supernatants were analyzed for TNF-α, the early liberation of this cytokine (4 hrs poststimulation) was significantly diminished in IL-10-treated neutrophils, whereas the late phase of TNF-α liberation was not affected (Fig. 5, *bottom*).

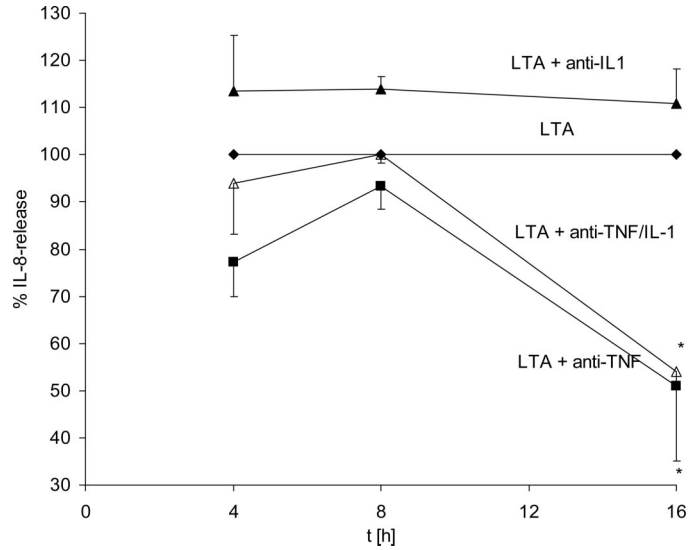

Figure 3. Autocrine role of tumor necrosis factor (*TNF*)-α in mediating lipoteichoic acid (*LTA*)-induced neutrophil interleukin (*IL*)-8 release. Isolated neutrophils were challenged with LTA (10 μg/mL) in the absence or presence of neutralizing antibodies targeting TNF-α, IL-1β (each at 5 μg/mL), or a combination of both antibodies. After 4, 8, and 16 hrs of stimulation, release of IL-8 was quantified, and quantity is expressed here as percentage of LTA-induced mediator release (100%). Mean ± SEM values of four independent experiments each are shown. Values marked (*) differ significantly from LTA-induced chemokine release.

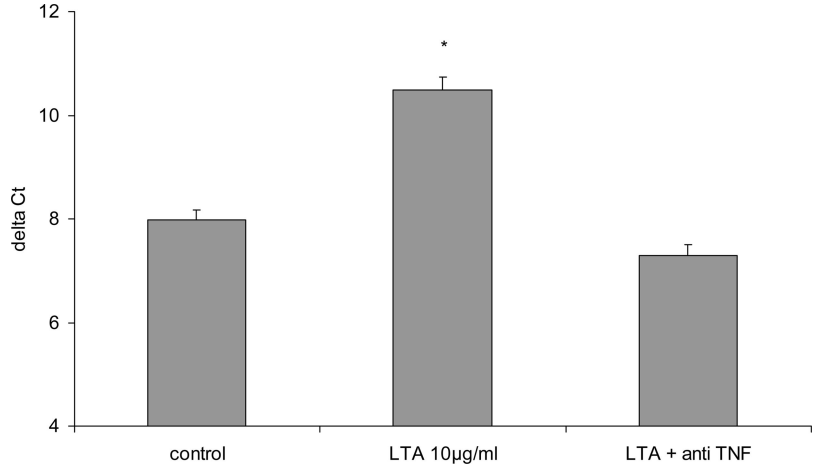

Figure 4. Neutrophil interleukin-8 mRNA expression in response to lipoteichoic acid (*LTA*): effect of tumor necrosis factor (*TNF*)-α antagonism. Neutrophils (10^7 polymorphonuclear lymphocytes/mL) were challenged with LTA (10 μg/mL) in the absence or presence of neutralizing anti-TNF-α antibodies (5 μg/mL), or sham incubation was performed (*control*). After 8 hrs, aliquots of 2×10^7 cells were collected, and mRNA was extracted and subjected to quantitative reverse transcriptase polymerase chain reaction. *Delta CT* (*threshold cycle*) values represent log-fold relative expression of IL-8 mRNA normalized to the reference gene HPRT mRNA. Means ± SEM values, originating from duplicate values from two different blood donors, are given. Values marked (*) differ significantly from controls as well as from cells incubated with LTA in the presence of anti-TNF.

Interference with CD14 but Not with TLR 2 or TLR4 Inhibits LTA-Induced Neutrophil Activation. To investigate the involvement of CD14, TLR2, and TLR4 in LTA-induced neutrophil activation, function-blocking antibodies were employed. Pretreatment of isolated neutrophils with anti-TLR2 (MAb TL2.1, 10 μg/mL) or anti-TLR4 (MAb HTA125, 10 μg/mL) did not inhibit the production of IL-8 upon stimulation with LTA (Fig. 6, *top*). In contrast, a significant reduction in IL-8 synthesis was observed in the presence of the function-blocking anti-CD14 antibody MY-4 (10 μg/mL). Combined inhibition of CD14 and TLR2 or TLR4 did not provoke further reduction of IL-8 synthesis, and interference with β_2-integrin-dependent adhesion by the function-blocking antibody MHM23 (10 μg/mL) did not affect LTA-induced cytokine synthesis, with IL-8 liberation in the presence of anti-CD18-antibodies corresponding to 116 ± 11.2% of mono-LTA-stimulated cells (n = 4).

For the LPS stimulation of neutrophils (Fig. 6, *bottom*), a comparable dependence on CD14 was observed, since MY-4 was able to depress the LPS-induced IL-8 liberation to 25 ± 0.8%. It is interesting that blocking TLR2 with TL2.1 reduced LPS-mediated IL-8 synthesis to 49 ± 8.3%, and combined blocking of CD14 and TLR.2 was even more effective (reduction of IL-8 to 17.9 ± 1.26% of controls). In contrast, targeting of TLR4 with HTA 125 had no effect on LPS-mediated neutrophil activation (IL-8 liberation corresponding to 99.1 ± 1.3% of control LPS-stimulated cells). In control experiments, anti-TLR as well as anti-CD14 antibodies did not elicit neutrophil IL-8 liberation *per se* (data not shown).

DISCUSSION

The current study demonstrates that highly purified LTA from *S. aureus*, a most relevant pathogen in Gram-positive sepsis (30), induces IL-8 secretion from isolated neutrophils in a biphasic manner, with TNF-α being centrally involved in endogenous induction of the late IL-8 response. Moreover, IL-10 blocked this inflammatory response, and the stimulatory effects of LTA were dependent on CD14, but independently of the Toll-like receptors 2 and 4.

The proinflammatory features of LTA have been questioned, and previous reports described inconsistent results concerning the capacity of LTA to induce cytokine secretion in differing models (18–20). These inconsistencies may be attributable to diverse biological activities of LTA from different species or simply due to the fact that commercially available LTA contains significant contaminations, particularly LPS (24). However, LTA used in the present study was purified by a novel procedure that preserves the critical stimulatory alanine substituents (25), ensuring that neutrophil cytokine generation was caused by pure and structurally intact LTA.

In previous reports, cytokine secretion in response to LTA was observed primar-

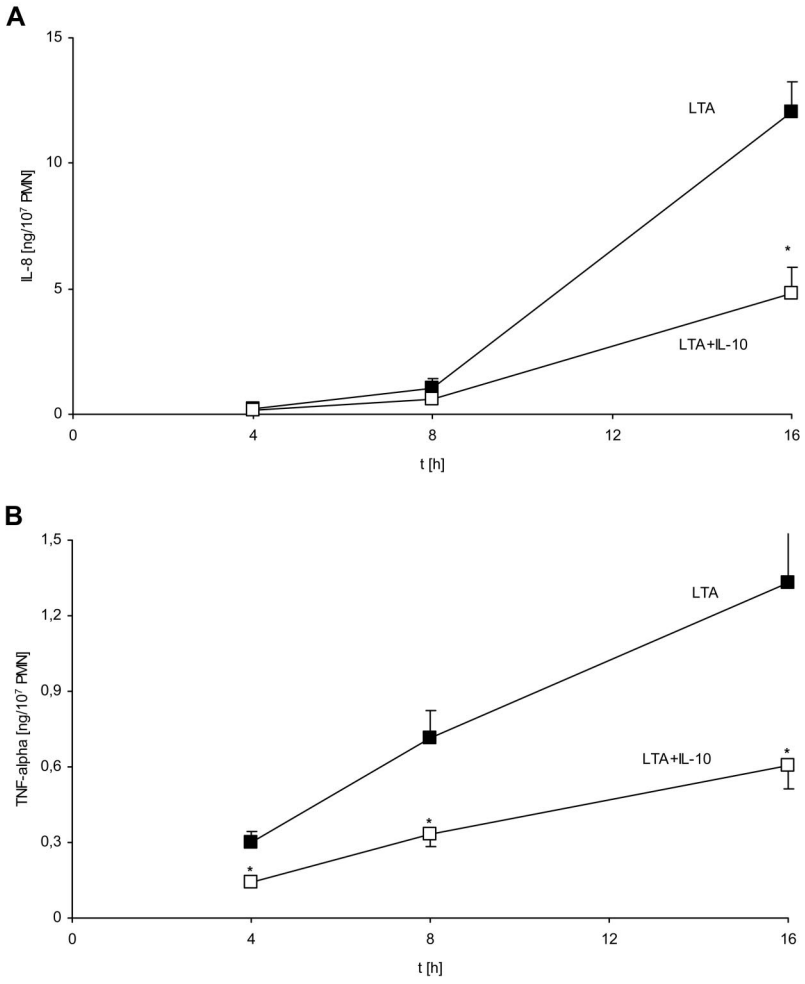

Figure 5. Inhibitory effect of interleukin (*IL*)-10 on lipoteichoic acid (*LTA*)-induced neutrophil tumor necrosis factor (*TNF*)-α and IL-8 generation. Isolated neutrophils were challenged with LTA (10 μg/mL) in the absence or presence of 10 ng/mL IL-10. After 4, 8, and 16 hrs of incubation, cell supernatants were harvested, and release of IL-8 (*top*) and TNF-α (*bottom*) was quantified by enzyme-linked immunosorbent assay. Mean ± SEM values for four independent experiments each are shown. Values marked (*) differ significantly from mono-LTA-incubated cells.

ily in peripheral blood mononuclear cells, and in a recent study, LTA-stimulated neutrophils did not exhibit substantial transcription of cytokine-encoding genes (26) or cytokine liberation (29) In contrast, in the present study, we demonstrated effective induction of neutrophil cytokine gene transcription and protein synthesis by LTA. These discrepancies may be explained largely by the different experimental setups of the respective studies. For example, Ellingsen et al. (26) used a whole-blood assay, in which other CD14-expressing cells, such as monocytes, display a much stronger CD14 expression than do neutrophils and would therefore compete with neutrophils for LTA-binding. Moreover, in whole blood, substantial amounts of soluble CD14 are present, which were recently described to interfere with cellular activation by LTA (34). Although a neutrophil purity of 97% does not totally exclude trace contaminations by mononuclear cells, the absence of IL-6 synthesis, which is strongly induced by LTA in monocytes (26), strongly suggests that monocytes were no significant contaminants of our preparations (35, 36). In line with our results, Lotz et al. (21) demonstrated the delay of apoptosis and effective induction of IL-8 and secretion of granulocyte-colony stimulating factor by highly purified granulocytes in response to LTA.

In our study, LTA-induced cytokine generation was dose-dependent, with maximal IL-8 secretion at 10 μg/mL LTA. This amount of LTA is substantially lower than that required in full-blood assays (26) and corresponds very well with recent studies in isolated monocytes and neutrophils (21, 37). In our model, small quantities of TNF-α and IL-1β but no IL-6 was recovered from LTA-stimulated PMNs. Nevertheless, "early" TNF-α (accumulating 4 hrs after LTA exposure), considerably stimulated the late phase of IL-8 secretion in an autocrine manner. This was demonstrated by the inhibitory effect of neutralizing anti-TNF-antibodies on both IL-8 mRNA induction and the sustained IL-8 secretion.

The early release of IL-8 by LTA appears to be provoked directly, as it was not affected by blocking TNF-α activity. Such a biphasic IL-8 synthesis has also been demonstrated in LPS-stimulated neutrophils (38). In these studies, LPS-activated TNF-α and IL-1β synergized in supporting IL-8 generation. In contrast to this, inhibition of IL-1β bioactivity in our study did not diminish LTA-induced IL-8 liberation, although effective blockage of IL-1β activity by our anti-IL-1-antibodies has been established in our previous studies (31).

It is interesting that IL-10 selectively inhibited the late phase of IL-8 liberation. This inhibition was comparable to that observed for the anti-TNF-α antibody. Furthermore, IL-10 also inhibited early TNF-α liberation, thus suggesting that IL-10 inhibited LTA-provoked IL-8 secretion by blocking the TNF-α-mediated autocrine stimulation. A comparable inhibition of TNF-α and IL-1β–mediated IL-8 liberation has been attributed to IL-10 in LPS-stimulated neutrophils (38). IL-10 has been successfully applied in animal models of sepsis (39, 40), and some of the molecular mechanisms underlying the actions of IL-10 in neutrophils have been recently characterized; both activation of STAT (signal transducers and activators of transcription) proteins and expression of SOCS (suppression of cytokine signaling) seem to be relevant in this context (41, 42).

Previous reports suggest that LTA-induced cell activation proceeds via Toll-like receptors and CD14, and current data favor a role for TLR2 rather than TLR4 in LTA-mediated signaling (21, 22, 26, 37). Employing the function-blocking anti-CD14 antibody MY-4, we confirmed that in neutrophils CD14 does mediate LTA signaling. However, antibodies targeting TLR2 and TLR4 were totally ineffective. One could speculate that the antibodies TL2.1 and HTA 125 were not effective blockers of Toll-mediated signaling. However, other studies have clearly dem-

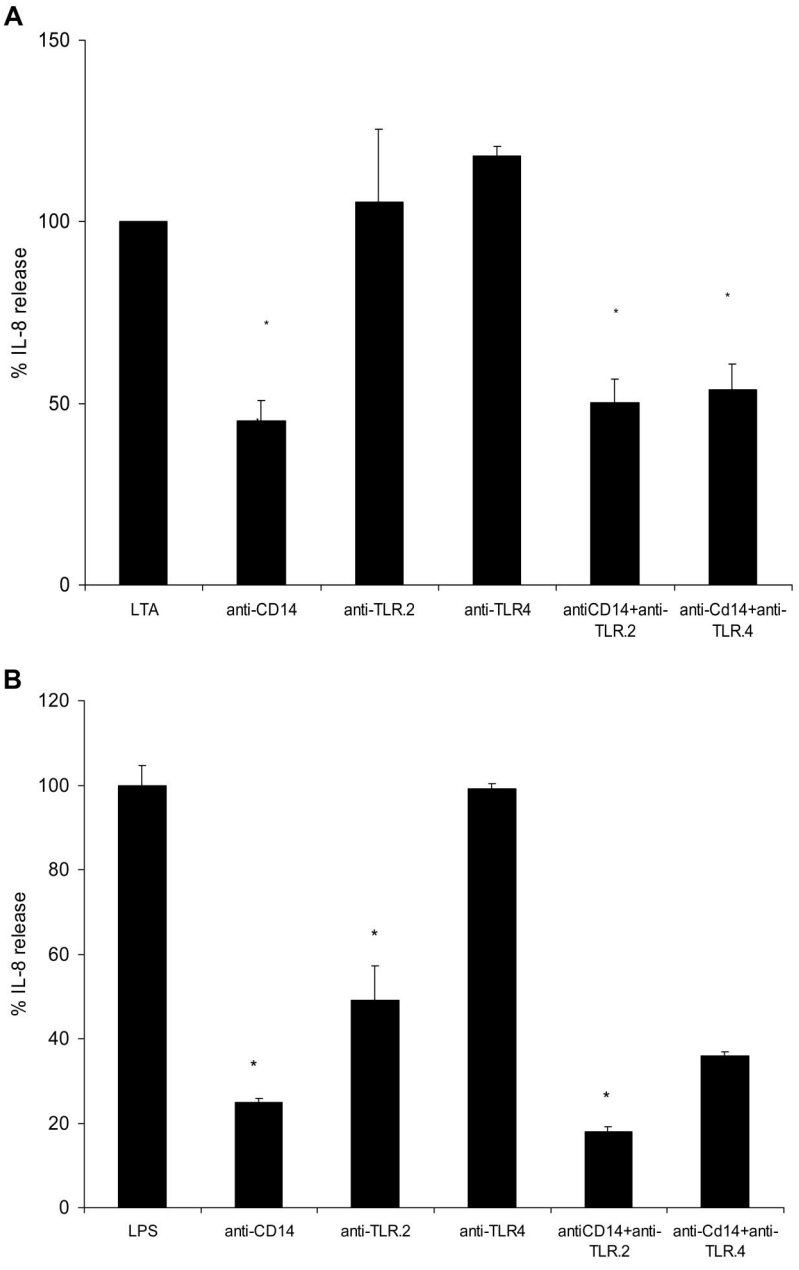

Figure 6. Effect of anti-CD14, anti-Toll-like-receptor (*TLR*)2 and anti-TLR4 antibodies on lipoteichoic acid (*LTA*)-induced neutrophil activation: comparison with lipopolysaccharide (*LPS*)-induced chemokine generation. Isolated neutrophils were challenged with LTA (10 μg/mL) (*top*) or LPS (10 ng/mL) (*bottom*) in the absence or presence of function-blocking antibodies targeting CD14 (MY-4), TLR2 (TL2.1), or TLR4 (HTA125) or a combination of these antibodies. All antibodies were used at 10 μg/mL. Release of IL-8 was quantified after 16 hrs of incubation, and quantity is expressed as percentage of LTA or LPS-induced mediator release in control cells (set at 100%). Mean ± SEM values of at least three independent experiments each are shown. Values marked (*) differ significantly from chemokine release induced by LTA or LPS in the absence of the respective antibodies. *IL*, interleukin.

onstrated that HTA-125 blocks TLR4-dependent cellular activation by LPS (12), and TL2.1 inhibits cellular activation by another Gram-positive bacterium, *Listeria monocytogenes*, as well as by lipoproteins and lipopeptides (43, 44). More recently, it was reported that the TL2.1 antibody attenuated the LTA-induced delay of neutrophil apoptosis and, albeit to a lesser extent, LTA-induced IL-8 induction (21). Thus, these antibodies are effective blockers of Toll-mediated signaling. In our hands, blocking of TLR2 but not TLR4 inhibited LPS-induced neutrophil IL-8 generation, which is generally considered a TLR4-dependent process (11, 12, 23). In support of our data, LPS-induced activation of neutrophil granulocytes was shown to proceed by lipopeptides and was dependent on TLR2 (45). The inhibition of LPS-mediated IL-8 generation by TL2.1 also demonstrates the inhibitory capacity of TL2.1 in the concentrations currently used. Previous studies have suggested that TLR2 may be sufficient to confer cellular responsiveness to LTA (22, 26); however, in the presence of other surface ligands such as CD14, it may not be mandatory for LTA-induced cellular activation.

Our data demonstrate that highly purified lipoteichoic acid from S. aureus*, an important pathogen in severe Gram-positive infections, is a potent inducer of neutrophil interleukin-8 synthesis.*

In conclusion, our data demonstrate that highly purified LTA from *S. aureus*, an important pathogen in severe Gram-positive infections, is a potent inducer of neutrophil IL-8 synthesis. The IL-8 formation was induced by endogenously formed TNF-α in an autocrine fashion, and this mechanism was inhibited by IL-10. Engagement of CD14 but not TLR2 or TLR4 was essential for activation of human neutrophils by LTA. These findings are relevant to our understanding of the inflammatory sequela in severe Gram-positive infection and sepsis.

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Anlage 5

Hattar K, Fink L, Fietzner K, Himmel B, Grimminger F, Seeger W, Sibelius U.

Cell density regulates neutrophil IL-8 synthesis: role of IL-1 receptor antagonist and soluble TNF receptors.

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Cell Density Regulates Neutrophil IL-8 Synthesis: Role of IL-1 Receptor Antagonist and Soluble TNF Receptors

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Cell Density Regulates Neutrophil IL-8 Synthesis: Role of IL-1 Receptor Antagonist and Soluble TNF Receptors

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Although cytokine synthesis in polymorphonuclear leukocytes (PMN) was shown to be modulated by soluble mediators, the impact of microenvironmental conditions has not been elucidated. In this study, we investigated the effect of cell density on cytokine release from human neutrophils. PMN were cultured at various cell densities (10×10^6 PMN/ml; 60×10^6 PMN/ml), and LPS-induced release of cytokines was quantified by ELISA technique. Upon an increase in PMN density, secretion of the CXC chemokine IL-8 was progressively reduced. This effect was paralleled by a decrease in IL-8 mRNA. In contrast, TNF-α and IL-1β rose proportionally with increasing cell density. The inhibition of IL-8 secretion was reproduced by conditioned media of PMN at high cell density, but was not affected by blocking β_2 integrin-dependent adhesion. When analyzing the supernatant of LPS-challenged neutrophils, large amounts of soluble TNFRs p55 and p75 (sTNFRI, sTNFRII), and IL-1R antagonist (IL-1RA), rising constantly with the cell density, were detected. Interestingly, combined blocking of the bioactivities of these mediators completely restored neutrophil IL-8 secretion at high cell densities, with the anti-IL-1RA Ab being the more potent agent. Moreover, combined application of exogenous IL-1RA and sTNFRs to 10×10^6 PMN/ml reproduced the suppression of IL-8 generation. We conclude that neutrophil IL-8 synthesis is autoregulated, being suppressed under conditions of high cell density. IL-1RA and sTNFRs, accumulating under these circumstances, seem to be centrally involved in this regulatory mechanism by interfering with the IL-1β- and TNF-α-dependent IL-8 generation. This feedback mechanism may control further neutrophil recruitment and activation in a neutrophil-rich environment, thereby preventing tissue destruction. ***The Journal of Immunology*****, 2001, 166: 6287–6293.**

The recruitment and activation of polymorphonuclear leukocytes (PMN)[2] are essential for nonspecific host defense against infectious agents. Neutrophil recruitment is directed by a number of exogenous and endogenous peptide and lipid mediators, such as fMLP, C5a, and leukotriene B_4. Additionally, chemotactic cytokines, the so-called chemokines, act as potent neutrophil chemoattractants (1). Among the neutrophil-attracting chemokines, IL-8 is the best characterized. IL-8 belongs to the CXC family of chemokines, and was originally described as a monocyte-derived neutrophil chemoattractant (2, 3). In addition, IL-8 stimulates neutrophil adherence, degranulation, respiratory burst, and lipid mediator synthesis (4). Although traditionally defined as terminal effector cells with little capacity of de novo protein synthesis, neutrophils themselves recently became known to be capable of synthesizing IL-8, and other cytokines, including TNF-α, IL-1β, M-CSF, and G-CSF, as well as IL-1R antagonist (IL-1RA) (5–7). While TNF-α, IL-1β, and growth factors represent classic proinflammatory cytokines, IL-1RA inhibits the proinflammatory effects of IL-1α and IL-1β by competitive binding to IL-1Rs (8, 9). Moreover, neutrophils have the capacity to release soluble receptors for TNF-α (sTNFRs), which block TNF activity by competing for it with cell surface TNFRs (10–12). As neutrophils predominate over other cell types in many variants of acute and chronic inflammatory conditions, PMN-derived cytokines may be centrally involved in the regulation of inflammatory and immune processes in vivo (13).

Although much attention has been paid to the onset of neutrophil recruitment and activation, little is known about mechanisms limiting neutrophil activity. Such mechanisms are, however, of major importance for the control of inflammation in vivo, as excessive neutrophil activation may cause severe tissue destruction (14, 15). Pneumococcal pneumonia is an example of initial massive neutrophil accumulation and subsequent complete resolution of pulmonary injury with maintenance of lung structure (16, 17). The neutrophil microenvironment may be a critical factor for the control of neutrophil kinetics and activity. In the present study, we investigated whether the neutrophil density per se regulates inflammatory cell functions, focusing on neutrophil cytokine release. In essence, an increase in cell density (from 10×10^6 to 60×10^6 PMN/ml) was found to dramatically down-regulate the release of the CXC chemokine IL-8 upon stimulation with LPS. Suppression of neutrophil chemokine generation was not attributable to β_2 integrin-dependent adhesion, but was mediated by soluble factor(s) arising in the supernatant of neutrophils cultured at high cell density. Biochemical analysis and function-blocking Abs identified IL-1RA and sTNFRs as suppressor agents. The observed down-regulation of neutrophil IL-8 generation at high cell density may represent a negative feedback mechanism helping to control neutrophil inflammatory activity by preventing further neutrophil recruitment and activation in a neutrophil-rich microenvironment.

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[2] Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; α_1AT, α_1-antitrypsin; ADA, adenosine deaminase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IL-1RA, IL-1R antagonist; s, soluble; SOD, superoxide dismutase.

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Materials and Methods

Materials

Ficoll-Paque was purchased from Pharmacia (Uppsala, Sweden), FCS from Greiner (Frickenhausen, Germany), and all other media and supplements were from Life Technologies (Eggenstein, Germany), unless otherwise indicated. LPS (*Escherichia coli*, 0111:B4), indomethacin, α_1-antitrypsin (α_1AT), superoxide dismutase (SOD), and adenosine deaminase (ADA) were purchased from Sigma (Deisenhofen, Germany), while MK-886 and CV-6209 were from Calbiochem (La Jolla, CA). All Abs used for cytokine ELISAs as well as recombinant cytokines were purchased from R&D Systems (Wiesbaden, Germany): mAbs against TNF-α (mAb 610), IL-1β (mAb 601), IL-8 (mAb 208), and IL-10 (mAb 217); biotinylated Abs against TNF-α (BAF 210), IL-1β (BAF 201), IL-8 (BAF 208), and IL-10 (BAF 417); as well as recombinant human TNF-α (210-TA), IL-1β (201-LB-005), IL-8 (208-TA-010), and IL-10 (217-IL-005). For the detection of IL-1RA, sTNFRs, IL-4, IL-13, and IFN-γ, commercial ELISA kits were used (R&D Systems). Neutralizing Abs targeting IL-1RA (AF-280-NA) and sTNFRs (mAb 225 for sTNFRI(p55) and mAb 226 for sTNFRII(p75)) were also from R&D Systems, as well as recombinant IL-1RA (280-RA-010) and sTNFRs (636-R1-025; 226-B2-025). Peroxidase-conjugated streptavidin (HRP) and ABTS were purchased from Zymed Laboratories (San Francisco, CA), while the anti-CD18 Ab MHM23 was from Dako (Hamburg, Germany). The Dynabeads mRNA direct kit was from Dynal (Oslo, Norway), and the SYBR Green PCR Core Reagents were from Applied Biosystems (Weiterstadt, Germany). Cell culture plasticware was purchased from Falcon (Mannheim, Germany).

Isolation of human neutrophils

Neutrophils were isolated from venous blood of healthy donors by centrifugation over a Ficoll-Paque gradient, as previously described (18). In brief, EDTA-anticoagulated blood was layered over Ficoll-Paque and centrifuged at 400 $\times$ *g* for 35 min. After removal of mononuclear cells, erythrocytes were sedimented in 1% polyvinyl alcohol. Residual erythrocytes were removed by hypotonic lysis, and cells were washed twice in Ca^{2+}/Mg^{2+}-free PBS and finally resuspended in RPMI containing 1% FCS at 10×10^6 to 60×10^6 PMN/ml. Cell purity was >97%, as quantified by flow cytometry, and cell viability was >96%, as assessed by trypan blue dye exclusion.

Cell culture and stimulation

In the standard protocol, neutrophils were resuspended in RPMI supplemented with 1% FCS, plated in 24-well tissue culture plates at 10×10^6 to 60×10^6/ml (500 μl/well), and incubated at 37°C in a 5% CO_2-humidified atmosphere. Neutrophils were cultured with media alone (control) or stimulated with 10 ng/ml LPS. In experiments designed to investigate the effects of IL-1RA or sTNFRs, neutralizing Abs were coapplied with LPS and used at 20 μg/ml. All other agents were also coapplied with LPS: ADA was used at 400 U/ml; the platelet-activating factor (PAF) receptor antagonist CV-6209, the cyclooxygenase-inhibitor indomethacin, and the 5-lipoxygenase inhibitor MK-886 were used at 10 μM; whereas the anti-CD18 Ab (MHM23) was used at 50 μg/ml. α_1AT was applied at 100 μg/ml, and the O_2^- scavenger SOD was used at 100 U/ml. After 4, 8, and 16 h of incubation, cells and cell supernatants were separated by centrifugation at 13,000 $\times$ *g*, and cell supernatants were harvested and stored at −80°C until further processing. To assess the levels of intracellularly stored cytokines, cell pellets were resuspended in 500 μl RPMI. Cells underwent two freeze-thaw cycles using liquid nitrogen, debris was removed by centrifugation at 13,000 $\times$ *g*, and lysates were stored at −80°C.

Cytokine ELISA

Release of TNF-α, IL-1β, IL-8, and IL-10 was determined by self-developed direct sandwich ELISAs. In brief, immunoassay plates were coated with mouse anti-human TNF-α, IL-1β, IL-8, or IL-10 mAbs at a concentration of 4 μg/ml. After a blocking period with 1% BSA in PBS, samples were added. Recombinant human TNF-α, IL-1β, IL-8, and IL-10 were used for standard titration curves. To sandwich the trapped Ag, biotinylated Abs were applied at the following concentrations: 400 ng/ml anti-TNF-α, 200 ng/ml anti-IL-1β, 40 ng/ml anti-IL-8, or 200 ng/ml IL-10. Next, plates were incubated with HRP-conjugated streptavidin, followed by addition of substrate (H_2O_2 and ABTS). Absorbance was measured at 450 nm in a microplate reader using SLT Lab Instruments software (Creilsheim, Germany) to analyze the generated data. IL-8 and IL-10 ELISA were sensitive to 15 pg/ml, IL-1β and TNF-α ELISA to 7 pg/ml. IL-1RA, sTNFRs, IL-4, IL-13, and IFN-γ were determined in a commercial ELISA system, sensitive to 15 pg/ml each. In separate recovery experiments, the effect of cell debris on the determination of IL-8 was evaluated. Therefore, rIL-8 was dissolved in the supernatants of unstimulated neutrophils cultured at the various cell densities, and cytokine ELISAs were performed exactly as described. Equal values (deviation <5%) were obtained for the determination of IL-8 either if dissolved in the supernatant of 10×10^6 PMN/ml or in that of 60×10^6 PMN/ml.

mRNA extraction

Aliquots of 0.5×10^6 cells derived from neutrophils cultured at the various cell densities were transferred into 1.5-ml reaction tubes. After centrifugation at 300 $\times$ *g*, the supernatant was removed and the pellet was lysed in 300 μl lysis buffer of the Dynabeads mRNA direct kit. Based on magnetic separation, mRNA is caught by attachment to oligo(dT) fragments that are coupled to supermagnetic glass particles. Per sample, 150 μg beads were applied. Isolated mRNA was finally dissolved in 20 μl diethyl pyrocarbonate-treated H_2O.

cDNA synthesis and real-time PCR

For cDNA synthesis, reagents and incubation steps were applied as described (19). Ten microliters each of the isolated mRNA were applied for reverse transcription. The reactions were set up with the SYBR Green PCR Core Reagents, according to the manufacturer's protocol. Using the oligonucleotide primer pairs given in Table I, 1 μl of each primer (10 μM) and 2 μl cDNA were added to a final volume of 50 μl. Cycling conditions were adapted to 95°C for 6 min, followed by 40 cycles of 95°C, 20 s; 58°C, 30 s; and 73°C, 30 s. PCR products were routinely identified by 2.5% agarose gel electrophoresis.

Relative mRNA quantitation

Relative mRNA quantitation was performed by the Sequence Detection System 7700 (Applied Biosystems) and real time PCR. $(T_0/R_0) = K \times (1 + E)(CT,R - CT,T)$ in which T_0 is the initial number of target gene mRNA copies, R_0 is the initial number of standard gene mRNA copies, E is the efficiency of amplification, CT,T is the threshold cycle of target gene, CT,R is the threshold cycle of standard gene, and K is the constant.

On the basis of the given equation, we used comparative quantitation (ΔC_T), normalizing IL-8 to an unregulated internal standard gene (19).

Table I. *Sequences, amplicon sizes, and exon localization of the primers*[a]

Primer Name	Sequence (5′ to 3′)	Exon
1. HPRT amplicon size: 157 bp		
HPRT-forward	AAGGACCCCACGAAGTGTTG	e7
HPRT-reverse	GGCTTTGTATTTTGCTTTTCCA	e9
2. IL-8 amplicon size: 224 bp		
IL-8-forward	GCCTTCCTGATTTCTGCAGC	e1
IL-8-reverse	TGCACCCAGTTTTCCTTGG	e3

[a] Selecting suitable intron-spanning primers, the cDNA amplicon was much shorter than the genomic DNA amplicon. Excluding falsification by amplification of possible pseudogene sequences, both primer sets were shown to detect no genomic DNA with cDNA sequence. Thus, DNase digestion could be omitted. The primer sets were selected to work under identical PCR cycling conditions, so that simultaneous amplification of HPRT and IL-8 was obtained in the same run.

Therefore, mRNA transcribed from the gene encoding hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used. Afterward, values of relative mRNA expression were normalized to the value of 10×10^6 PMN/ml after 1 h of LPS challenge. In preliminary experiments, we could show that amplification efficiency of HPRT and IL-8 primer sets was approximately equal and amounted to 0.95 ± 0.02 ($= 95 \pm 2\%$). *K* is assumed to be equal within a definite primer system and thus does not influence the comparison of calculated relative ratios. Due to the nonselective dsDNA binding of the SYBR Green, gel electrophoresis was performed to confirm the exclusive amplification of the expected PCR product.

Statistics

For statistical comparison, one-way ANOVA was performed, followed by Tukey's honestly significant difference test when appropriate. A level of $p < 0.05$ was considered to be significant.

Results

IL-8 synthesis is suppressed upon increase in neutrophil density

Whereas unstimulated neutrophils released only small amounts of IL-8 into the cell supernatant (data not given in detail), stimulation of 10×10^6 PMN with 10 ng/ml LPS resulted in progressive liberation of this CXC chemokine into the cell supernatant (Fig. 1*A*). Significant accumulation of IL-8 was already noted after 4 h of endotoxin stimulation, and rose steadily up to 16 h of stimulation. Interestingly, with increasing cell density, the levels of IL-8 in the supernatant volume did not increase, but were instead dramatically reduced (Fig. 1*A*). As compared with 10×10^6 PMN/ml, IL-8 liberation was reduced to ~35% in 60×10^6 PMN/ml after 8 and 16 h of stimulation (1746 ± 316 vs 677.5 ± 180 pg/ml after 8 h, and 4350 +/− 1001 vs 1796 ± 448 pg/ml after 16 h, $n = 6$). When calculations were performed per cell number, this corresponds to a suppression of IL-8 release to <10% per single cell under conditions of high cell density. Importantly, any debris in the cell supernatants of neutrophils at high cell density did not affect the determination of IL-8 in the currently used ELISA system, as assessed in separate recovery experiments.

To assess whether inhibition of chemokine liberation rather than synthesis was underlying the observed reduction of IL-8 in supernatants of neutrophils at high cell density, cell lysates were analyzed. A corresponding decrease of IL-8 with increasing cell density was again detected (Fig. 1*B*), indicating that down-regulation of neutrophil IL-8 release at high cell density was attributable to reduced de novo synthesis of this chemokine. mRNA quantification by real-time RT-PCR, as performed after 1 and 4 h of LPS stimulation, revealed a comparable decrease in IL-8 mRNA as normalized to HPRT-mRNA in 60×10^6 PMN/ml (Fig. 2).

TNF-α and IL-1β are continuously liberated upon increase in neutrophil density

To assess whether the down-regulation of IL-8 synthesis was paralleled by the suppression of other neutrophil-derived cytokines, supernatants of LPS-stimulated PMN at various cell densities were analyzed for TNF-α and IL-1β. Contrary to IL-8, the liberation of these cytokines increased proportionally with increasing PMN density (Fig. 3). After 16 h of incubation, LPS-induced release of IL-1β from 10×10^6 PMN/ml was 165 ± 32.5 pg/ml, while 838 ± 201 pg/ml IL-1β was liberated from 60×10^6 PMN/ml (Fig. 3*A*). When calculating IL-1β synthesis per single cell, this corresponds to 165 ± 32.5 pg/10×10^6 PMN at the lowest cell density and 138 ± 19.45 pg/10×10^6 PMN at the highest cell density, indicating that the liberation of this cytokine was not undergoing any substantial down-regulation by cell density. Similarly, LPS-induced liberation of TNF-α, quantified after 16 h of

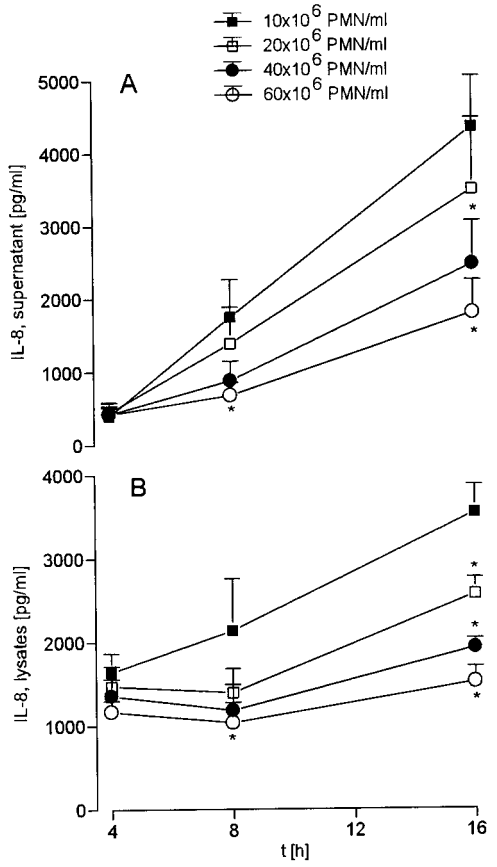

FIGURE 1. Effect of cell density on neutrophil IL-8 synthesis in response to LPS. Increasing concentrations of neutrophils (10×10^6 PMN/ml to 60×10^6 PMN/ml) were challenged with LPS (10 ng/ml). At indicated time points, reactions were stopped, and cell supernatants (*A*) and cell lysates (*B*) were collected. IL-8 is given in picograms per milliliter. Means ± SEM of at least six independent experiments are shown. Values marked differ significantly from corresponding values with 10×10^6 PMN/ml.

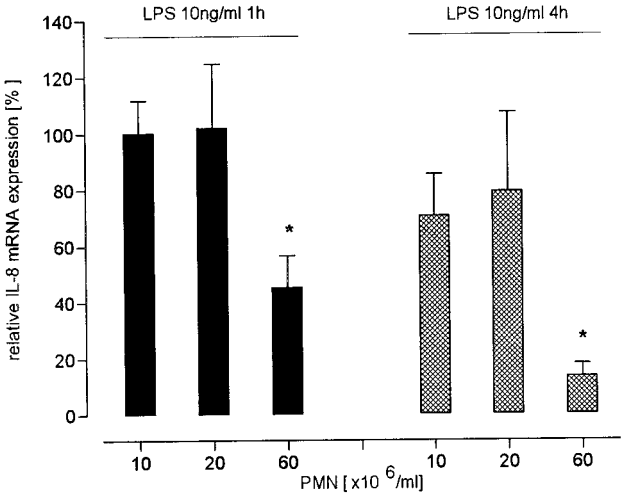

FIGURE 2. Effect of cell density on neutrophil IL-8 mRNA expression in response to LPS. Increasing concentrations of neutrophils (10×10^6 PMN/ml to 60×10^6 PMN/ml) were challenged with LPS (10 ng/ml). After 1 and 4 h, aliquots of 0.5×10^6 cells were collected, and mRNA was extracted and subjected to quantitative RT-PCR. Relative expression of IL-8 mRNA is normalized to the number of IL-8 transcripts in 10×10^6 PMN/ml after 1 h of LPS stimulation (100%). Values marked differ significantly from corresponding values with 10×10^6 PMN/ml.

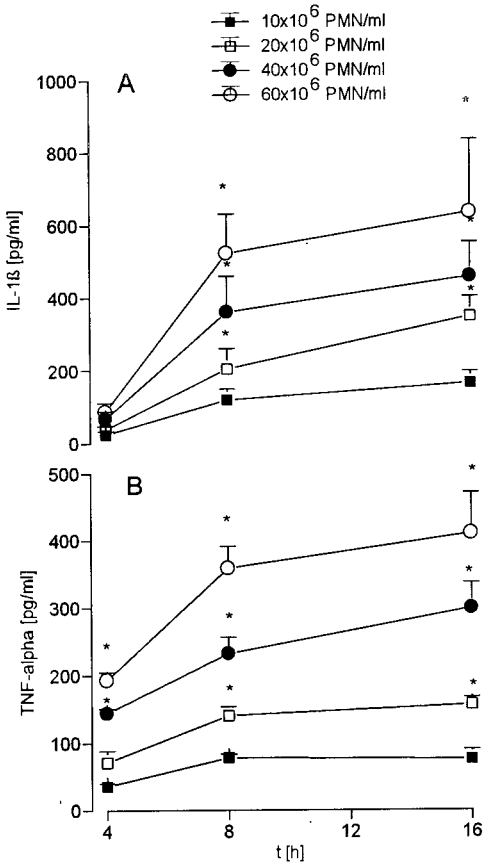

FIGURE 3. Effect of cell density on neutrophil IL-1β and TNF-α synthesis in response to LPS. Increasing concentrations of neutrophils (10×10^6 PMN/ml to 60×10^6 PMN/ml) were challenged with LPS (10 ng/ml). At indicated time points, reactions were stopped, and cell supernatants were collected. IL-1β (*A*) and TNF-α (*B*) are given in picograms per milliliter. Means ± SEM of at least four independent experiments are shown. Values marked differ significantly from corresponding values with 10×10^6 PMN/ml.

stimulation, was 75.6 ± 14.3 pg/ml from 10×10^6 PMN/ml, and increased to 410.3 ± 60.6 pg/ml in 60×10^6 PMN/ml (Fig. 3*B*), corresponding to a TNF-α liberation of 75.6 ± 14.3 pg/10×10^6 PMN cultured at the lowest cell density, and 68.4 ± 10.1 pg/10×10^6 PMN kept in the highest cell density.

Down-regulation of neutrophil IL-8 synthesis at high cell density is mediated by a soluble mediator arising in the cell supernatant

Next, we investigated whether the selective down-regulation of neutrophil IL-8 generation was caused by direct cell-to-cell interactions, or was due to a soluble mediator arising in the cell supernatant of neutrophils cultured at high cell density. Inhibition of β_2 integrin-dependent adhesion with the function-blocking Ab MHM23, targeting the β-chain of LFA-1, did not restore neutrophil IL-8 generation at high cell density (Table II), indicating that integrin-mediated cell-to-cell interactions were not underlying neutrophil down-regulation. Therefore, a transfer of the putative down-regulatory mediator derived from neutrophils at high cell density was undertaken to neutrophils at constant density. For this purpose, constant concentrations of neutrophils (10×10^6 PMN/ml) were stimulated for 8 h with LPS in conditioned medium derived from neutrophils (originating from the same donor) cultured at the varying cell densities (10×10^6–60×10^6 PMN/ml). Conditioned media were obtained after an incubation period with 10 ng/ml LPS for 8 h. As depicted in Fig. 4, inhibition of IL-8 secretion was reproduced with conditioned media from neutrophils cultured at high cell density: when stimulated in conditioned medium derived from neutrophils at 60×10^6/ml, IL-8 release of 10×10^6 PMN was reduced to ~20% of that upon use of conditioned medium from PMN at the lowest cell density (431 ± 33 pg IL-8/10×10^6 PMN cultured in conditioned medium from 60×10^6 PMN/ml vs 1808 ± 220 pg IL-8/10×10^6 PMN cultured in conditioned medium from 10×10^6 PMN/ml).

Table II. *Lack of impact of other neutrophil-derived metabolites on the down-regulation of IL-8 synthesis with increasing cell density*[a]

	IL-8 Secretion (pg/ml)	
	10×10^6 PMN/ml	60×10^6 PMN/ml
LPS, 10 ng/ml	3497.5 ± 443.3	686.2 ± 77.6
+ anti-CD18	4435 ± 887.3	804.9 ± 103.3
+ indomethacin	4028 ± 543.2	743 ± 82.5
+ MK-886	3884.2 ± 302	633.9 ± 92.3
+ CV-6209	3158.4 ± 646.9	596.4 ± 102
+ α_1AT	4224 ± 702.3	776.6 ± 89.9
+ SOD	2996.3 ± 334	546.1 ± 64
+ ADO	3648.8 ± 602.6	702 ± 119.3
LPS, 1 μg/ml	8338.3 ± 995	2525.4 ± 366.3
LPS, 0.1 ng/ml	886 ± 102	182.5 ± 30.3

[a] Neutrophils were incubated with LPS (10 ng/ml) at different cell densities (10×10^6 and 60×10^6 PMN/ml) in the absence or presence of the anti-CD18 Ab MHM23 (50 μg/ml), the cyclooxygenase inhibitor indomethacin (10 μM), the 5-LO inhibitor MK-886 (10 μM), the PAF receptor antagonist CV-6209 (10 μM), the protease inhibitor α_1AT (100 μg/ml), the O_2^- scavenger SOD (100 U/ml), or the adenosine-degrading enzyme ADA (400 U/ml). No significant differences were obtained between untreated or pharmaco-treated groups. Additionally, neutrophils were incubated with 1 μg/ml LPS or 0.1 ng/ml LPS at different cell densities (10×10^6 and 60×10^6 PMN/ml). At both LPS concentrations, IL-8 release from 60×10^6 PMN/ml was significantly reduced compared with 10×10^6 PMN/ml.

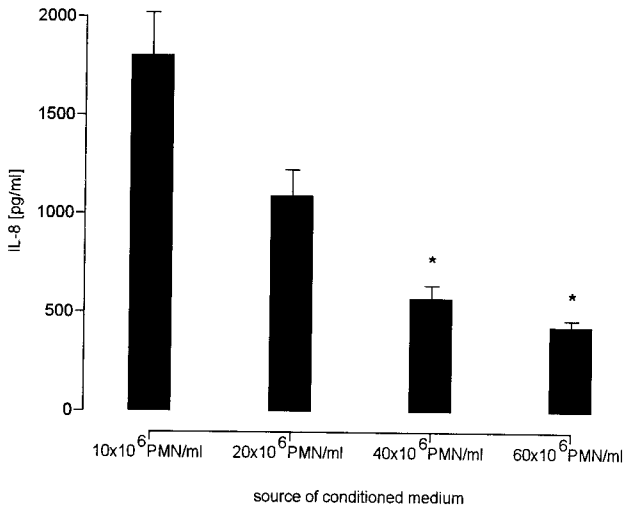

FIGURE 4. Effect of conditioned medium from neutrophils cultured at increasing cell density on LPS-induced IL-8 release from a separate set of neutrophils. Conditioned medium was obtained by incubating neutrophils at increasing cell density (10×10^6 PMN/ml to 60×10^6 PMN/ml) for 8 h with 10 ng/ml LPS. Constant PMN concentrations (10×10^6 PMN/ml) of the same donor were then stimulated with LPS (10 ng/ml) in the presence of the respective conditioned media for an additional 8 h. Cell supernatants were collected, and IL-8 release was determined separately for the conditioned media, and for the additional 8-h stimulation of 10×10^6 PMN in the respective media. By calculating the difference, this figure gives the IL-8 release of 10×10^6 PMN/ml stimulated in the conditioned media for 8 h. Means ± SEM of at least three different experiments are shown. Values marked differ significantly from neutrophils cultured in conditioned medium from 10×10^6 PMN/ml.

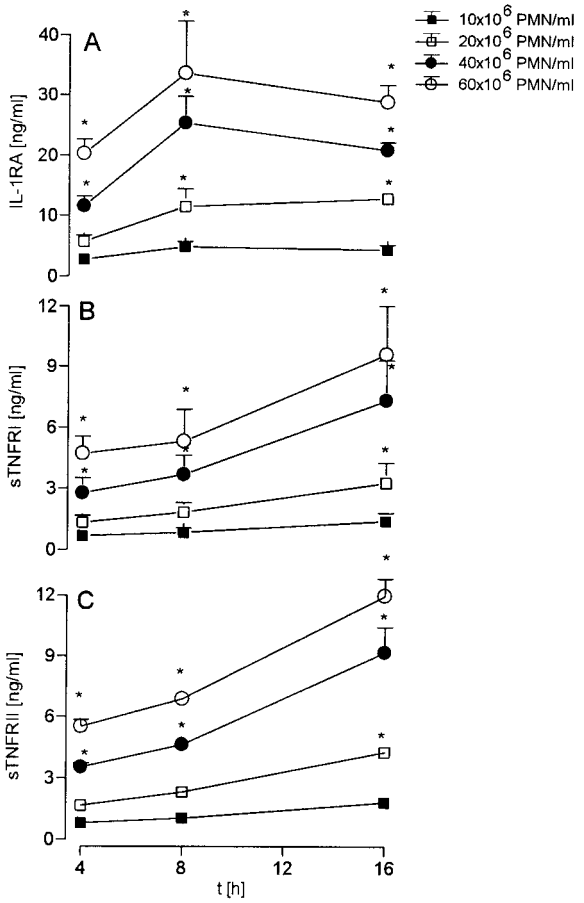

FIGURE 5. Accumulation of IL-1RA and sTNFRs in the supernatants of LPS-treated neutrophils upon increase in cell density. Increasing concentrations of neutrophils (10×10^6 PMN/ml to 60×10^6 PMN/ml) were challenged with LPS (10 ng/ml). At indicated time points, reactions were stopped, and cell supernatants were collected. IL-1RA (*A*), sTNFRI (*B*), and sTNFRII (*C*) are given in ng/ml. Means ± SEM of at least four independent experiments are shown. Values marked differ significantly from corresponding values with 10×10^6 PMN/ml.

Down-regulation of neutrophil IL-8 generation at high cell density is mediated by IL-1RA and sTNFRs

To identify the soluble factor(s) involved in the down-regulation of neutrophil IL-8 generation at high cell density, cell supernatants were analyzed for potentially inhibitory cytokines or cytokine antagonists. While the antiinflammatory cytokines IL-4, IL-10, and IL-13 were not detected (data not given), excessive quantities of IL-1RA (>30 ng/ml for 60×10^6 PMN/ml) and both types of soluble TNFRs (>10 ng/ml for 60×10^6 PMN/ml), rising dramatically upon increase in cell density, were recovered from LPS-treated neutrophils (Fig. 5).

As LPS-induced IL-8 formation in neutrophils is largely dependent on endogenously formed TNF-α and IL-1β (20), we speculated that down-regulation of neutrophil IL-8 generation was due to blockage of this autocrine loop by IL-1RA and sTNFRs. Indeed, when blocking the bioactivity of these mediators, LPS-induced synthesis of IL-8 was restored, rising proportionally with increasing cell density: as depicted in Fig. 6, combined application of neutralizing Abs (20 μg/ml) targeting IL-1RA and both types of soluble TNFRs was the most efficient approach, provoking a ~20-fold increase in IL-8 liberation from neutrophils cultured at the highest cell density (12,024 ± 1,028 pg/ml in the presence of neutralizing Abs vs 572 ± 36.4 pg/ml in the absence of neutralizing Abs). Sole neutralization of IL-1RA induced a ~15-fold increase in IL-8 liberation from 60×10^6 PMN/ml, while sole inhibition of sTNFR bioactivity was less efficient, but still resulted in the liberation of 4,323 ± 663 pg/ml IL-8 from 60×10^6 PMN vs 572 ± 36.4 pg/ml IL-8, thus corresponding to a ~7.5-fold increase. Application of neutralizing Abs per se in the absence of LPS challenge did not provoke neutrophil IL-8 release (data not given).

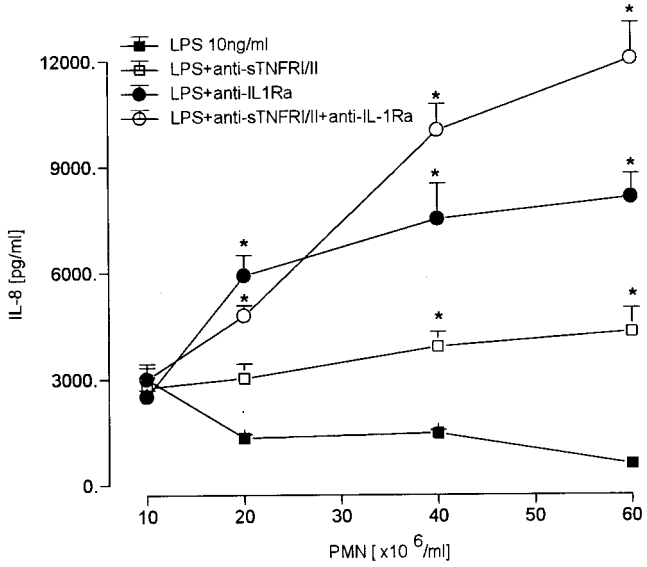

FIGURE 6. Effect of neutralization of IL-1RA and sTNFRs on the down-regulation of LPS-induced neutrophil IL-8 generation upon increase in cell density. Increasing concentrations of neutrophils (10×10^6 PMN/ml to 60×10^6 PMN/ml) were challenged with LPS (10 ng/ml) in the absence or presence of neutralizing Abs targeting IL-1RA, sTNFRI, or sTNFRII. All Abs were used at 20 μg/ml. Reactions were stopped after 16 h, and cell supernatants were collected. IL-8 is given in pg/ml. Means ± SEM of at least four independent experiments are shown. Values marked differ significantly from corresponding values without neutralizing Abs.

Exogenous IL-1RA and sTNFRs suppress LPS-induced IL-8 release from neutrophils at constant cell density

To further prove the inhibitory effect of IL-1RA and sTNFRs on LPS-induced neutrophil IL-8 release, these mediators were added to neutrophils at constant concentrations (10×10^6 PMN/ml) exposed to LPS challenge, with the amounts of exogenous IL-1RA (40 ng/ml) and sTNFRs (20 ng/ml) corresponding to the levels of these mediators secreted from neutrophils at high cell density. As depicted for IL-8 release after 16 h of LPS challenge, combined application of IL-1RA and both types of soluble TNFRs resulted in a most impressive inhibition of LPS-induced IL-8 generation, decreasing from 3585.3 ± 522.6 pg/ml IL-8 in the absence of cytokine antagonists to 1003.3 ± 206.6 pg/ml in their presence (Fig. 7). As anticipated from the studies with function-blocking Abs, sole application of IL-1RA exerted more efficient inhibition of IL-8 release than sole sTNFR challenge.

Control experiments

In separate experiments, we addressed further neutrophil-derived mediators concerning a putative role in the down-regulation of IL-8 generation upon increase in cell density. Although the cyclooxygenase product PGE_2 was continuously liberated from neutrophils upon increase in cell density (data not given in detail), application of the cyclooxygenase inhibitor indomethacin (10 μM) did not restore chemokine synthesis at high cell density. Moreover,

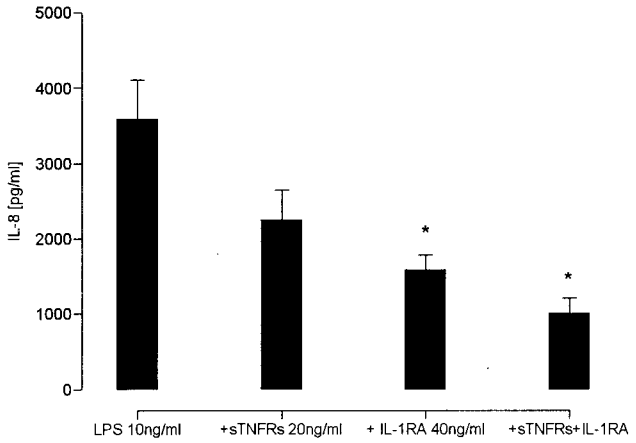

FIGURE 7. Effect of exogenous IL-1RA and sTNFRs on LPS-induced IL-8 release from neutrophils incubated at constant cell density. Constant concentrations of neutrophils (10×10^6 PMN/ml) were challenged with LPS (10 ng/ml) in the absence or presence of exogenous IL-1RA, sTNFRs, or a combination of IL-1RA and sTNFRs. IL-1RA was applied at 40 ng/ml, while each sTNFR was given at 20 ng/ml. Reactions were stopped after 16 h, and cell supernatants were collected. IL-8 is given in pg/ml. Means ± SEM of at least three independent experiments are shown. Values marked differ significantly from mono-LPS-stimulated PMN.

interference with neutrophil 5-LO metabolism by using the specific inhibitor MK-886 (10 μM), or inhibition of PAF activity by the receptor antagonist CV-6209 (10 μM) was ineffective. Furthermore, neither scavenging oxygen radicals with SOD (100 μM), nor antagonizing neutrophil proteases with α_1AT (100 μg/ml) restored neutrophil IL-8 synthesis, and a potential inhibitory role for endogenous adenosine was excluded by addition of appropriate concentrations of ADA (400 U/ml). Also upon stimulation of neutrophils with higher (1 μg/ml) or lower (0.1 ng/ml) LPS concentrations, a down-regulation of IL-8 generation was observed. All data are given in Table II.

Discussion

The present study focused on mechanisms that control the recruitment and activation of polymorphonuclear neutrophils. Microenvironmental conditions may be of major importance in this aspect, and some very recent studies demonstrated that upon contact of neutrophils with physiological matrices, e.g., adherence to endothelial cells, inflammatory neutrophil functions are profoundly modulated (10, 21, 22). The main finding of the present study is that neutrophil density per se represents an important variable controlling phagocyte inflammatory activity. Upon increase in neutrophil density, a dramatic down-regulation of LPS-induced IL-8 generation was noted. In contrast, TNF-α and IL-1β secretion were not inhibited by the cell density. Supernatant transfer experiments suggested that the down-regulation of IL-8 secretion was attributable to secreted factor(s), and these were identified as IL-1RA and sTNFRs by immunodetection, by experiments with specific blocking, and by studies employing exogenous IL-1RA and sTNFRs.

Enhancing neutrophil density, achieved by increasing the total number of neutrophils in a given volume, exerted a profound influence on the LPS-induced liberation of the CXC chemokine IL-8, which decreased to <10% per single neutrophil at the highest (60×10^6 PMN/ml) vs the lowest (10×10^6 PMN/ml) cell density. As intracellular levels of IL-8 were equally diminished, the down-regulation of IL-8 was obviously not due to reduced liberation, but was caused by reduced de novo synthesis of the chemokine. Well in line with this reasoning, a corresponding decrease in IL-8 message was obtained for the highest neutrophil density (60×10^6 PMN/ml) when analyzed by quantitative RT-PCR. As in 20×10^6 PMN/ml, a significant decrease in IL-8 mRNA could not be detected; a posttranscriptional mechanism may additionally be involved in the regulation of IL-8 synthesis at that cell density. Importantly, any debris possibly present in the cell supernatant of more densely cultured neutrophils did not affect the determination of IL-8 in the currently used ELISA system, as assessed in separate recovery experiments. Moreover, the reduced IL-8 generation was unlikely to be an unspecific toxic effect of the high neutrophil density, as release of lactate dehydrogenase was consistently below 5% of the total cellular content, and as other neutrophil metabolic properties demanding cellular integrity, such as TNF-α and IL-1β formation, were unaffected by PMN density.

When attempting to elucidate the mechanism(s) involved in the down-regulation of chemokine generation upon increase in cell density, supernatant transfer experiments clearly indicated that a soluble agent arising from the densely cultured neutrophils was responsible for the suppression of IL-8 generation. Neutrophil IL-8 generation was previously shown to be regulated by a wide variety of soluble agonists. Among the cytokines, IL-4, IL-10, IL-13, and IFN-γ (23–25) have been implicated in inhibition of LPS-induced IL-8 formation. However, these cytokines were not detected in the cell supernatants of LPS-treated neutrophils at any cell density; therefore, an inhibitory role for these agonists could be excluded for the present experimental system. Interestingly, IL-10 was found in the lysates of neutrophils, but was not liberated, and addition of neutralizing anti-IL-10 Abs did not restore neutrophil chemokine generation (data not given). Besides antiinflammatory cytokines, prostanoids have been shown to down-regulate neutrophil IL-8 generation (26). Although PGE_2 was continuously secreted from neutrophils at increasing cell densities, the cyclooxygenase inhibitor indomethacin had no effect on IL-8 generation. Moreover, interference with other neutrophil-derived mediators, namely 5-LO-metabolites, PAF, proteases, and reactive oxygen species, was ineffective. Endogenous adenosine, an autacoid known to accumulate in neutrophil suspensions (27), has recently been implicated in the regulation of a variety of neutrophil functions, including cytokine generation (28), and was thus considered as a further candidate contributing to the presently observed down-regulation of neutrophil IL-8 generation. However, the lack of effect of ADA, added at high concentrations to neutrophil suspensions, seriously questions a role of endogenous adenosine in the present setup.

Compelling evidence was, however, provided that the inhibition of IL-8 synthesis was centrally linked to IL-1RA and soluble TNFRs, secreted progressively upon neutrophil culturing in high cell densities. First, excessive quantities of IL-1RA (>30 ng/ml) and sTNFRs (>10 ng/ml), rising in parallel with the increase in cell density, were recovered from the supernatants of LPS-treated PMN. Second, blocking the bioactivity of these mediators by application of neutralizing Abs completely restored chemokine generation under conditions of high neutrophil density, thus resulting in a proportional increase in IL-8 with increasing cell density. These studies with function-blocking Abs suggested that the different cytokine antagonists synergized in inhibiting IL-8 release, with IL-1RA being a more efficacious agent than the soluble TNFRs, as antagonizing sole IL-1RA activity provoked a ~15-fold increase in IL-8 secretion from 60×10^6 PMN, vs a ~7.5-fold increase in IL-8 observed after neutralizing sTNFRs. And third, exogenous addition of recombinant IL-1RA and sTNFRs, in concentrations comparable with those detected in the supernatants of LPS-treated neutrophils at high cell density, reproduced the suppression of LPS-induced IL-8 liberation, thus confirming the key

role previously suggested for TNF-α and IL-1β as autocrine facilitators of LPS-induced formation of IL-8 (20, 29). Thus, at high neutrophil cell density, this autocrine loop is apparently blocked, as although IL-1β and TNF-α are present, their cell surface receptor occupancy is competitively inhibited by IL-1RA and sTNFRs.

IL-1RA was produced in an ~80-fold excess over IL-1β, and sTNFRs were in ~40-fold excess over TNF-α in populations with high neutrophil density. Such an excess over the respective agonists is well known to be necessary to block the physiological actions of IL-1β and TNF-α (8, 9), probably due to decreased affinity of IL-1RA to IL-1Rs as compared with the agonist IL-1β, and due to preferred engagement of cell surface TNFRs by circulating TNF-α. Hence, in 10×10^6 PMN/ml, function-blocking Abs targeting IL-1RA and sTNFRs did not amplify LPS-induced neutrophil IL-8 generation (Fig. 6), indicating that this regulatory loop was only operative when a certain excess of the cytokine antagonists over the respective agonists was given, just as occurring in high neutrophil cell density. In vivo, such excessive amounts of neutrophil-derived cytokine antagonists should not only down-regulate inflammatory neutrophil functions, but exert an overall anti-inflammatory effect on various cellular immune effector cells. In this respect, release of sTNFR p75 by neutrophils has recently been postulated to be implicated in the regulation of TNF bioactivity in vivo (30), and exogenous application of IL-1RA and sTNFRs has been shown to reduce the mortality of endotoxin shock in numerous animal models (31–33).

Neutrophils represent the first line of defense against infections or nonself agents, and a wide variety of systemic mediators was shown to regulate neutrophil activity. In the present study, we found that the neutrophil density per se represents a critically variable-limiting excessive neutrophil activation. IL-1RA and soluble TNFRs, accumulating massively under conditions of high neutrophil density, are centrally involved in this regulatory mechanism by interfering with the IL-1β- and TNF-α-dependent amplification of IL-8 generation. As a result, IL-8 synthesis and release are dramatically reduced at high neutrophil density. This negative feedback mechanism is to be assumed to result in the inhibition of further neutrophil recruitment and activation in a neutrophil-rich microenvironment, thereby contributing to the control of inflammatory functions in vivo.

Acknowledgments

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Anlage 6

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Anti-proteinase 3 antibodies (c-ANCA) prime CD14-dependent leukocyte activation.

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Anti-proteinase 3 antibodies (c-ANCA) prime CD14-dependent leukocyte activation

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Abstract: In Wegener's granulomatosis (WG), a pathogenetic role has been proposed for circulating anti-neutrophil-cytoplasmic antibodies (ANCA) targeting proteinase 3 (PR3). Disease activation in WG appears to be triggered by bacterial infections. In the present study, we characterized the effect of anti-PR3 antibodies on in vitro activation of isolated monocytes and neutrophils by the bacterial cell-wall components lipopolysaccharide (LPS) and lipoteichoic acid (LTA). Although sole incubation of monocytes and neutrophils with monoclonal anti-PR3 antibodies induced the release of minor quantities of the chemokine interleukin-8 (IL-8), preincubation with anti-PR3 antibodies, but not with isotype-matched control immunogloblin G (IgG), resulted in a markedly enhanced IL-8 liberation upon LPS challenge. The priming response was evident after 2 h of preincubation with anti-PR3 and peaked after 6 h. The anti-PR3-related priming was also observed for tumor necrosis factor α (TNF-α) and IL-6 synthesis. Comparable priming occurred when leukocytes were preincubated with ANCA-IgG derived from WG serum but not with normal IgG. The priming effect of the anti-PR3 antibody pretreatment was reproduced for LTA challenge of monocytes and neutrophils but not for leukocyte stimulation with TNF-α. Flow cytometric analysis revealed an increase in monocyte and neutrophil membrane CD14 expression during the anti-PR3 priming. We conclude that cytoplasmic ANCA specifically prime CD14-dependent monocytes and neutrophils for activation. The resulting enhanced responsiveness to bacterial pathogens may contribute to the development and maintenance of inflammatory lesions during active WG. *J. Leukoc. Biol.* 78: 992–1000; 2005.

Key Words: *monocytes/macrophages · neutrophils · autoantibodies · cell surface molecules · cytokines*

INTRODUCTION

Wegener's granulomatosis (WG) is characterized by granulomatous inflammation of the upper and lower respiratory tract and necrotizing vasculitis, primarily involving the kidneys. The vasculitic lesions are characterized by an early phase of neutrophil accumulation, followed by a monocytic infiltrate [1, 2]. As the degree of leukocyte activation was found to correlate with the extent of the disease [3–5], activated neutrophils and monocytes appear to be the major effector cells in the development of inflammatory lesions in WG [6].

Is is proposed that disease activation is triggered by bacterial infections, as the onset of symptoms peaks in the winter [7], and antibiotics have a beneficial effect in the treatment of refractory WG [8, 9]. Moreover, chronic nasal carriage of *Staphylococcus aureus* is associated with higher relapse rates of the disease [10]. The immunostimulatory cell-wall components of *S. aureus* include lipoteichoic acid (LTA), a macroamphiphile found in the cell membranes of virtually all Gram-positive bacteria [11], whereas lipopolysaccharides (LPS) are well-characterized, pathogenic factors of Gram-negative bacteria. LPS and LTA are capable of activating a wide variety of inflammatory functions in neutrophils and monocytes, including the induction of adhesion, the activation of the respiratory burst, and cytokine secretion [12–15]. Cellular activation by LPS and LTA is largely mediated by ligation of the CD14 molecule [16, 17], a glycosylphosphatidylinositol (GPI)-linked membrane protein, and involves the activation of Toll-like receptors (TLRs) [18, 19]. Whether an inadequate or overwhelming inflammatory response to invading microorganisms is responsible for the association between infection and disease exacerbations in WG or whether these bacterial cell-wall components directly stimulate activation of autoreactive B- and T-lymphocytes, thus further triggering the autoimmune process, remains unclear.

An established association does, however, exist between the occurrence of anti-neutrophil-cytoplasmic antibodies (ANCA), especially those targeting proteinase 3 (PR3), and the development of active WG [20]. The PR3 ANCA are reported to be causally involved in the pathogenesis of the disease, as the autoantibody titer correlates with the disease activity in vivo

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[21], and ANCA directly activate a wide variety of inflammatory functions in neutrophils in vitro, such as secretion of oxygen radicals, proteases, and lipid mediators, once the autoantigen PR3 is expressed on the leukocyte cell surface [22–25] under inflammatory conditions. Additionally, in isolated monocytes, anti-PR3 antibodies stimulate the release of proinflammatory cytokines [26, 27].

In the present study, we focused on the interaction between anti-PR3 antibodies and bacterial cell-wall components in leukocyte activation. We used LPS as the major immunostimulatory component of Gram-negative and LTA as a prototype of Gram-positive cell-wall components. Both agents are activators of leukocyte interleukin-8 (IL-8) generation [13, 15], which is pertinent, as a pathogenetic role has recently been attributed to this CXC chemokine in ANCA-associated glomerulonephritis [28]. Dual stimulation with anti-PR3 and LPS as well as LTA was performed. It is most interesting that the WG autoantibody caused a strong priming of the leukocyte cytokine response triggered upon subsequent stimulation with both prototype bacterial cell-wall components, and an up-regulation of membrane CD14 was proposed as one underlying mechanism.

MATERIALS AND METHODS

Materials

Ficoll-Paque and Protein G Sepharose were purchased from Pharmacia (Uppsala, Sweden), fetal calf serum (FCS) was from Greiner (Frickenhausen, Germany), and all media and supplements were from Gibco (Eggenstein, Germany), unless otherwise indicated. LPS *(Escherichia coli*, 0111:B4), fluorescein isothiocyanate (FITC)-conjugated LPS, polymyxin B (PMB) immobilized on agarose, and isotype control mouse immunoglobulin G_1 (IgG_1c; MOPC-21) were from Sigma (Deisenhofen, Germany). Antibodies used for tumor necrosis factor α (TNF-α), IL-6, and IL-8 enzyme-linked immunosorbent assay (ELISA; MAB 208; BAF 208) as well as recombinant human cytokines were purchased from R&D Systems (Wiesbaden, Germany). Peroxidase-conjugated streptavidin [horseradish peroxidase (HRP)] and 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate; ABTS) were from Zymed Laboratories (San Francisco, CA). The murine monoclonal anti-CD-14 antibody (MY4) was from Coulter Immunotech (Hamburg, Germany), and the phycoerythrin (PE)-labeled anti-CD14 antibody LeuM3 and the PE/FITC-labeled irrelevant control antibodies (Simultest IgG2ac/IgG1) were from Becton Dickinson (Heidelberg, Germany). PE-labeled anti-human TLR2 (clone TL2.1) and anti-TLR4 (clone HTA25) were purchased from eBioscience (San Diego, CA). Pooled human IgG (Octagam) was obtained from Octapharma (Langenfeld, Germany), and the *Limulus* amebocyte cell lysate (LAL) test for the detection of endotoxin was from Chromogenix (Mölndal, Sweden). Cell culture plasticware was purchased from Falcon (Mannheim, Germany). All other chemicals were from Merck (Darmstadt, Germany).

Purification of LTA from *S. aureus*

LTA was purified by a structure-preserving isolation procedure as described [29]. Briefly, the classic phenol-water extraction at 68°C and subsequent dialysis were replaced by butanol extraction at room temperature and lyophilization. *S. aureus* (DSM 20233) was grown in a 42 L fermenter and harvested by centrifugation at 4°C. The pelleted bacteria were sonicated (Branson, Danbury, CT) on ice or disrupted with a cell mill (Büchi, Uster, Switzerland). LTA was negative for endotoxins in the chromogenic LAL assay; i.e., the LAL reactivity was lower than that for 1 pg LPS/ml.

Anti-PR3 antibodies

Murine monoclonal antibodies targeting PR3 were prepared by hybridoma technology, as described previously [30]. The clone WGM_2 (IgG_1) was chosen for further experiments. Cytoplasmic (c)-ANCA were purified from the sera of patients with monospecific, anti-PR3-positive WG by adsorption on a protein G column, as described [31], and human IgGc was prepared similarly using sera from five healthy volunteers. The IgG concentration of the eluates was determined according to standard procedures. PR3 specificity of the monoclonal and serum-derived antibodies was assessed by antigen-specific ELISA. Using the kinetic-OLC LAL test, endotoxin contamination of the murine antibody WGM_2 and human anti-PR3 antibodies was below 15 pg/ml. The monoclonal anti-PR3 antibody 12.8 was purchased from Research Diagnostics (Flanders, NY) and was applied to a polymyxin-agarose column to remove contaminating endotoxin. After PMB treatment, endotoxin contamination of antibody preparation was below 15 pg/ml.

Isolation of neutrophils

Neutrophils were isolated from venous blood of healthy donors by centrifugation over a Ficoll-Paque gradient as described previously [23]. In brief, EDTA-anticoagulated blood was layered over Ficoll-Paque and centrifuged at 400 *g* for 35 min. After removal of mononuclear cells, erythrocytes were sedimented in 10% polyvinyl alcohol. Residual erythrocytes were removed by hypotonic lysis, and cells were washed twice in Ca^{++}/Mg^{++}-free phosphate-buffered saline (PBS) and finally, resuspended in RPMI containing 1% FCS at 10×10^6 polymorphonuclear neutrophil/ml. Cell purity was >97%, as quantified by flow cytometry, and cell viability was >96%, as assessed by trypan blue dye exclusion.

Isolation of monocytes

Monocytes were isolated by countercurrent centrifugal elutriation from leukocyte-enriched buffy coats, kindly provided by the local blood bank. Initially, peripheral blood mononuclear cells were separated by density gradient centrifugation on Ficoll-Paque gradients (400 *g*, 35 min). Then, monocytes were purified by elutriation in a Beckmann centrifugal elutriator (JE-5.0 elutriation system). Purity was determined by fluorescence-associated cell sorting, and only fractions containing ≥90% monocytes, <1% granulocytes, and <10% lymphocytes were used for experiments. Cell viability was always > 97%, as assessed by the trypan blue dye exclusion test.

Cell culture and stimulation

Monocytes and neutrophils were resuspended in RPMI supplemented with 1% FCS, plated in 24-well tissue-culture plates at 10^6/ml (monocytes) or 10^7/ml (neutrophils), each at 500 μl/well, and incubated at 37°C in a 5% CO_2-humidified atmosphere. To induce surface expression of PR3 on neutrophils, cells were stimulated with TNF-α (2 ng/ml) for 30 min, whereas isolated monocytes were constitutively expressing surface PR3. Prior to stimulation with LPS (10 ng/ml), LTA (1 μg/ml), or TNF-α (10 ng/ml), cells were preincubated with media alone, with murine monoclonal anti-PR3 antibodies (1 μg/ml), or with purified c-ANCA IgG (100 μg/ml), originating from WG serum for various time periods (2–12 h). Mouse IgG_1 isotype control (1 μg/ml) and normal human IgG (100 μg/ml) from healthy donors were used as control antibodies. In experiments designed to investigate the effects of anti-CD14 antibodies on agonist-induced IL-8 generation, the antibody MY-4 (5 μg/ml) was added simultaneously with LPS, LTA, or TNF-α. After 18 h of incubation, cell supernatants were harvested, debris was removed by centrifugation, and samples were stored at –20°C until further processing.

Cytokine ELISAs

Release of TNF-α, IL-6, and IL-8 was determined in a direct sandwich ELISA. In brief, immunoassay plates were coated with mouse monoclonal anti-human TNF-α, IL-6, or IL-8 antibodies at a concentration of 4 μg/ml. After a blocking period, samples were added. Recombinant human TNF-α, IL-6, and IL-8 were used for standard titration curves. To sandwich the antigen, biotinylated antibodies were applied at the following concentrations: 400 ng/ml polyclonal anti-TNF-α, 50 ng/ml anti-IL-6, or 40 ng/ml anti-IL-8. Plates were subsequently incubated with HRP-conjugated streptavidin followed by addition of substrate (H_2O_2 and ABTS). Absorbance was measured at 450 nm in a microplate reader using SLT LabInstruments software (Creilsheim, Germany) to analyze the generated data. IL-8 and IL-6 ELISA were sensitive to 15 pg/ml and TNF-α to 7 pg/ml.

Flow cytometry

For the determination of CD14, TLR2, and TLR4 surface expression, flow cytometry was performed. In brief, after incubation with anti-PR3 antibodies or IgGc, leukocytes were washed twice in ice-cold PBS containing 0.1% bovine serum albumin and 0.02% sodium acid. Then, 2×10^5 cells were distributed to each well of flexible round-bottom microtiter plates. Prior to the addition of a PE-labeled monoclonal anti-CD-14 antibody (LeuM3, 10 μg/ml), PE-labeled antibodies targeting TLR2 (TL2.1; 20 μg/ml) or TLR4 (HTA 25; 20 μg/ml), FITC-labeled LPS, or equal concentrations of the respective isotype-matched control antibodies, 20 μl pooled human Ig (100 μg/ml), were added to block leukocyte Fc_{IgG} receptors. As a negative control, incubation with a PE/FITC-labeled irrelevant antibody was performed. After 30 min of incubation with the specific antibodies or LPS, three washes were performed, and cells were resuspended in PBS and kept on ice until flow cytometric analysis, which was performed on a FACScan (Becton Dickinson, Mountain View, CA) using forward and orthogonal light-scatter to select viable cells. CellQuest® research software (Becton Dickinson, Mountain View, CA) was used to analyze the generated data.

Statistics

For statistical comparison, one-way ANOVA was performed, followed by Tukey's honestly significant difference test when appropriate. A level of $P < 0.05$ was considered to be significant.

RESULTS

Anti-PR3 antibodies amplify LPS-induced, proinflammatory cytokine secretion in monocytes and neutrophils

Incubation of isolated monocytes and neutrophils with various concentrations of murine monoclonal anti-PR3 antibodies (WGM_2; 0.01–10 μg/ml) for 24 h resulted in a dose-dependent release of IL-8 into the cell supernatants of monocytes and neutrophils, and equal concentrations of an isotype-matched mouse IgGc were ineffective (**Fig. 1**). However, when compared with leukocyte activation by 10 ng/ml LPS (111.62±23.13 ng/ml IL-8 for monocytes and 7.11±1.89 ng/ml IL-8 for neutrophils), the quantities of IL-8 formation elicited, even by treatment with high concentrations of anti-PR3 antibodies (10 μg/ml), were comparatively low (25.3±4.6 ng/ml IL-8 for monocytes and 1.1±0.22 ng/ml IL-8 for neutrophils).

When leukocytes were preincubated with 1 μg/ml WGM_2 for 6 h and subsequently challenged with LPS (10 ng/ml) for a further 18 h, a massive amplification of LPS-induced IL-8 formation was noted in monocytes and neutrophils (**Fig. 2**). The anti-PR3-elicited priming could be reproduced by a second monoclonal anti-PR3 antibody (12.8) targeting a different epitope of the autoantigen but not by an isotype-matched mouse IgGc.

Apart from amplification of leukocyte IL-8 secretion, a comparable priming effect for monocyte TNF-α and IL-6 release occurred in anti-PR3-pretreated cells (**Fig. 3**). In fact, release of TNF-α increased from 4.2 ± 0.95 ng/ml to 9.0 ± 3.6 ng/ml in WGM_2- and to 12.3 ± 1.4 ng/ml in 12.8-pretreated cells in response to LPS stimulation (n=4). In contrast, both anti-PR3 antibodies elicited the release of only low quantities of this proinflammatory cytokine in the absence of LPS (0.25±0.03 ng/ml for WGM_2 and 0.43±0.09 ng/ml for 12.8, n=4). Comparable results were obtained for IL-6: The LPS-induced synthesis of this cytokine was increased from 34 ± 0.7 ng/ml to 82.9 ± 1.3 ng/ml upon priming with WGM_2 and to 95.4 ± 3.7 ng/ml upon priming with 12.8 (n=4), and IL-6 release induced by the anti-PR3 antibodies per se was low (1.25±0.34 ng/ml for WGM_2 and 1.86±0.24 ng/ml for 12.8, n=4). These data again support the concept of primed leukocyte behavior upon anti-PR3 challenge.

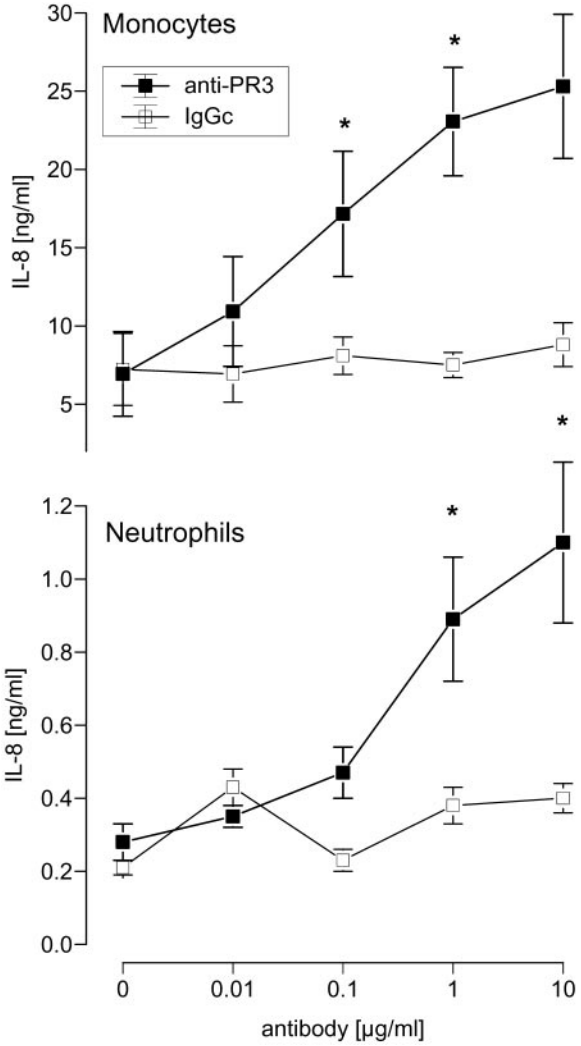

Fig. 1. Dose-dependent release of IL-8 from leukocytes in response to anti-PR3 antibodies. Isolated monocytes (0.5×10^6/ml) or neutrophils (5×10^6/ml) were exposed to various concentrations of monoclonal anti-PR3 antibodies (WGM_2, anti-PR3; 0.01–10 μg/ml) or equal concentrations of isotype-matched IgGc (MOPC-21) for 24 h, or sham incubation was performed. IL-8 released into the supernatant is given in ng/ml. Data reflect mean ± SEM of at least three independent experiments. Values marked * differ significantly from IgGc-incubated leukocytes.

When leukocytes were preincubated with three different human ANCA-IgG (100 μg/ml) preparations, purified from the sera of patients with monospecific anti-PR3-positive WG, amplification of LPS-induced IL-8 generation was reproduced (**Fig. 4**), and normal human IgG was ineffective. Similar to monoclonal anti-PR3 antibodies, treatment with ANCA IgG per se elicited the release of some minor quantities of IL-8 from monocytes (20.2±6.8 ng/ml) and neutrophils (1.1±0.33 ng/ml), thus confirming that the increase in LPS-induced IL-8

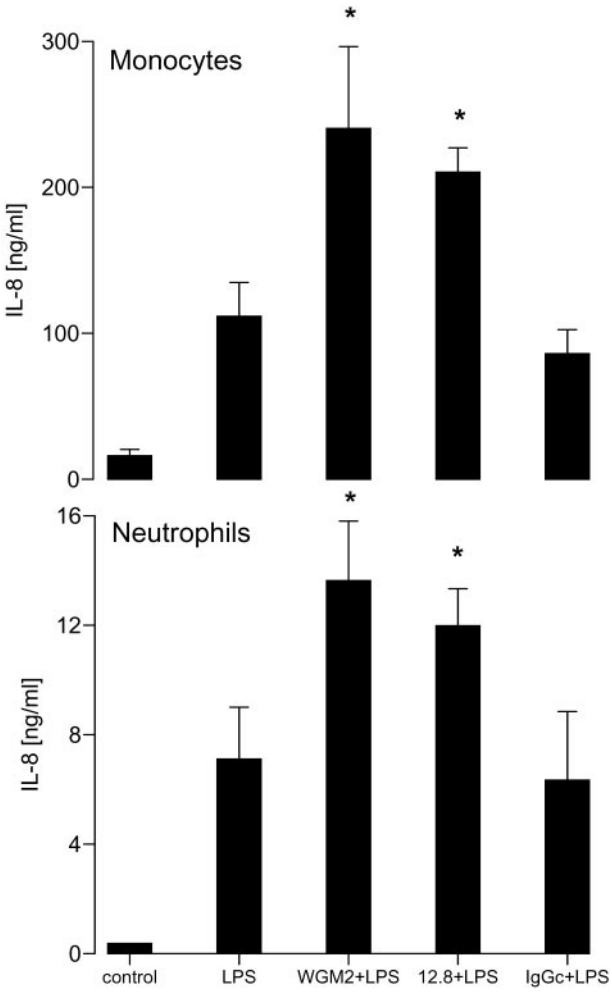

Fig. 2. Amplification of LPS-induced IL-8 release after preincubation with anti-PR3 antibodies. Isolated monocytes (0.5×10^6/ml) or neutrophils (5×10^6/ml) were preincubated for 6 h with two different monoclonal anti-PR3 antibodies (WGM_2 or 12.8; 1 μg/ml) with equal amounts of an isotype-matched IgGc, or sham incubation was performed (control). Subsequently, leukocytes were challenged with LPS (10 ng/ml) for 18 h. IL-8 released into the supernatant is given in ng/ml. Data reflect mean ± SEM of at least five independent experiments. Values marked differ significantly from sham-incubated leukocytes.

generation upon ANCA preincubation was not simply a result of an additive effect but a result of a priming phenomenon.

Time course of the anti-PR3-elicited priming response

Next, analysis of the kinetics of the anti-PR3-elicited priming response was performed. Therefore, monocytes and neutrophils were incubated with monoclonal anti-PR3 antibodies (WGM_2) for various time periods prior to LPS challenge. Although similar with respect to the magnitude of the priming reaction, the kinetics of the leukocyte response to anti-PR3 pretreatment differed between monocytes and neutrophils (**Fig. 5**): Although monocytes responded only with a slight elevation of IL-8 release after a 2-h priming period with anti-PR3, LPS-induced IL-8 generation was already significantly elevated at this time-point in neutrophils. In monocytes and neutrophils, priming was maximally enhanced after 6 h of anti-PR3 treatment. Longer priming periods (12 h) were still effective in monocytes but not in neutrophils.

Reproduction of the anti-PR3-related priming for LTA but not for TNF-α stimulation

When investigating whether the anti-PR3-related priming was also true for other agonists, an impressive amplification of monocyte and neutrophil IL-8 generation upon stimulation with *S. aureus* LTA was obtained (**Fig. 6**). After a 6-h pretreatment period with monoclonal anti-PR3 antibodies but not with isotype-matched IgGc, the LTA-induced IL-8 liberation from monocytes and neutrophils was approximately doubled in anti-PR3-pretreated cells, corresponding well with the priming response previously observed for LPS-induced leukocyte activation. In contrast, when the proinflammatory cytokine TNF-α was used for leukocyte activation, no priming for IL-8 generation was observed after anti-PR3 pretreatment (Fig. 6).

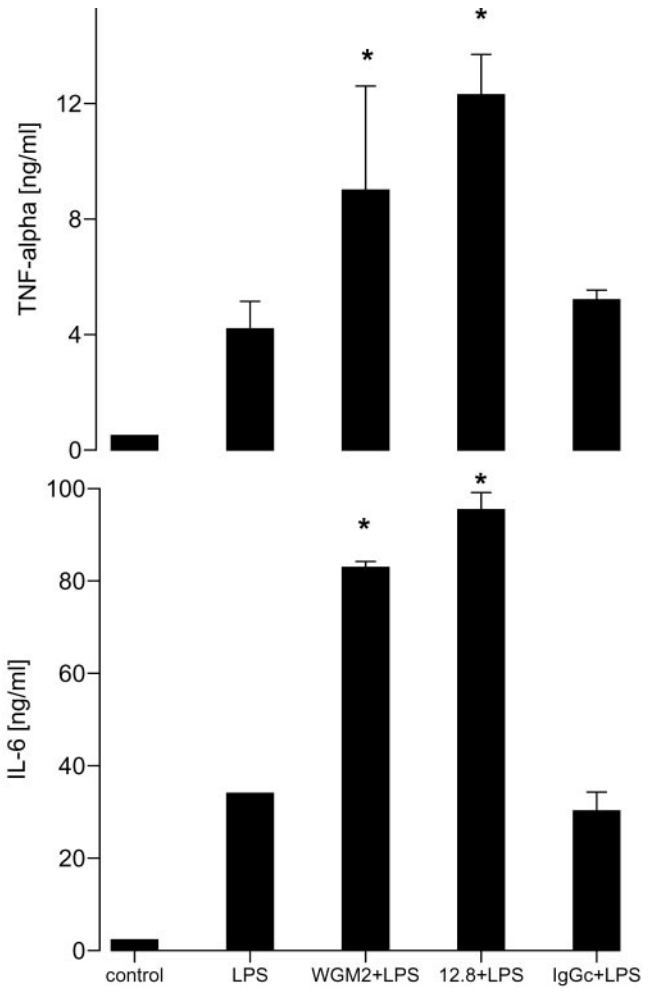

Fig. 3. Reproduction of the anti-PR3-related priming for monocyte TNF-α and IL-6 synthesis. Isolated monocytes (0.5×10^6/ml) were preincubated for 6 h with two different monoclonal anti-PR3 antibodies (WGM_2 or 12.8; 1 μg/ml) with equal amounts of an isotype-matched IgG_c, or sham incubation was performed (control). Subsequently, leukocytes were challenged with LPS (10 ng/ml) for 18 h. TNF-α (upper figure) and IL-6 (lower figure) released into the supernatant are given in ng/ml. Data reflect the means ± SEM of at least four independent experiments given. Values marked differ significantly from sham-incubated leukocytes.

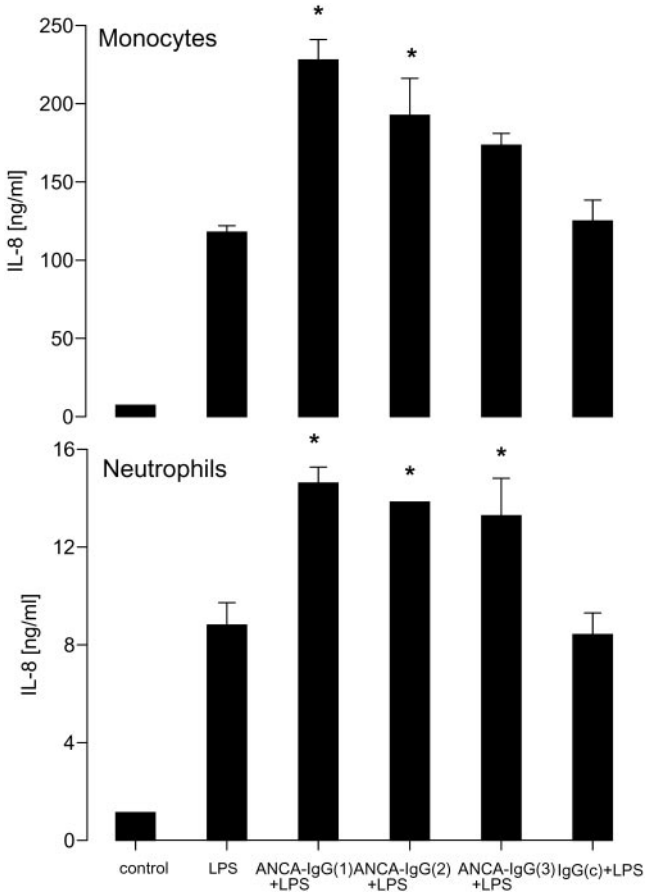

Fig. 4. Amplification of LPS-induced IL-8 release after preincubation with c-ANCA IgG. Isolated monocytes (0.5×10^6/ml) or neutrophils (5×10^6/ml) were preincubated for 6 h with three different c-ANCA-IgG preparations (100 μg/ml) or with equal amounts of normal human IgG (IgG_c), or sham incubation was performed (control). Subsequently, leukocytes were challenged with LPS (10 ng/ml) for 18 h. IL-8 released into the supernatant is given in ng/ml. Data reflect the mean ± SEM of at least three independent experiments. Values marked differ significantly from sham-incubated leukocytes.

Anti-PR3 antibodies prime leukocytes by up-regulation of CD14 expression

As it is well known that LPS- and LTA- but not TNF-α-induced leukocyte activation proceeds via CD14, we speculated that the autoantibodies modified the leukocyte response to the bacterial cell-wall components via regulation at the receptor level. Therefore, monocytes and neutrophils were assessed for CD14 surface expression by flow cytometry after anti-PR3 or IgGc challenge. As observed for CD14 expression after a priming period for 6 h, anti-PR3 antibodies markedly increased the membrane expression of this CD14 in both leukocyte types as compared with isotype-matched IgGc (**Fig. 7**).

The dependence on CD14 of LPS- or LTA-induced IL-8 generation was evident from the inhibitory capacity of the function-blocking anti-CD14 antibody MY-4 (5 μg/ml) on agonist-induced IL-8 generation. When MY-4 was admixed prior to LPS challenge, IL-8 generation was inhibited by 63 ± 16.3% in monocytes and by 75.1 ± 5.9% in neutrophils (**Table 1**). Comparable effects were obtained for LTA-induced IL-8 generation: 64.9 ± 22.3% inhibition in monocytes and 53.08 ± 6.1% inhibition in neutrophils was observed in MY-4-treated cells. In contrast, leukocyte preincubation with MY-4 did not affect the TNF-α-related generation of IL-8 in both leukocyte types (data not shown).

Moreover, when the anti-CD14 antibody MY-4 was used in experiments with monocytes and neutrophils undergoing preceding anti-PR3 priming for 6 h, strong inhibition of the LPS-induced IL-8 generation was again noted (Table 1).

It is interesting that the kinetics of CD14 up-regulation, as assessed in monocytes and neutrophils after 2, 6, and 12 h of anti-PR3 treatment by flow cytometry, correlated well with the previously assessed time dependence of the priming of the cytokine response: Corresponding to priming of LPS-induced IL-8 generation in monocytes, we found a maximal up-regulation of CD14 after 6 h of preincubation, which was still evident after 12 h in this leukocyte type, versus a faster and more transient response in neutrophils, with up-regulation of CD14 already evident after 2 h of anti-PR3 treatment, peaking at 6 h and rapidly declining thereafter (**Fig. 8**).

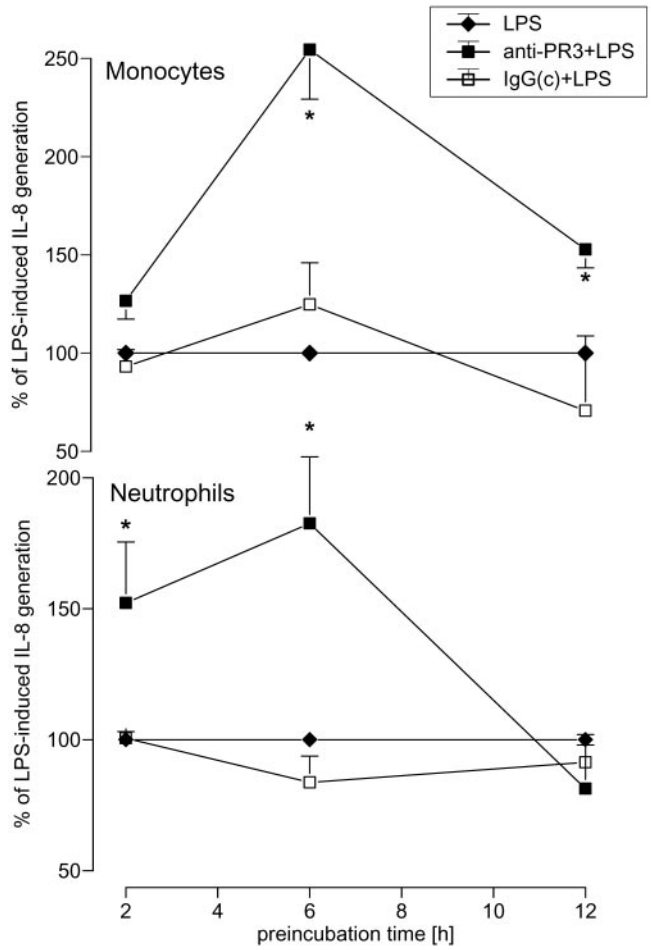

Fig. 5. Time course of the anti-PR3-related priming for LPS-induced IL-8 generation. Isolated monocytes (0.5×10^6/ml) or neutrophils (5×10^6/ml) were preincubated for various time periods with 1 μg/ml monoclonal anti-PR3 antibodies (WGM_2) with equal amounts of an isotype-matched IgG_c, or sham incubation was performed. Subsequently, leukocytes were challenged with LPS (10 ng/ml) for 18 h. IL-8 released into the supernatant is given as percentage of LPS-induced IL-8 generation in sham-primed cells. Data reflect the mean ± SEM of at least four independent experiments. Values marked differ significantly from sham-incubated leukocytes.

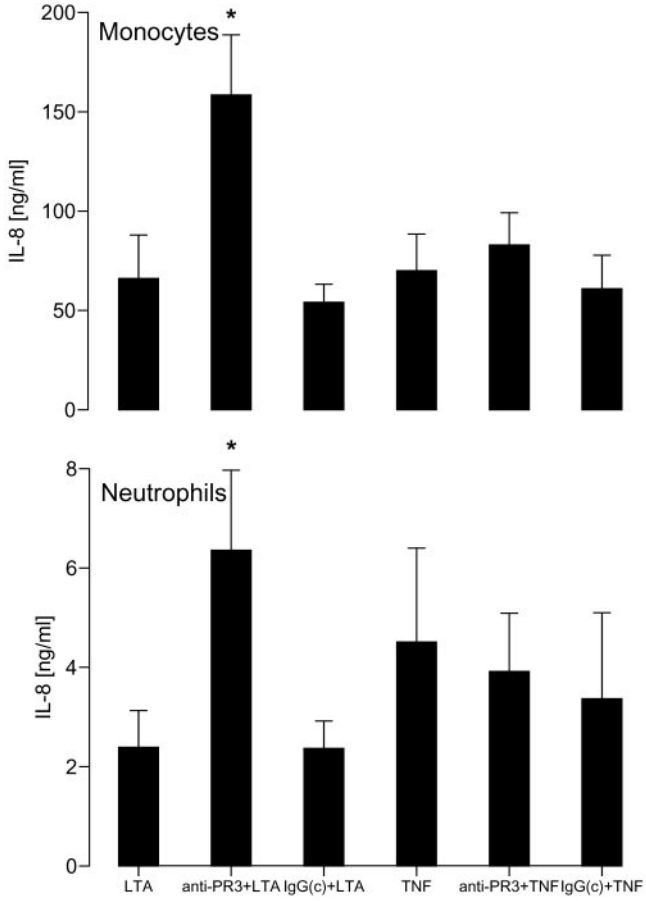

Fig. 6. Anti-PR3 priming of LTA-induced IL-8 release—comparison with TNF-α challenge. Isolated monocytes (0.5×10^6/ml) or neutrophils (5×10^6/ml) were preincubated for 6 h with monoclonal anti-PR3 antibodies (WGM_2; 1 μg/ml) with equal amounts of an isotype-matched IgG_c, or sham incubation was performed. Subsequently, leukocytes were challenged with LTA (1 μg/ml) or TNF-α (10 ng/ml) for 18 h. IL-8 released into the supernatant is given in ng/ml. Data reflect the means ± SEM of at least four independent experiments. Values marked differ significantly from sham-incubated leukocytes.

Anti-PR3 antibodies do not affect surface expression of TLR2 or TLR4 on monocytes

As the subtypes of TLRs TLR2 and TLR4 have recently been identified as key molecules in mediating LPS (TLR4)- and LTA (TLR2)-induced cellular activation [18, 19], we investigated whether the increased leukocyte response to bacterial factors corresponded with an increased expression of these molecules on the leukocyte surface. As monocytes, as opposed to neutrophils, represent the prototype of a TLR-expressing cell [32], studies about TLR expression were done in these cells. However, preatreatment with anti-PR3 antibodies did not affect the expression of TLR2 and TLR4 on monocytes (**Table 2**).

TABLE 1. Effect of Anti-CD14 Antibodies on the Anti-RP3-Induced Priming of Leukocyte IL-8 Generation in Response to LPS

	Control	LPS	LPS + MY-4
Monocytes			
Control	7.15 ± 2.25	124.23 ± 3.54	21.43* ± 1.54
WGM_2	28.69 ± 1.56	208.32 ± 16.94	28.25* ± 1.25
Neutrophils			
Control	0.45 ± 0.12	7.54 ± 0.86	1.88* ± 0.14
WGM_2	0.93 ± 0.24	14.2 ± 2.01	4.43* ± 0.41

DISCUSSION

Among the ANCA, those targeting PR3 have a strong and specific association with WG [20]. Besides their significance as a seromarker, a pathogenetic role has been proposed for these autoantibodies as a result of their capacity to directly activate leukocytes in vitro [22–25]. In the present study, an alternative approach was chosen to define the effect of PR3 ANCA on inflammatory leukocyte functions: Preincubation of isolated monocytes and TNF-pretreated neutrophils with substimulatory concentrations of anti-PR3 antibodies were performed, and the impact of this priming on subsequent leukocyte activation with bacterial cell-wall components was evaluated. Generation of IL-8, which is recognized to play a pathogenetic role in ANCA-associated vasculitis [28], and other proinflammatory cytokines, such as TNF-α and IL-6, were noted to be strongly up-regulated by anti-PR3 priming in monocytes and neutrophils.

The background for this experimental rationale is based on the fact that disease activation in WG, which is paralleled by a rising c-ANCA titer [20, 21], appears to be triggered by bacterial infections [7–10]. Therefore, simultaneous exposure

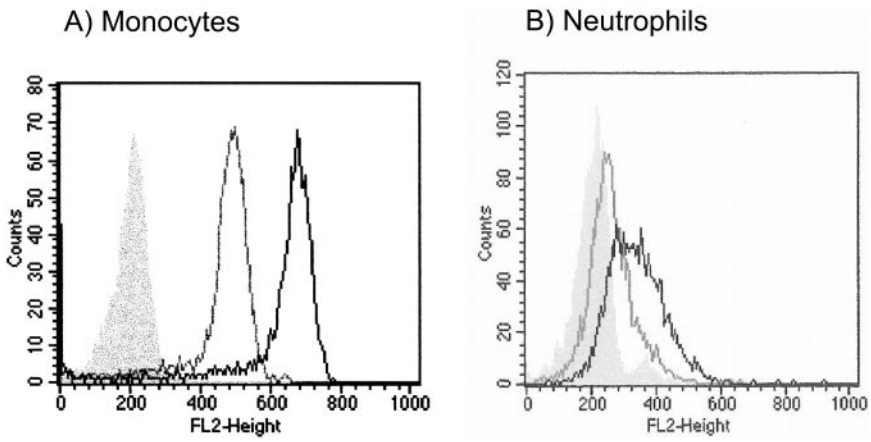

Fig. 7. Up-regulation of leukocyte membrane CD14 in response to anti-PR3. Isolated monocytes (A; 0.5×10^6/ml) or neutrophils (B; 5×10^6/ml) were incubated with IgGc (1 μg/ml; gray line) or anti-PR3 antibodies (WGM_2, 1 μg/ml; black line). After 6 h of incubation, CD14 membrane expression was assessed by flow cytometry. Filled graphs represent isotype control-labeled cells. Representative data of three independent flow cytometric studies are given.

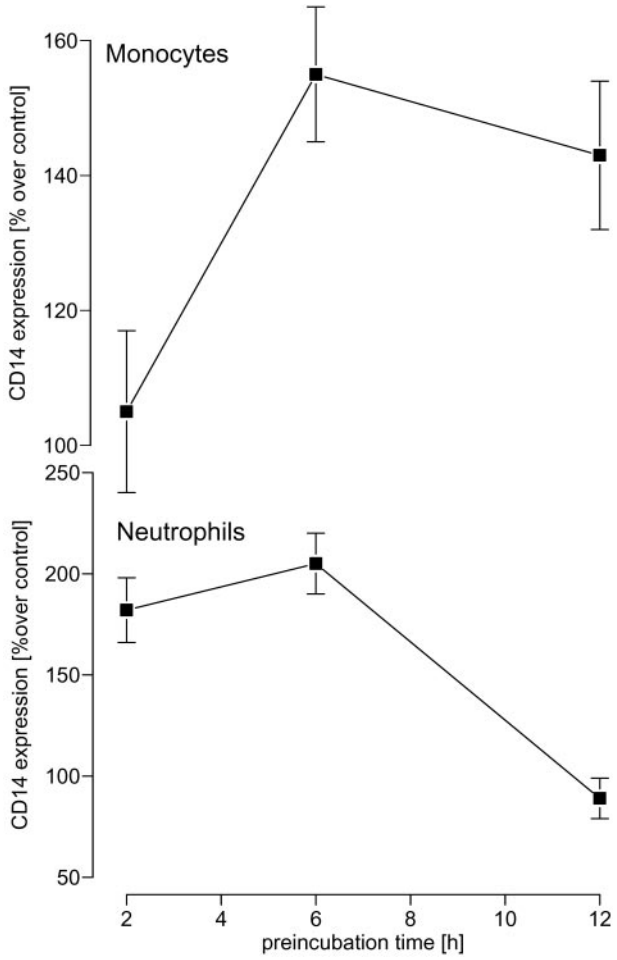

Fig. 8. Time course of up-regulation of CD14 expression in response to anti-PR3. Isolated monocytes (0.5×10^6/ml) or neutrophils (5×10^6/ml) were incubated with IgGc (1 μg/ml) or anti-PR3 antibodies (WGM_2, 1 μg/ml). After 2, 6, or 12 h of incubation, CD14 membrane expression was assessed by flow cytometry. Data are expressed as CD14 expression as a percentage over controls, and controls represent IgGc-incubated cells. Data reflect the mean ± SEM of at least three independent experiments.

of leukocytes to proinflammatory cytokines, inducing surface expression of the ANCA-target antigen PR3, such as TNF-α used for neutrophil pretreatment in the current study circulating anti-PR3 antibodies and bacterial cell-wall components, actively secreted or liberated as a result of antibiotic treatment [33], may likely occur in active WG. In vivo, the leukocyte response to bacterial pathogens depends on the state of cellular activation, varying from "dormant" via "primed" to "fully activated." Priming is a key mechanism involved in the regulation of the leukocyte-dependent host defense: Although not directly activating secretory neutrophil and monocyte functions, priming agents induce a "sensitization" of the leukocytes for subsequent stimulation with naturally occurring agonists, such as bacterial-derived products [34, 35]. In case of local or systemic microbial invasion, full-blown leukocyte activation would then follow priming. As a result of its important role in the regulation of inflammatory leukocyte functions, the phenomenon of leukocyte priming may also be relevant to the pathogenesis of vasculitis. Indeed, in active WG, neutrophils and monocytes display a phenotype attributable to a state of cellular preactivation with enhanced surface expression of activation markers such as CD11b and CD64 [36, 37].

In the present study, we hypothesized that c-ANCA, apart from their direct prosecretory capacity, may induce leukocyte priming and sensitize these effector cells for subsequent activation with cell-wall components of Gram-negative (LPS) and Gram-positive (LTA) bacteria. After a 6-h pre-exposure of both leukocyte types to two different murine monoclonal anti-PR3 antibodies, markedly enhanced generation of IL-8 in response to stimulation with LPS was indeed noted. This phenomenon was not observed when isotype-matched IgGc was used in place of the anti-PR3 antibodies. Moreover, in isolated monocytes, an impressive priming of TNF-α and IL-6 synthesis was also observed. The priming response for IL-8 was reproduced by ANCA IgG, using IgG preparations from three different patients with monospecific, anti-PR3-positive WG but not with IgG from healthy donors. Therefore, specific targeting of PR3, present on the surface of isolated leukocytes, most likely underlies the ANCA-mediated priming response. Although anti-PR3 antibodies have previously been described to directly induce neutrophil [28] and monocyte [26] cytokine generation, the presently observed, strong increase in agonist-induced chemokine generation after anti-PR3 pretreatment was clearly not merely a result of an additive effect but rather, was caused by a real priming phenomenon. When compared with LPS-induced leukocyte activation, resulting in the liberation of large amounts of IL-8 from monocytes (~150 ng/ml) and neutrophils (~10 ng/ml), the quantities of IL-8 release elicited by the currently used anti-PR3 antibodies per se were comparatively low (~20 ng/ml for monocytes and ~1 ng/ml for neutrophils), corresponding well to previous investigations [26, 28]. It is important that endotoxin contamination of the murine and human anti-PR3 antibodies, which has to be considered, as even low doses of LPS are capable of inducing leukocyte priming, was excluded in the highly sensitive kinetic *Limulus* assay system.

When attempting to reproduce the ANCA-induced priming by using agonists other than endotoxin, it became evident that the priming response was restricted to leukocyte activation by bacterial cell-wall components. After anti-PR3 pretreatment, a marked amplification of leukocyte IL-8 generation was noted upon stimulation with LTA derived from *S. aureus*, whereas chemokine synthesis elicited by TNF-α (and other cytokines, such as IL-1β

TABLE 2. Lack of Regulation of TLR2 or TLR4 Expression by Anti-PR3 Antibodies

	CD14	TLR2	TLR4	LPS	Control-PE	Control-FITC
Control	441 ± 88	363 ± 35	281 ± 20	389 ± 88	196 ± 11	195 ± 7
WGM_2	651 ± 102	353 ± 54	288 ± 29	389 ± 95	207 ± 14	205 ± 6

and granulocyte macrophage-colony stimulating factor; data not given) was not influenced by the anti-PR3 antibodies.

It is interesting that in this context, we have recently described a short-term priming effect by anti-PR3 antibodies on neutrophil activation with the bacterial-derived chemotactic peptide formyl-Met-Leu-Phe (fMLP) [38], which resulted in enhanced leukotriene synthesis and chemotactic movement toward the peptide. It is important that the priming response to LPS and LTA stimulation, which we describe here, displays some fundamental differences from our previous data. Priming for subsequent stimulation with fMLP required lower concentrations of anti-PR3 antibodies and had a rapid and transient character, occurring within minutes of anti-PR3 challenge. These differences may be a result of the different mechanisms underlying the priming phenomena. Changes in the affinity of the formyl peptide receptor most probably affect the priming toward the chemotactic peptide [38], which takes place within seconds to minutes, and up-regulation of CD14 requires mobilization from intracellular pools and/or activation of transcriptional processes.

In contrast to TNF-α-mediated IL-8 secretion, which involves ligation of TNF receptor p55 and p75, LPS- and LTA-induced leukocyte activation is known to proceed via initial binding to the CD14 receptor, expressed abundantly by monocytes and by a subset of neutrophils [39]. In the present study, the CD14 dependence of leukocyte activation by the bacterial cell-wall components was again proven by the inhibitory capacity of the function-blocking antibody MY-4. In contrast, MY-4 did not inhibit TNF-α-induced leukocyte activation, thus arguing against any nonspecific inhibitory effect of the CD-14 antibody.

As LPS- and LTA-mediated leukocyte activation was largely dependent on CD14, whereas TNF-α-elicited cellular activation was an independent event, we speculated that the anti-PR3 antibodies modified the leukocyte response to the bacterial cell-wall components at the receptor level. Indeed, when analyzed for CD14 membrane expression after a 6-h anti-PR3 pretreatment versus IgGc, an up-regulation of this receptor molecule was observed on neutrophils and monocytes.

CD14, initially described as a marker to identify cells of the monocyte/macrophage lineage, was identified in 1990 as a receptor for endotoxin [16]. Besides recognizing LPS, recent studies demonstrated that CD14 equally confers cellular responsiveness to Gram-positive cell-wall components, including LTA, peptidoglycan [17, 19, 40], and recently isolated molecules derived from *S. aureus* [41]. Corresponding to its receptor function, the expression of this GPI-linked protein is not stationary but subject to distinct regulatory processes, and several agents have been described to up-regulate membrane CD14 expression by de novo synthesis or by mobilization from preformed intracellular stores [42, 43]. In monocytes, de novo synthesis of CD14 is believed to be the more important pathway involved in the regulation of CD14 membrane expression, whereas in neutrophils, CD14 is stored in an intracellular compartment, thus rapidly available for translocation to the membrane surface [44]. The different kinetics of the leukocyte responses to anti-PR3 priming with a rapid reaction by neutrophils and a more prolonged response by monocytes may thus be attributable to translocation of CD14 from an intracellular store in neutrophils and a de novo synthesis of this molecule in monocytes. In line with this reasoning, Nowack et al. [45] recently described an increase in CD14 mRNA in monocytes following incubation with c-ANCA with comparable kinetics. Besides acting as a surface receptor for bacterial wall components, CD14 has been implicated in the regulation of adherence and apoptosis in leukocytes, and an increase is associated with a proadhesive state and prolonged survival [46, 47]. Such events might additionally promote the persistence of inflammatory events and granuloma formation.

However, the observation that anti-CD14 antibody MY-4 blocked LPS-induced IL-8 secretion by ~70% but not completely may reflect the fact that other surface molecules in addition to CD14 may confer leukocyte responsiveness to bacterial cell-wall components. Therefore, from the current data, displaying an approximate twofold increase in surface CD14 on monocytes and a weaker up-regulation on neutrophils but a constant two- to threefold augmentation of the cytokine response, one could speculate that CD14 is not the only cell surface receptors for bacterial cell-wall components to be up-regulated by anti-PR3 antibodies. An up-regulation of TLR2 and TLR4, key molecules conferring cellular responsiveness to LPS and LTA, has been excluded in the current study. It is interesting that up-regulation of CD18, which has been implicated as a LPS receptor [48], was recently demonstrated to occur in ANCA-stimulated monocytes [45]. Alternatively, post-CD14 receptor signal transduction pathways, such as the activation of protein kinases, may be stimulated by ANCA [49, 50].

In conclusion, c-ANCA, being only a weak, direct activator of monocyte and neutrophil cytokine release per se, exert a major priming effect on these leukocytes, enhancing their responsiveness to secondary stimulation with bacterial cell-wall components of Gram-positive and Gram-negative bacteria. Up-regulation of the CD14 molecule, acting as a cellular receptor for LPS and LTA, was characterized as one mechanism underlying the anti-PR3-elicited priming response. Such cooperation between anti-PR3 antibodies and bacterial cell-wall components may well trigger exacerbations of disease activity during infections and contribute to the persistence of inflammatory lesions and granuloma formation in WG.

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Anlage 7

Sibelius U, Hattar K, Hoffmann S, Mayer K, Grandel U, Schenkel A, Seeger W, Grimminger F.

Distinct pathways of lipopolysaccharide priming of human neutrophil Respiratory Burst: role of lipid mediator synthesis and sensitivity to IL-10**.**

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Distinct pathways of lipopolysaccharide priming of human neutrophil respiratory burst: Role of lipid mediator synthesis and sensitivity to interleukin-10

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Objective: **Exposure of neutrophils to low doses of bacterial lipopolysaccharides enhances their readiness to respond with inflammatory mediator generation including oxygen radical formation to a subsequently applied inflammatory stimulus ("priming"). In the present study, we investigated the role of lipid mediator synthesis and the impact of the anti-inflammatory cytokine interleukin-10 on the lipopolysaccharide-dependent priming of human neutrophils in response to N-formyl-methionyl-leucyl-phenylalanine.**

Design: **Prospective, experimental study.**

Setting: **Research laboratory at a university hospital.**

Subjects: **Isolated neutrophils from healthy volunteers.**

Interventions: **Incubation of isolated neutrophils with endotoxin.**

Measurements and Main Results: **Evidence for two distinct priming mechanisms was obtained. The first was strictly serum component dependent, proceeded via CD14, and was not inhibited by even high concentrations of interleukin-10. The second priming mechanism was serum component independent but nevertheless proceeded via CD14. It was linked with neutrophil synthesis of the platelet activating factor and resulted in the appearance of leukotrienes, in particular leukotriene B_4, as far as exogenous arachidonic acid was provided. The employment of a platelet-activating factor receptor antagonist (WEB 2086) blocked leukotriene synthesis, and both WEB 2086 and a 5-lipoxygenase inhibitor (MK-886) suppressed the respiratory burst linked with this second priming pathway. This sequence of priming events was inhibited by interleukin-10, when this cytokine was coadministered with the priming agent lipopolysaccharide, whereas late interleukin-10 admixture was ineffective.**

Conclusions: **We conclude that two mechanisms of lipopolysaccharide priming of human neutrophil respiratory burst can be differentiated. One displays serum component dependence, is independent of neutrophil lipid mediator generation, and is not affected by interleukin-10. The other is serum independent although being operated via CD14, employs autocrine loops of platelet-activating factor and leukotriene B_4 synthesis, and is sensitive to the inhibitory capacity of interleukin-10. These features may be relevant when the goal is to pharmacologically modify neutrophil functions in septic events. (Crit Care Med 2002; 30:2306–2312)**

KEY WORDS: shock; sepsis; neutrophils; neutrophil activation; endotoxin; lipid mediators; leukotrienes; platelet-activating factor; respiratory burst; CD14 antigen; interleukin-10

Polymorphonuclear leukocyte (PMN)-mediated defense mechanisms are an important component of the host response to bacterial infection. Neutrophils are well equipped to destroy invading microorganisms. In response to several stimuli, they generate superoxide anion and derived oxygen radicals and secrete a variety of proteolytic enzymes (1). Moreover, neutrophils are a rich source of inflammatory lipid mediators, in particular leukotriene (LT)B_4 and platelet-activating factor (PAF). LTB_4 causes increased chemotactic and chemokinetic migration of neutrophils and stimulates neutrophils to release lysosomal enzymes and to produce superoxide (2–4). PAF is a potent mediator of a variety of inflammatory reactions (5, 6) and was described to synergize with LTB_4 by amplifying LT synthesis in neutrophils as a positive feedback loop (7). Selective PAF antagonists inhibit endotoxin effects in some models of endotoxemia, suggesting that PAF is a mediator involved in endotoxin shock (8–10).

Neutrophils are major targets for the action of lipopolysaccharides (LPS), the major immunostimulatory component of the outer membrane of Gram-negative organisms. The exposure of PMN to LPS primes the cells for enhanced release of microbicidal metabolites (11, 12). When neutrophils were exposed to a variety of secondary stimuli after preceding LPS incubation, many neutrophil functions were shown to be up-regulated (13–16). These functions include the respiratory burst and the synthesis of LTB_4 and PAF. The priming effect of LPS was suggested to be largely dependent on ligation of CD14, a glycosyl-phosphatidylinositol-linked membrane protein, and this reaction is facilitated by first complexing LPS with a serum component, the LPS-binding protein (17, 18). CD14 does, however, lack a transmembrane domain necessary for intracellular signaling. Additional molecules thus have been postulated to interact with CD14 to induce cell activation, and toll-like receptors are recently recognized candidates in this field (19–21). Although the LPS-related synthesis of inflammatory mediators is crit-

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ical for the host defense capacity of neutrophils, such mediators can cause severe tissue damage when excessively or inappropriately generated (22, 23).

Numerous studies have demonstrated that activation, recruitment, and destructive functions of neutrophils can be controlled by soluble and cell-associated mediators, including cytokines. Interleukin (IL)-10, which is mainly synthesized by activated monocytes, macrophages, and helper T cells, is known to attenuate a wide range of inflammatory reactions in LPS-primed neutrophils (24). Inhibition of LPS-induced prostanoid synthesis and suppression of cytokine and PAF synthesis in response to LPS have been reported for IL-10 (25–28). However, controversial data exist as to the ability of IL-10 to modulate the oxidative burst of neutrophils (29–31), and the influence of IL-10 on neutrophil LT metabolism is largely unknown.

When we investigated the superoxide anion synthesis in LPS-primed neutrophils in the present study, two different priming mechanisms were separated. Both were shown to proceed via CD14, but one was serum dependent and independent of lipid mediators, and the other was serum independent and involved autocrine stimulation by PAF and LTs. Moreover, differential sensitivity to IL-10 was noted. These features may be relevant when that aim is to modulate neutrophil functions by anti-inflammatory cytokines under conditions of sepsis and severe inflammatory events.

MATERIALS AND METHODS

Materials. Ficoll-Paque was purchased from Pharmacia (Upsalla, Sweden). LPS (*Escherichia coli*, serotype 0111:B4), arachidonic acid (AA), cytochrome *c*, superoxide dismutase, and N-formyl-methionyl-leucyl-phenylalanine (fMLP) were obtained from Sigma (Deisenhofen, Germany), whereas IL-10 was purchased from Boeringer Mannheim (Mannheim, Germany). The PAF-antagonist WEB2086 was obtained from Boeringer Ingelheim (Ingelheim, Germany). The leukotrienes LTC_4, LTD_4, LTE_4, LTB_4, 20-OH, and 20-COOH-LTB_4 and the synthetic LTA_4-methyl ester were purchased from Biomol (Hamburg, Germany). Additional LTs were graciously supplied by Dr. W. Bartmann, Hoechst AG (Frankfurt, Germany). In addition, 5-, 8-, 9-, 11-, 12-, and 15-hydroxyeicosatetraenoic acid (HETE), 5(S), 12(S)-diHETE, 5,15-diHETE, and 12-HHT, as well as the nonenzymatic hydrolysis products of LTA_4 (6-*trans* diasteromeric pairs of LTB_4 and 5,6-diHETEs), were obtained from Paesel AG (Frankfurt, Germany). All LTs were checked for purity and quantified spectrophotometrically before use, as described (32). [^{3}H]-acetate was obtained from New England Nuclear (Boston, MA). Chromatographic supplies included an analytical high-pressure liquid chromatography (HPLC) column (250 mm length × 4 mm inner diameter; Shandon, Astmoor, UK) filled with ODS-Hypersil (particle size 3 μM; pore size 100 Å; Machery Nagel, Düren, Germany). HPLC-grade solvents distilled in glass were purchased from Fluka (Heidelberg, Germany). All other biochemicals were obtained from Merck (Darmstadt, Germany).

Isolation of Human Neutrophils. Neutrophils were isolated from venous blood of healthy donors by centrifugation over a Ficoll-Paque gradient as previously described (33). In brief, EDTA-anticoagulated blood was layered over Ficoll-Paque and centrifuged at 400 × *g* for 35 mins. After removal of mononuclear cells, erythrocytes were sedimented in 10% polyvinyl alcohol. Residual erythrocytes were removed by hypotonic lysis; cells were washed twice in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and finally resuspended in PBS containing $CaCl_2$ (1 mmol/L) and $MgSO_4$ (1 mmol/L) at 10×10^6 PMN/mL. Cell purity was >97%, as quantified by flow cytometry, and cell viability was >96%, as assessed by trypan blue dye exclusion.

Measurement of Leukotrienes. LTs and HETEs were extracted from cell supernatants by octadecylsilyl solid-phase extraction columns, as described (32, 33). Reversed-phase HPLC was carried out on octadecylsilyl columns (Hypersil, 5-μm particles), with a mobile phase of methanol/water/acetic acid (72:28:0.16, pH 4.9) (32). In addition to the conventional ultraviolet detection at 270 nm (LTs) and 237 nm (HETEs), a photodiode array detector (Waters 990, Milford, MA) was used, which provided full ultraviolet spectra (190–600 nm) of eluting compounds and allowed us to check for peak purity and subtract possible coeluting material. All data obtained by the different analytical procedures were corrected for the respective recoveries and are given in pmol/mL throughout the experiments. Recovery was determined by separate recovery experiments using different quantities of the individual compounds in the appropriate concentration range. Factors for recovery were further confirmed by addition of 0.2 μCi of [^{3}H]LTB_4 and [^{3}H]5-HETE to buffer medium as internal standards in selected experiments. For quantification of LTs and 5-HETE, correspondence of values calculated from ultraviolet absorbency in two different chromatographic procedures was required (deviation <10%).

Measurement of PAF. Neutrophil PAF production was quantified by incorporation of labeled acetate. PMNs were stimulated in the presence of 50 μCi/mL of [^{3}H]acetate according to the method of Tessner et al. (34). Reactions were stopped by addition of three volumes of chloroform/methanol (1:2, v/v), and extraction was performed according to the method of Bligh and Dyer (35). The entire lipid extract was evaporated to dryness, redissolved in 60 μL of mobile phase, and subjected to straight phase HPLC separation. The column (25 × 0.46 cm) was packed with silica gel (5-μm) particles and eluted isocratically with acetonitrile/methanol/phosphoric acid at a flow rate of 1.8 mL/min. Eluate fractions corresponding to appropriate standard retention times were collected and assayed for radioactivity by liquid scintillation counting. In selected experiments, the elution of radiolabel was monitored by using a radiochromatogram imaging system (5LS Raytest, Pittsburgh, PA).

Superoxide Anion Generation. Neutrophil O_2^- generation was assessed as superoxide dismutase-inhibitable reduction of cytochrome *c* according to Cohen (36). Duplicate reaction mixtures containing neutrophils (10×10^6/mL) and 75 μM ferricytochrome *c* were incubated at 37°C in the presence or absence of 10 μg/mL superoxide dismutase. Incubations were terminated by centrifugation at 4°C at 1200 × *g*. O_2^- release was quantified as nanomoles of cytochrome *c* reduction, by using an extinction coefficient of 21 mM^{-1} cm^{-1} at 550 nm in an Uvicon Spectrophotometer.

Experimental Procedure. Before experimental use, PMNs were resuspended in PBS to obtain a PMN concentration of 10×10^6 PMN/mL. For PAF measurement, 0.1% bovine serum albumin in PBS was used as assay buffer. In the standard protocol, PMNs were preincubated with LPS in the presence or absence of serum or IL-10 and subsequently stimulated with 1 μM fMLP (10 μM for PAF measurement due to the presence of bovine serum albumin in the assay buffer) for 10 mins. For induction of LT synthesis, exogenous AA (10 μM) was supplied together with fMLP. Reactions were stopped by centrifugation at 4°C for 5 mins at 1200 × *g* (LTs and respiratory burst) or by admixing three volumes of chloroform/methanol (1:2, v/v; PAF).

Statistics

For statistical comparison, one-way analysis of variance was performed, followed by Tukey's honestly significant difference test when appropriate. A level of $p < .05$ was considered to be significant.

RESULTS

LPS Priming of the Neutrophil LTB_4 Metabolism and Respiratory Burst—Differential Serum Dependence. The LPS priming of fMLP-induced LT metabolism affected all metabolites (LTB_4, 20-OH, and 20-COOH-LTB_4, nonenzymatic hydrolysis products of LTA_4 and 5-HETE) to a similar extent, and representative data for LTB_4-synthesis are given (Fig. 1*A*). Preincubation of 10×10^6 PMNs/mL with

different concentrations of LPS for various time periods in the presence or in the absence of serum was undertaken, followed by subsequent stimulation of the PMN with fMLP (1 μM) and arachidonic acid (10 μM). Maximum enhancement of LTB_4 generation—approximately threefold values above control—was noted after preincubation with 1 ng/mL LPS for 30 mins. Preincubation periods less or more than 30 mins were less effective (data not shown). Interestingly, there was no difference between LPS-priming in the presence or absence of serum. In contrast, the LPS priming of the neutrophil respiratory burst was found to be strictly serum dependent. Maximal amplification of superoxide anion release by fMLP, fivefold over control, was achieved by preincubation of PMN with 10 ng/mL LPS for 30 mins in the presence of 1% serum. In the absence of serum, the preincubation with LPS was entirely ineffective in priming the respiratory burst (Fig. 1*B*).

IL-10 Inhibits LPS Priming of Neutrophil LTB_4 Liberation But Not LPS Priming of the Respiratory Burst. To investigate the influence of the anti-inflammatory cytokine IL-10 on LPS-priming of LTB_4 metabolism, 10×10^6 PMNs/mL were preincubated simultaneously with LPS (1 ng/mL) and different concentrations of IL-10 for 30 mins and subsequently stimulated with fMLP (1 μM) and arachidonic acid (10 μM) for 10 mins. As depicted in Figure 2*A*, the LPS-induced priming for LTB_4 generation was completely suppressed in the presence of 10 units/mL IL-10 as optimal concentration. The time point of IL-10 application was of major importance. Only when applied before or simultaneously with LPS, IL-10 inhibited the LPS-induced priming of LTB_4 generation. In contrast, when IL-10 was administered 10 mins after the LPS challenge, the inhibitory effect was completely lost. Contrary to the LTB_4 metabolism, the LPS priming of the respiratory burst was unaffected by IL-10 (Fig. 2*B*).

Both Priming of LTB_4 Secretion and Respiratory Burst Depend on Ligation of CD14. Although displaying completely different features as to serum dependence and sensitivity to inhibition by IL-10, both the LPS priming of LTB_4 secretion and respiratory burst were completely abrogated by neutrophil preincubation with the anti-CD14-antibody MY-4 (5 μg/mL; Fig. 3).

PAF-Receptor Antagonism Inhibits Only the LPS Priming of Neutrophil LTB_4 Synthesis. We then investigated the role of PAF in LPS priming of neutrophil LTB_4 secretion. PMNs were preincubated with LPS (1 ng/mL) for 30 mins and subsequently activated with fMLP (1 μM) plus AA (10 μM) for 10 mins in the presence or absence of the PAF-receptor antagonist WEB 2086 (10 μM). As shown in Figure 4*A*, the priming effect of LPS on fMLP-induced LTB_4 secretion in neutrophils was totally blocked in the presence of this PAF receptor antagonist. In contrast, priming of the respiratory burst was entirely unaffected by WEB 2086 (Fig. 4*B*).

LPS Primes the Neutrophil PAF Secretion in Response to fMLP. Next, the effect of LPS on neutrophil PAF metabolism was investigated. While LPS challenge per se (100 pg to 100 μg/mL) did not induce activation of neutrophil PAF synthesis, LPS-preincubated PMNs responded differently to subsequent stimulation with fMLP. As depicted in Figure 5, the fMLP-induced PAF secretion was maximally enhanced after a preincubation period of 30 mins with 1 ng/mL LPS, to values nearly three-fold increased over

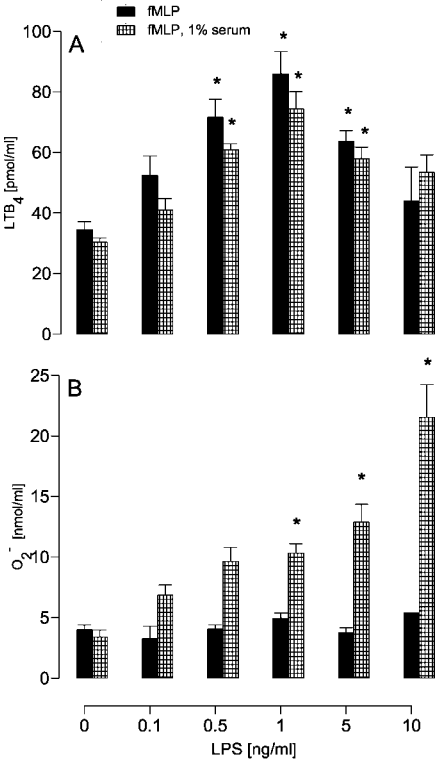

Figure 1. Influence of serum on lipopolysaccharide (*LPS*) priming of leukotriene (*LT*)B_4 metabolism and respiratory burst. Isolated neutrophils (10×10^6/mL) were preincubated for 30 mins with different concentrations of LPS either in the presence or absence of serum (1% v/v), or sham priming was performed. Subsequently, polymorphonuclear leukocytes (PMNs) were stimulated with N-formyl-methionyl-leucyl-phenylalanine (*fMLP*; 1 μM) plus arachidonic acid (10 μM) for induction of LTB_4 synthesis *(A)* or with fMLP (1 μM) alone for activation of the respiratory burst *(B)* for 10 mins. Mean ± SEM of five independent experiments each are given. *Significantly different from sham-primed PMNs.

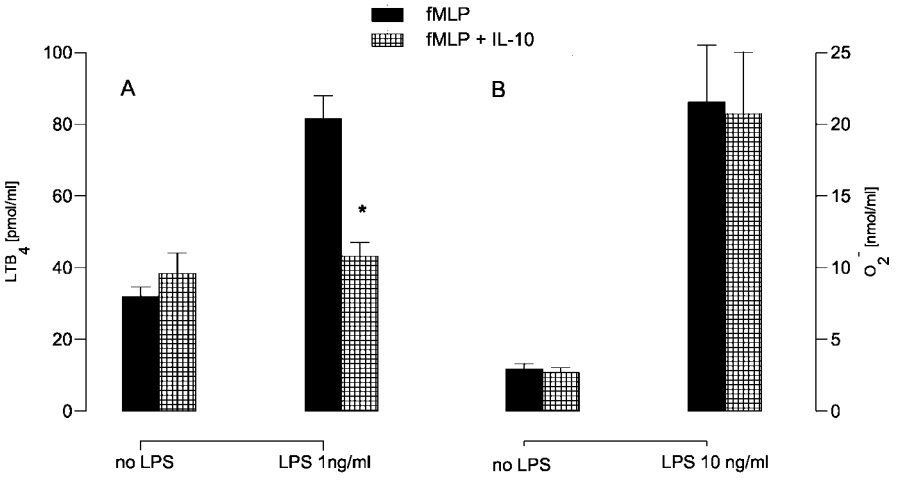

Figure 2. Impact of interleukin (*IL*)-10 on lipopolysaccharide (*LPS*) priming of leukotriene (*LT*)B_4 synthesis and respiratory burst. Isolated neutrophils (10×10^6/mL) were simultaneously preincubated with LPS (1 ng/mL for leukotriene generation in the absence of serum and 10 ng/mL for the respiratory burst in presence of serum) in the absence or presence of IL-10 (10 units/mL) for 30 mins, or sham priming was performed. Then, polymorphonuclear leukocytes were stimulated for 10 mins with N-formyl-methionyl-leucyl-phenylalanine (*fMLP*; 1 μM) plus arachidonic acid (10 μM) for induction of LTB_4 metabolism *(A)* or with fMLP (1 μM) alone for activation of the respiratory burst *(B)*. Mean ± SEM of six independent experiments each are given. *Significantly different from corresponding values without IL-10.

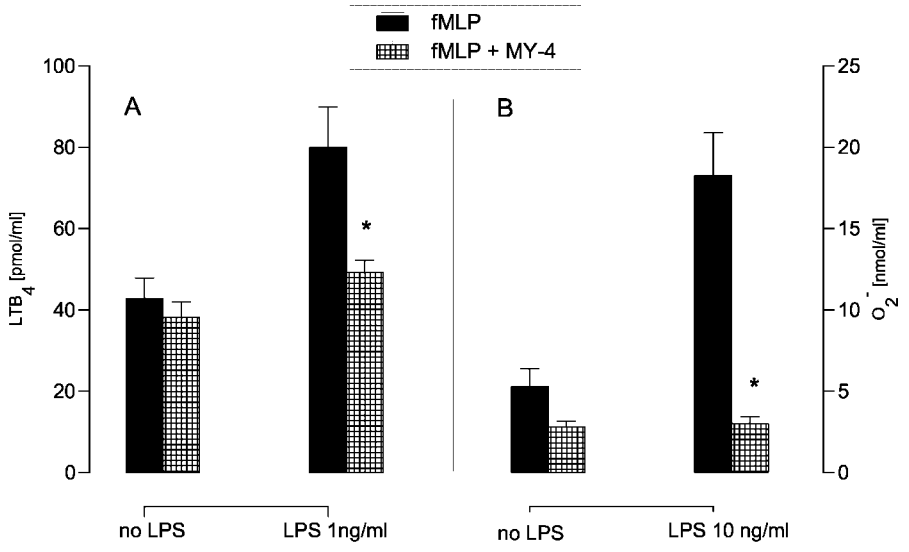

Figure 3. Influence of anti-CD14 antibodies on lipopolysaccharide (*LPS*) priming of leukotriene (*LT*)B_4 metabolism and respiratory burst. Polymorphonuclear leukocytes (PMNs; 10×10^6/mL) were preincubated with LPS (1 ng/mL for leukotriene generation in the absence of serum and 10 ng/mL for the respiratory burst in the presence of serum) for 30 mins either in the presence or absence of anti-CD14-antibodies (MY-4, 5 μg/mL), or sham priming was performed. Subsequently, PMNs were challenged with N-formyl-methionyl-leucyl-phenylalanine (*fMLP*; 1 μM) plus arachidonic acid (10 μM) for induction of LTB_4 synthesis *(A)* or with fMLP (1 μM) alone for activation of the respiratory burst *(B)* for 10 mins. Mean ± SEM of seven independent experiments each are given. *Significantly different from corresponding values without MY-4.

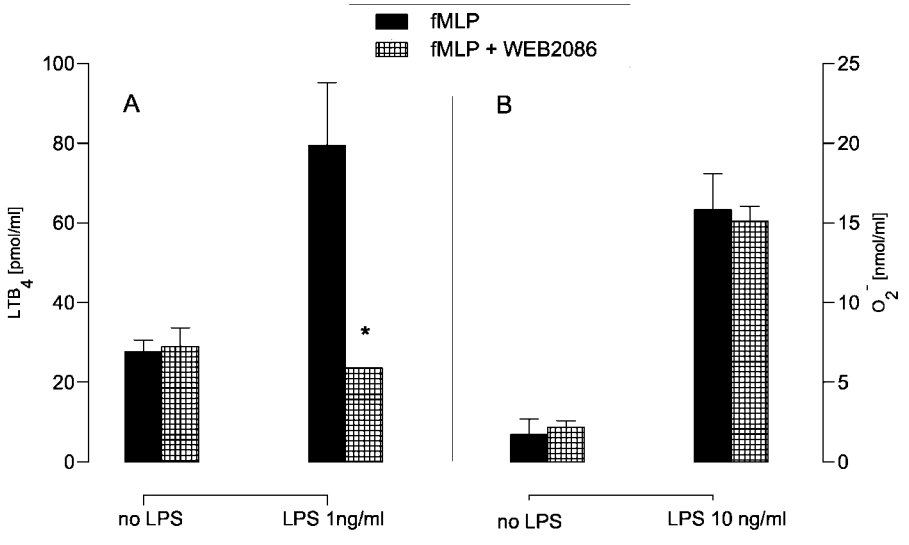

Figure 4. Influence of the platelet-activating factor receptor antagonist WEB 2086 on lipopolysaccharide (*LPS*) priming of leukotriene (*LT*)B_4 synthesis and respiratory burst. Isolated neutrophils (10×10^6/mL) were preincubated with LPS (1 ng/mL for leukotriene generation in the absence of serum and 10 ng/mL for the respiratory burst in the presence of serum) for 30 mins either in the presence or absence of WEB 2086 (10 μM) or sham priming was performed. Then, neutrophils were stimulated for 10 mins with N-formyl-methionyl-leucyl-phenylalanine (*fMLP*; 1 μM) and arachidonic acid (10 μM) for induction of LTB_4 synthesis *(A)* or with fMLP (1 μM) alone for activation of the respiratory burst *(B)*. Mean ± SEM of five independent experiments each are given. *Significantly different from corresponding values without WEB 2086.

control (fMLP-activated PMNs without LPS-preincubation). The LPS priming of neutrophil PAF metabolism in response to fMLP was again completely abrogated in the presence of IL-10 (10 units/mL), applied before or simultaneously with LPS.

Enhanced Leukotriene Secretion Amplifies the Respiratory Burst Via an Autocrine Loop. We preincubated 10×10^6 PMNs/mL with different concentrations of LPS for 30 mins in the absence of serum and subsequently stimulated them with fMLP (1 μM) for 10 mins in the absence or in the presence of AA (10 μM). As depicted in Figure 6, control stimulation of neutrophils with fMLP provoked a respiratory burst of ~4 nmol/mL. As shown previously, preincubation of the neutrophils with 1 ng/mL LPS for 30 mins in the absence of serum showed no priming effect on fMLP-induced respiratory burst. However, when arachidonic acid (10μM) was coapplied with fMLP, thus giving rise to LTB_4 generation, the respiratory burst was enhanced nearly four-fold over control (sole fMLP challenge). This effect was completely blocked in the presence of the 5-lipoxygenase inhibitor MK-886 (10 μM).

DISCUSSION

When analyzing the generation of lipid mediators and oxygen radicals in LPS-primed human neutrophils in the present study, we obtained evidence for two independent priming mechanisms (Fig. 7). One proceeded via CD14, was serum component dependent, and enhanced the fMLP-elicited respiratory burst independent of lipid mediator generation. This priming mechanism was not inhibited by IL-10. The second did not demand the presence of serum components, again employed CD14, and was linked with both PAF and LT biosynthesis. This priming mechanism was fully suppressed in the presence of IL-10.

The first interesting finding of the present study was the fact that LPS priming of LT synthesis in human neutrophils occurred at very low LPS concentrations independent of serum components, suggesting that humoral factors such as LPS-binding protein are not mandatory for this type of priming response. In contrast, the LPS priming of the respiratory burst was strictly serum dependent. Both pathways required ligation of CD14, as evidenced by blocking studies with anti-CD14 antibodies. The same phenomenon recently was described for human macrophages, which respond to LPS with tumor necrosis factor release and upregulation of procoagulant activity in a serum-independent but CD14-dependent manner (37). In that study, it was hypothesized that during their differentiation, macrophages acquire a functional substitute for the serum factor LPS-binding protein (37). In the mature neutrophils

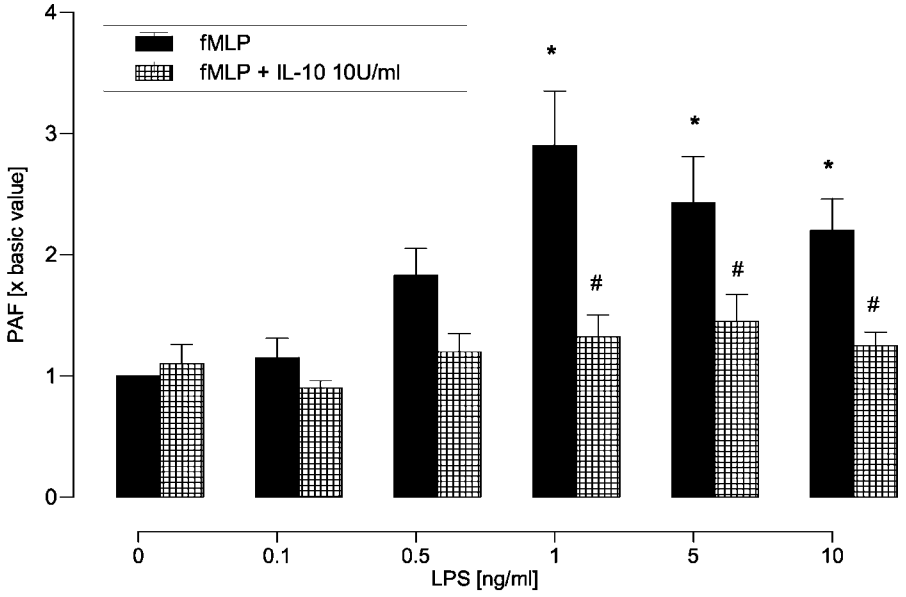

Figure 5. Influence of lipopolysaccharide (*LPS*) priming on neutrophil platelet-activating factor (*PAF*) metabolism—impact of interleukin (*IL*)-10. Isolated neutrophils (10×10^6/mL) were preincubated for 30 mins with different concentrations of LPS in either the presence or absence of IL-10 (10 units/mL), or sham priming was performed. Subsequently, the polymorphonuclear leukocytes (PMNs) were stimulated with N-formyl-methionyl-leucyl-phenylalanine (*fMLP*; 10 μM) for induction of PAF synthesis for 10 mins. Mean ± SEM of four independent experiments each are given. *Significantly different from sham-primed PMN; #significantly different from corresponding values without IL-10.

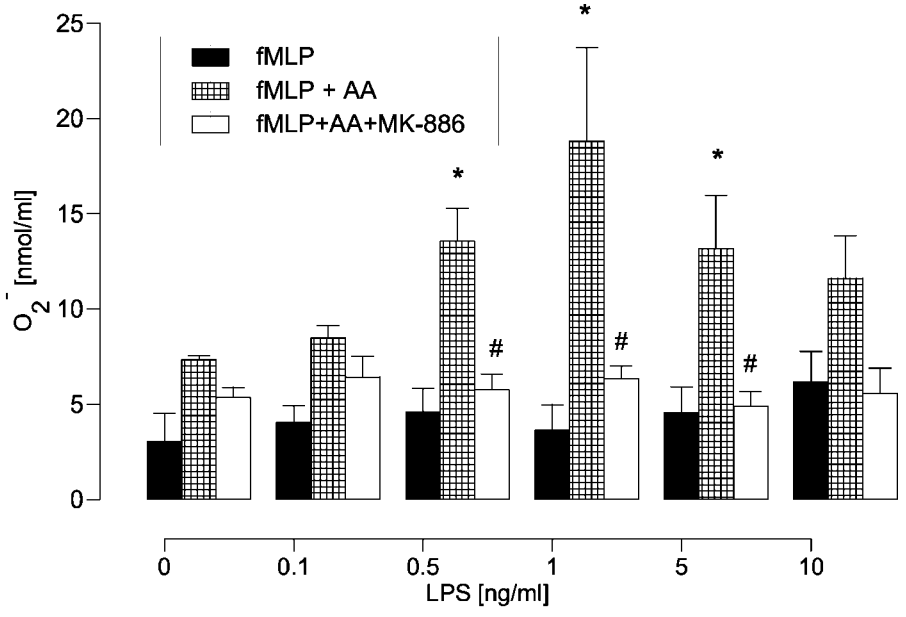

Figure 6. Influence of leukotriene generation on lipopolysaccharide (*LPS*)-induced priming of neutrophil respiratory burst. Polymorphonuclear leukocytes (PMNs; 10×10^6/mL) were preincubated with different concentrations of LPS (without serum) for 30 mins, or sham incubation was performed. For induction of the respiratory burst, PMNs were subsequently stimulated with N-formyl-methionyl-leucyl-phenylalanine (*fMLP*; 1 μM) either in the presence or absence of arachidonic acid (*AA*; 10 μM) and the 5-LO inhibitor MK-885 (10 μM). Mean ± SEM of five independent experiments each are given. *Significantly different from sham-primed PMN; #significantly different from corresponding values without MK-886.

currently investigated, two independent "priming cascades" apparently are linked to CD14. Whether different subtypes of this surface molecule, or different epitopes on the same molecule linked to different cell functions, are responsible for this phenomenon requires further investigation. Responsiveness to low LPS concentrations in a milieu devoid of serum components may enable neutrophils to trigger further PMN recruitment via LTB_4 and PAF liberation, thereby probably also promoting the generation of an inflammatory focus with leakage of serum components, which might then provide the basis for further serum-dependent LPS-priming phenomena.

Different pathways of lipopolysaccharide-induced priming of inflammatory neutrophil functions apparently exist.

The second important finding was that the LPS-priming of fMLP-induced LTB_4 secretion was completely inhibited by the PAF receptor antagonist WEB 2086, whereas the respiratory burst was entirely unaffected. These results are in line with a recent study where the PAF receptor antagonist Y-24180 diminished the lipid A-induced elevation of intracellular Ca^{2+} levels in guinea-pig neutrophils but not the priming effects of lipid A on O_2^- synthesis (38). The authors speculated that at least two distinct pathways are involved in the actions of LPS, one via cross-recognition of LPS with PAF receptors and the other being independent of a PAF receptor. To discriminate how WEB2086 acts on LPS priming, we subdivided the process of PMN activation into two steps—the first step of LPS preincubation, and the second step of responsiveness to fMLP—by either coincubating WEB2086 with LPS or by applying WEB2086 together with fMLP. The LPS priming of the fMLP-induced LTB_4 release was blocked under both experimental conditions, indicating that PAF developed its effect in the further sequence of PMN activation.

LPS-stimulated PMNs were described to produce PAF with a pattern characterized by an early and a delayed peak of synthesis (28). The early peak was shown to result from a direct effect of LPS, while the second peak was attributed to LPS-induced IL-8 and tumor necrosis factor-α liberation. IL-10 was previously noted to

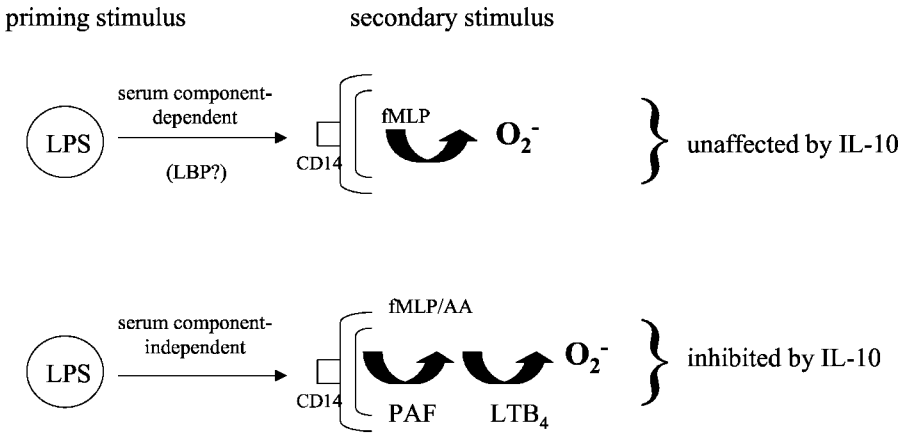

Figure 7. Hypothetical representation of two independent lipopolysaccharide (*LPS*) priming mechanisms of the respiratory burst in human neutrophils. The first priming mechanism is serum component dependent (possibly mediated via LPS binding protein) and CD14 dependent but is not inhibited by interleukin (*IL*)-10. The second mechanism is serum-component independent and employs CD14, an autocrine platelet-activating factor (*PAF*) loop, as well as a leukotriene (*LT*)B_4-loop. It is not inhibited by IL-10. *LBP*, LPS-binding protein; *fMLP*, N-formyl-methionyl-leucyl-phenylalanine; *AA*, arachidonic acid.

suppress PAF-synthesis, although only that corresponding to the second, cytokine-dependent peak (28). With the low concentrations of LPS used for priming in the present study, we did not detect PAF in response to the endotoxin *per se*, but we noted a marked increase of PAF synthesis induced by the second stimulus fMLP. Similar to the inhibition of IL-8 and tumor necrosis factor-α release, this effect was suppressed in the presence of IL-10. IL-10 thus may modulate PAF metabolism in LPS-challenged neutrophils in different ways: by inhibition of LPS-induced cytokine synthesis linked to secondary PAF generation, and by inhibition of the LPS priming of PAF secretion in response to the second stimulus fMLP.

The initial step in PAF biosynthesis is the activation of a phospholipase A_2, which removes a long-chain fatty acid, for the most part arachidonic acid, from the B position of the PAF precursor, and the arising lyso-PAF is then acetylated by acetyltransferase to form PAF (5, 6). Doerfler et al. (39) reported that priming of neutrophils by LPS results in the phosphorylation of an AA-phospholipid-selective cytosolic phospholipase A_2, which is dissociated from activation until a second stimulus is applied. In their study, the LPS incubation of PMN enhanced the release of AA in response to opsonized zymosan, whereas the influence on PAF liberation was not investigated. Moreover, it has been shown that high concentrations of LPS prime neutrophil LT generation by enhancing arachidonic acid availability in a CD14-dependent manner (40). Together, these previous and the present findings suggest that LPS developed the effect on fMLP-evoked PAF synthesis by phosphorylation of the cytosolic phospholipase A_2, a process that may be antagonized by IL-10. However, in the present study, secondary stimulation with fMLP did not provoke detectable quantities of LTs, unless exogenous AA was provided, as known to occur in an inflammatory focus (41) and, for example, in the plasma of septic patients (42). Thus, the more relevant step in the currently observed LPS-priming of LT synthesis apparently lies downstream of phospholipolysis and may in particular include prolonged activation of 5-lipoxygenase, as recently suggested (43). Moreover, increased PAF generation apparently adds to the up-regulation of the LT-biosynthesis pathway, as suggested by the inhibitory capacity of the PAF receptor antagonist WEB 2086. As similarly discussed for PAF, LTB_4 is known to boost the secretion of inflammatory mediators via an autocrine loop (4, 7, 33). In such a "late" event in a sequence involving LPS priming, enhanced PAF synthesis and increased LTB_4 formation again can stimulate the respiratory burst, as strongly indicated by the efficacy of the 5-lipoxygenase-inhibitor on the LPS-induced priming of the respiratory burst in the presence of the 5-LO-substrate arachidonic acid. LPS thus may exert its priming effect on fMLP-induced respiratory burst in different ways: one serum-dependent pathway may be operative, probably via up-regulation of the nicotinamide adenine dinucleotide phosphate-oxidase assembly, as recently described (44), and one serum-independent pathway apparently involves autocrine activities of enhanced formation of the lipid mediators PAF and LTB_4.

These two pathways display differential sensitivity to IL-10, which was found to be a potent suppressor of the sequence linked to PAF and LTB_4 synthesis but was ineffective on the respiratory burst associated with the serum component-related LPS-priming. Investigators should consider these facts when planning to employ IL-10 for dampening of inflammatory events related to increased neutrophil activities.

CONCLUSION

Different pathways of LPS-induced priming of inflammatory neutrophil functions apparently exist. Differential dependence on serum components, the role of autocrine loops of lipid mediator generation, and differential sensitivity to the inhibitory capacity of IL-10 are key features in this finding. These aspects may be relevant when investigators aim to control neutrophil functions in inflammatory and septic events and should be taken into consideration when designing therapeutic approaches in this area.

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Anlage 8

Grandel U, Heygster D, Sibelius U, Fink L, Sigel S, Seeger W, Grimminger F, Hattar K.

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Amplification of Lipopolysaccharide-Induced Cytokine Synthesis in Non–Small Cell Lung Cancer/Neutrophil Cocultures

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Abstract

Proinflammatory cytokines are centrally involved in tumor progression and survival in non–small cell lung cancer, and both the presence of infiltrating neutrophils and bacterial infection in the lung may indicate a poor prognosis. Against this background, we investigated the effect of the bacterial cell wall component lipopolysaccharide (LPS) on interleukin (IL)-6 and IL-8 synthesis in the non–small cell lung cancer line A549 and in A549-neutrophil cocultures. The LPS induced a dose-dependent and time-dependent release of IL-8 from A549 cells, whereas IL-6 could not be detected. Interestingly, in A549-neutrophil cocultures, IL-8 synthesis was massively amplified and IL-6 was also released, compared with the respective monocultures. The A549 cells were identified as the primary cellular source of these cytokines, as enhanced cytokine mRNA transcription was detected in this cell type, although not in neutrophils in the coculture system. Experiments done in transwells indicated that direct cell-cell contact was a prerequisite for the increased cytokine generation. Inhibition of tumor necrosis factor-α bioactivity by neutralizing antibodies and blocking cyclooxygenase-2 activity blunted the enhanced cytokine generation in the coculture system. Amplification of LPS-induced cytokine secretion could be reproduced when the small cell lung cancer cell line H69 was cocultured with neutrophils. When the Gram-positive cell wall component lipoteichoic acid was used instead of LPS, cytokine synthesis was also amplified in A549-neutrophil cocultures, to a similar extent to that observed with LPS. These data indicate that interaction between bacterial pathogens, neutrophils, and tumor cells might amplify the release of proinflammatory cytokines which may promote tumor growth *in vivo*. (Mol Cancer Res 2009;7(10):1729–35)

Introduction

Lung cancer is the leading cause of cancer deaths in the Western world, with non–small cell lung cancer (NSCLC) histology predominating over small cell lung cancer (SCLC). Regardless of the histologic type, patients with lung cancer frequently suffer from pulmonary infections which are associated with a reduction in median survival (1). The most frequent pathogens found in patients with lung cancer are Gram-negative bacteria such as *Haemophilus influenzae* and *Escherichia coli*, and an increase in Gram-positive infections, especially with *Streptococcus pneumoniae* and *Staphylococcus aureus* has been observed recently (2, 3). The use of antibiotics, especially clarithromycin, has profitable effects on survival and tumor progression in NSCLC (4). It is not clear whether bacterial infections are only a simple epiphenomenon of advanced lung cancer, or whether they promote cancer growth and metastasis formation (5). However, *in vivo* and *in vitro* observations have indicated that bacterial pathogens promote malignant transformation of cells and cancer growth. Chronic *Helicobacter pylori* infection is known to cause gastric cancer (6), and in lung cancer, an association of *Chlamydia pneumoniae* infection and tumorigenesis has been postulated (7).

In general, bacterial pathogenicity is mediated by bacterial toxins. The most prominent virulence factor of Gram-negative bacteria is endotoxin, which consists of lipopolysaccharides (LPS) from the bacterial cell wall. Gram-positive bacteria generate endotoxin-like substances such as lipoteichoic acid (LTA) and peptidoglycan. Both LPS and LTA activate a variety of inflammatory reactions in multiple target cells (8-10).

During the course of bacterial infections in the lung, neutrophils are recruited into the alveolar space and this mechanism is driven by chemotactic cytokines, in particular, interleukin (IL)-8 (11). Apart from attracting and activating neutrophils, IL-8 is a potent proangiogenic and proproliferative agent in NSCLC. In animal models, IL-8 facilitates tumorigenesis *in vivo* (12), whereas *in vitro*, IL-8 induces the proliferation of NSCLC cells (13, 14). Moreover, in patients with NSCLC, elevated levels of IL-8 are associated with a poor prognosis (15). Another predictor of survival in patients with NSCLC are elevated levels of IL-6 (16) and a strong association between tumor cell proliferation and IL-6 production has been found *in vitro* (17, 18). These findings suggest a decisive role for IL-6 and IL-8 in the progression of NSCLC (19). In adenocarcinoma of the lung, tumor cells themselves may be the primary cellular source of IL-8 (20, 21). *In vitro*, IL-8 and IL-6 mRNA and protein are induced in response to proinflammatory cytokines, such

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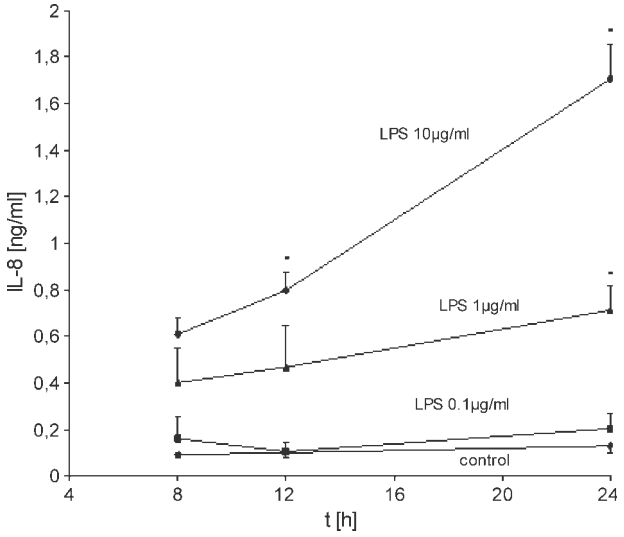

FIGURE 1. Effect of LPS on IL-8 release in A549 cells. A549 cells were incubated with LPS at various concentrations or sham-incubated (control). At the indicated time points, cell supernatants were harvested and IL-8 release was quantified by ELISA. Points, mean of at least four independent experiments, each done with duplicate values; bars, SEM. *, significantly different values from controls.

as tumor necrosis factor-α (TNF-α) in lung adenocarcinoma cells (22). Apart from cytokines, prostanoids derived from the inducible isoform of cyclooxygenase (COX-2) seem to play a decisive role in the development and progression of NSCLC (23, 24). Furthermore, in adenocarcinoma of the lung, marked COX-2 expression has been detected both *in vivo* and *in vitro* (25, 26).

Recently, it has been suggested that the tumor microenvironment (the interaction of tumor cells with surrounding stroma or infiltrating cells) may be pivotal in tumor promotion and progression (27). In NSCLC, the tumor microenvironment may contribute to the induction of cytokines (19, 28). During bacterial infection, leukocytes are a key component of the tumor microenvironment, and the outcome of NSCLC is negatively influenced by the number of infiltrating neutrophils (20, 29). Whether neutrophil-derived cytokines contribute to tumor growth has not been studied in detail.

Against this background, we cocultured the NSCLC line A549 with isolated human neutrophils and analyzed cytokine generation in response to LPS. In essence, we found that LPS induced IL-8 but not IL-6 liberation from A549 cells. Interestingly, LPS-induced IL-8 generation was massively amplified in coculture systems, and IL-6 was also detected. The amplification of cytokine generation in these cocultures was mediated by TNF-α and COX-2 activity.

Results

LPS Induces a Time-Dependent and Dose-Dependent Release of IL-8 in A549 Cells

The A549 monolayers were incubated with different concentrations of LPS (0.1, 1, and 10 μg/mL) for various time periods. Application of LPS stimulated the release of IL-8 from A549 cells in a time-dependent and dose-dependent manner, with significant amounts of IL-8 already accumulating 8 hours after stimulation with LPS (Fig. 1). In contrast to the IL-8 response, only trace amounts of IL-6 were liberated from A549 cells in response to LPS (Table 1).

LPS-Induced IL-8 Formation Is Massively Amplified in A549-PMN Cocultures

When A549 cells were cocultured with isolated human neutrophils (PMN, 5×10^6/mL), a massive amplification of LPS-induced IL-8 formation was observed (Fig. 2). As depicted for stimulation with 0.1 μg/mL of LPS for 24 hours, coculturing of these two cell types resulted in an ~10-fold increase in LPS-induced IL-8 formation compared with LPS-exposed monocultures of neutrophils or A549 cells (PMN, 3.4 ± 0.7 ng/mL IL-8; A549, 0.2 ± 0.06 ng/mL IL-8; PMN + A549, 49.6 ± 8.7 ng/mL IL-8). In the absence of LPS, no amplification of IL-8 synthesis was observed in the coculture system. Interestingly, the amplification of LPS-induced IL-8 synthesis was not observed when neutrophils and A549 cells were cocultured in a transwell system, preventing direct cell-cell contact (Fig. 2).

IL-6 Is Induced in A549-PMN Cocultures

When A549 cells were stimulated with LPS, only trace amounts of IL-6 could be detected (Table 1). In neutrophils, LPS also failed to activate IL-6 synthesis. However, in A549-neutrophil cocultures, large amounts of IL-6 were detected in the cell culture supernatants after endotoxin stimulation (Fig. 3). In contrast, in cocultures of A549 cells and neutrophils in the transwell system, in which direct cell-cell contact was prevented, no IL-6 was detected in response to LPS (Fig. 3).

A549 Cells Are the Cellular Origin of the Amplified Cytokine Generation Cocultures

In order to determine the cellular origin of the enhanced cytokine response in supernatants of A549 and neutrophil cocultures, we coincubated neutrophils with A549 cells for 8 hours, then harvested neutrophils and A549 cells separately, and quantified IL-8 and IL-6 mRNA by reverse transcription-PCR. By this experimental approach, A549 cells were clearly identified as the cellular source of the enhanced cytokine generation. In cocultures, transcription of the IL-8 mRNA in A549 cells was ~30-fold increased compared with monocultures of A549 cells (Fig. 4). In contrast, no significant regulation was evident in PMN in either system (data not shown). In A549 cells cocultured with neutrophils, an impressive upregulation of IL-6 mRNA synthesis was also observed (Fig. 4).

Table 1. IL-6 Release of A549 Cells in Response to LPS

t (h)	Control	LPS		
		0.1 μg/mL	1 μg/mL	10 μg/mL
8	12.2 ± 2.8	13.5 ± 4.5	30.0 ± 10.2	52.1 ± 11.9
12	17.74 ± 9.29	15.4 ± 3.5	30.6 ± 10.8	61.6 ± 18.5
24	28.66 ± 9.26	19.2 ± 2.9	35.7 ± 12.3	101.1 ± 15.4*

NOTE: A549 cells were plated on 24-well culture plates and incubated with LPS at various concentrations or sham-incubation was done. At the indicated time points, cell supernatants were harvested and IL-6 release is expressed in pg/mL. Data represent the mean ± SEM of at least four independent experiments, each done with duplicate values.
*Values which differed significantly from controls.

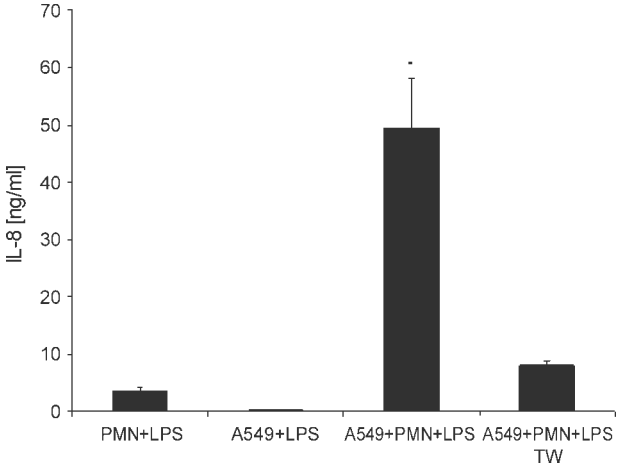

FIGURE 2. IL-8 release in response to LPS in A549-PMN cocultures. Neutrophils (PMN, 10^6/mL) and A549 cells were either cultured separately and stimulated with LPS (0.1 μg/mL) for 24 h or both cell types were cultured together and activated with LPS (0.1 μg/mL, A549 + PMN + LPS). In selected experiments, neutrophils were placed in the upper compartment of 0.4-μmol/L pore transwells and cocultured with A549 cells grown in the lower compartment of 24-well cell culture plates and stimulated with LPS (0.1 μg/mL, A549 + PMN + LPS, TW). After 24 h, cell supernatants were harvested and IL-8 synthesis was analyzed by ELISA. Columns, mean of at least five independent experiments, each done in duplicate; bars, SEM. *, $P < 0.05$, significantly different values.

Amplification of Cytokine Synthesis Was Dependent on TNF-α and COX-2 Activity

Although direct cell-to-cell contact was a prerequisite for the observed amplification of IL-8 and IL-6 synthesis in A549-PMN cocultures, we tried to identify soluble mediators possibly involved in this response. As depicted for LPS-activated IL-8 release in cocultures, blocking of TNF-α bioactivity by a neutralizing polyclonal anti–TNF-α antibody (1 μg/mL) resulted in an inhibition of IL-8 generation by ~50% (Fig. 5). Inhibition of COX was even more effective in this context: nonspecific inhibition of COX with indomethacin (100 μmol/L) resulted in the reduction of IL-8 synthesis in the coculture system to 27% and specific inhibition of COX-2 by NS-398 (10 μmol/L) was equally effective.

LPS-Induced IL-8 Synthesis Is Also Amplified in PMN-SCLC (H69) Cocultures

When neutrophils were cocultured with the SCLC-derived cell line H69, the amplification of IL-8 generation could be reproduced. As depicted in Fig. 6, significant amounts of IL-8 (5.2 ± 0.2 ng/mL) accumulated in the cell supernatant of this cell type. When cocultured with neutrophils, the LPS-induced IL-8 response was massively amplified (89.2 ± 35.6 ng/mL IL-8; Fig. 6). This ~10-fold amplification of LPS-induced IL-8 synthesis was comparable to the response observed in A549-PMN cocultures.

LTA Mimics the Effects of LPS in A549-PMN Cocultures

When LTA was used instead of LPS, amplified IL-8 synthesis was also detected in A549-PMN cocultures (Fig. 7). When A549 cells were incubated with LTA in the absence of PMN only trace amounts of IL-8 were detected (0.2 ± 0.1 ng/mL). Likewise, LTA was not a strong inducer of IL-8 synthesis in purified PMN (1.6 ± 0.4 ng/mL). However, when A549 cells were cocultured with PMN LTA induced marked liberation of IL-8 (8.1 ± 2.7 ng/mL).

Discussion

Frequent bacterial infections of the lung are associated with a poor prognosis in patients with lung cancer (1). Secretion of proinflammatory cytokines in response to bacterial toxins is part of the host response to bacterial infections, but has also been suggested to promote tumor cell proliferation and may thereby contribute to the poor prognosis of patients with lung cancer. In the present study, we showed that the capacity of lung cancer cells to release the proangiogenic and proproliferative cytokines IL-8 and IL-6 in response to the clinically relevant bacterial pathogens *E. coli* LPS and *S. aureus* LTA is critically determined by the presence of neutrophils. Moreover, we provide evidence that direct cell-cell contact is a prerequisite for enhanced cytokine generation, and TNF-α and COX-2 activity are major regulator molecules in this context.

The key finding of the present study is that massive amplification of cytokine synthesis in response to LPS was found in cocultures of A549 cells and neutrophils but not in monocultures. In monocultures of A549 cells or neutrophils, LPS induced a modest release of IL-8. However, in coculture, a massive (10-fold) amplification of IL-8 secretion was observed in response to LPS. To test whether this phenomenon was restricted to IL-8 synthesis, we also analyzed the production of IL-6. In A549 cells and in PMN in monocultures, no IL-6 was detected. In cocultures exposed to LPS, a massive induction of IL-6 was noted. This finding supports the notion that amplified cytokine synthesis is not limited to IL-8 production but may extend to other pathophysiologically relevant cytokines.

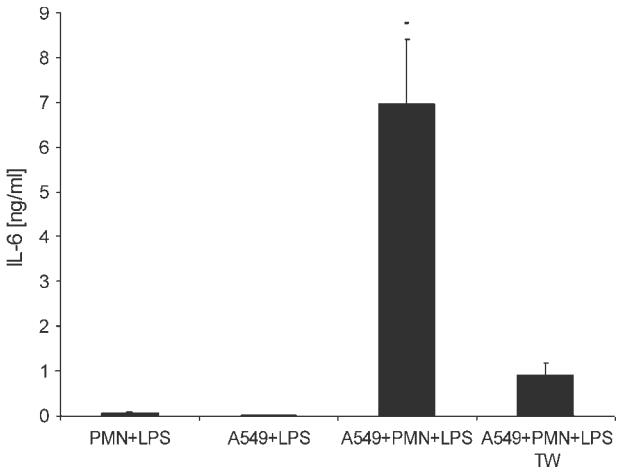

FIGURE 3. Induction of IL-6 in neutrophil-A549 cocultures. Neutrophils (PMN, 5×10^6/mL) and A549 cells were either cultured separately and stimulated with LPS (0.1 μg/mL) for 24 h or both cell types were cultured together and activated with LPS (0.1 μg/mL, A549 + PMN + LPS). In selected experiments, neutrophils were placed in the upper compartment of 0.4-μmol/L pore transwells and cocultured with LPS-activated A549 cells grown in the lower compartment of 24-well cell culture plates (A549 + PMN + LPS, TW). After 24 h, cell supernatants were harvested and IL-6 synthesis was analyzed by ELISA. Columns, mean of at least five independent experiments, each done in duplicate; bars, SEM. *, $P < 0.05$, significantly different values.

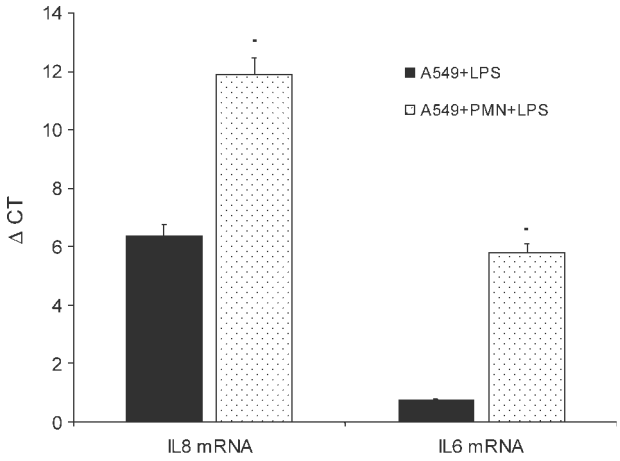

FIGURE 4. Effect of neutrophils on IL-8 and IL-6 mRNA expression in A549 cells. The A549 cells were activated with 0.1 μg/mL of LPS in the absence (A549 + LPS) or presence (A549 + PMN + LPS) of 5×10^6 PMN/mL. After 8 h of incubation, aliquots of 5×10^6 neutrophils and 10^5 A549 cells were harvested separately, and mRNA was extracted and subjected to quantitative reverse transcriptase PCR. The ΔC_T values, as depicted for A549 cells, represent relative expression of IL-8 and IL-6 mRNA normalized to the reference genes *HPRT* and *GAPDH*. Columns, mean, originating from three independent experiments, each done in duplicate; bars, SEM. *, $P < 0.05$, significantly different values from corresponding values without neutrophils.

Analysis of gene transcription clearly identified A549 cells as the cellular origin of enhanced cytokine generation in this coculture model. When A549 cells and neutrophils were separated at the end of the incubation period, a 30-fold upregulation of expression of IL-8 and IL-6 transcripts was observed in A549 cells. In contrast, expression of IL-8 mRNA was not enhanced, and no IL-6 mRNA was detected in neutrophils derived from these cocultures. This corroborates previous studies which reported on the lack of IL-6 gene expression in this cell type (30).

We have not elucidated the precise mechanisms of IL-8 amplification in our cocultures. However, direct cell-cell contact of neutrophils and A549 cells was a prerequisite for amplified IL-8 release in the LPS-stimulated cocultures. When A549 cells and neutrophils were cultured in transwells, which prevented direct cell-cell contact between the two cell types, the amplification of IL-8 release in response to LPS was abrogated. We do not have an explanation for this phenomenon. However, neutrophils have been shown to interact with A549 cells via binding of CD11/CD18 to intercellular adhesion molecule-1 (31), and ligation of intercellular adhesion molecule-1 on A549 cells might activate the mitogen-activated protein kinases ERK and JNK with subsequent secretion of chemokines such as IL-8 (32).

Apart from direct cell-cell interaction, TNF-α bioactivity was another prerequisite for amplified IL-8 release in our coculture model. When TNF-α was blocked by a neutralizing antibody, the amplified IL-8 synthesis was substantially reduced. Paracrine stimulation of A549 cells by TNF-α may offer an attractive explanation. First, TNF-α is known to stimulate the generation of IL-8 and IL-6 in NSCLC cells by paracrine pathways (19, 22, 33, 34). Second, although A549 cells do not produce TNF-α (35), they are known to express TNF receptor 1 (36). Third, the cellular source of TNF-α are most likely neutrophils which released TNF-α in response to LPS (151 ± 5 pg/mL upon stimulation with LPS, 0.1 μg after 24 hours) in the current study.

Apart from proinflammatory cytokines, COX-2 has been implicated as an important factor in lung cancer development and progression (23, 24). Against this background, we investigated whether amplified release of cytokines is modulated by COX-2 activity in our experimental setup. Interestingly, nonspecific inhibition of COX by indomethacin as well as specific inhibition of COX-2 activity by NS-398 equally blunted amplified IL-8 secretion in the coculture model. This is well in line with a recent report showing that COX-2 activity correlates with chemokine synthesis in NSCLC cell lines (37), and with another report showing that inhibition of COX-2 is associated with reduced activity of nuclear factor-κB activation and inhibition of JNK, p38MAPK, and ERK activity in NSCLC as a molecular prerequisite for cytokine generation (38). The particular importance of COX-2 in a coculture system challenged with LPS may be explained by the fact that LPS is known to activate phospholipase A_2 with subsequent release of free arachidonic acid from neutrophils (39). Adjacent A549 cells may internalize and metabolize AA by COX-2, thus synthesizing prostanoids such as prostaglandin E_2, which are relevant to tumor progression (40).

Interestingly, the amplification of cytokine release was not restricted to the NSCLC-derived A549 cells. In cocultures of SCLC-derived H69 cells, the release of IL-8 in response to LPS was also dramatically enhanced compared with neutrophils or H69 cells in monoculture. This is in line with a previous report which shows that elevated levels of IL-8 are actually found in patients with SCLC (41). At least *in vitro*, IL-8 may contribute to the rapid growth of SCLC cells (14).

Because pulmonary infections with Gram-positive bacteria are of increasing clinical relevance (2, 3), we further tested

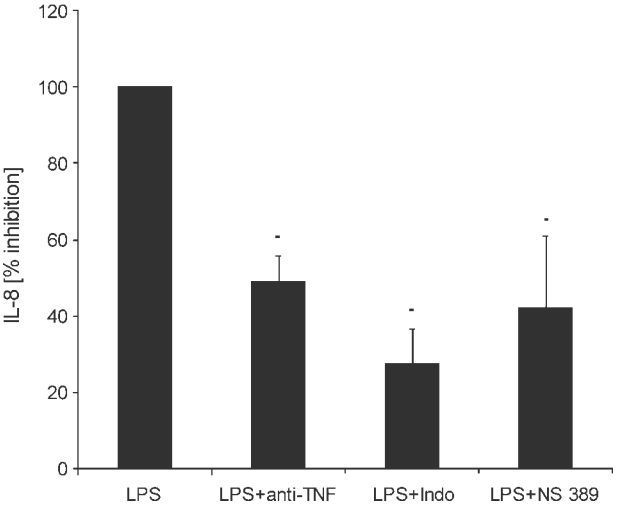

FIGURE 5. Effect of TNF and COX inhibitors on IL-8 synthesis in A549-PMN cocultures. Cocultures of neutrophils and A549 cells were activated with LPS (0.1 μg/mL) in the absence or presence of a neutralizing TNF antibody (anti-TNF, 10 μg/mL). COX was blocked by indomethacin (Indo, 100 μmol/L) or by the specific COX-2 inhibitor NS-398 (10 μmol/L). After 24 h, IL-8 release into the cell supernatant was analyzed. Values are given as percentage of LPS-induced IL-8 liberation in the absence of inhibitors. Columns, mean of at least four independent experiments, each done in duplicate; bars, SEM. *, $P < 0.05$, significantly different values from LPS-induced IL-8 release in the absence of inhibitors.

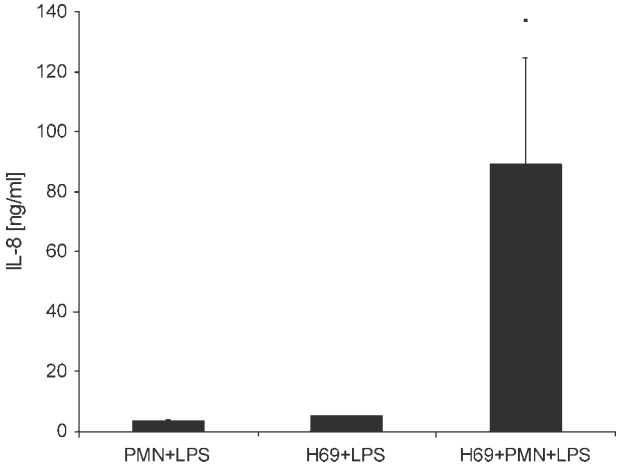

FIGURE 6. Effect of coculturing H69 cells and neutrophils on LPS-induced IL-8 release. Neutrophils (PMN, 5×10^6/mL) and cells from the SCLC line H69 (2×10^5/mL) were either cultured separately and stimulated with LPS (0.1 μg/mL) for 24 h or both cell types were cultured together and activated with LPS (H69 + PMN + LPS). After 24 h, cell supernatants were harvested and IL-8 synthesis was analyzed by ELISA. Columns, mean of at least five independent experiments, each done in duplicate; bars, SEM. *, $P < 0.05$, significantly different values.

whether amplification of IL-8 release also occurs in A549-PMN cocultures exposed to the Gram-positive pathogenic factor LTA, because LTA is probably the most important pathogen of Gram-positive germs, and is found in all clinically relevant strains. When incubated with LTA, a comparable upregulation of IL-8 release was observed in the cocultures. This effect is clearly attributable to LTA and not contaminating LPS because the isolation procedure of LTA renders a purity of >99% (42). The fact that higher concentrations of LTA compared with LPS were necessary to amplify IL-8 synthesis might reflect the fact that LTA requires costimulatory molecules to evoke full cellular activation (43).

In conclusion, we showed in the current study that cytokine generation of human lung cancer cells is strongly potentiated in the presence of neutrophils. Direct cell-to-cell contact is a prerequisite for this response, and key regulators of this amplified cytokine generation are TNF-α and COX activity. These findings further support the concept that the host response can promote tumor progression under conditions of inflammation and infection and may be taken into consideration when developing new therapies for lung cancer.

Materials and Methods

Materials

Ficoll-Paque was purchased from Pharmacia and FCS was from Greiner. All other media and supplements were purchased from Life Technologies unless otherwise indicated. The LPS (*E. coli*, 0111:B4) and indomethacin were purchased from Sigma, whereas NS-398 was from Calbiochem. All antibodies and recombinant cytokines used for cytokine ELISAs were purchased from R&D Systems: monoclonal antibodies against IL-6 (MAB 206) and IL-8 (MAB 208), biotinylated anti-human antibodies against IL-6 (BAF 260) and IL-8 (BAF 208), recombinant human IL-6 (206-IL-010) and IL-8 (208-TA-010), as well as the neutralizing anti-TNF antibody (AF-210-NA). Peroxidase-conjugated streptavidin (HRP) and ABTS were purchased from Zymed Laboratories. The RNeasy Mini Kit and QIAShredder columns were from Qiagen. The Platinum SYBR Green qPCR SuperMix UDG was purchased from Invitrogen. Cell culture plasticware was purchased from Falcon.

Isolation of Human Neutrophils

Polymorphonuclear cells (neutrophils, PMN) were isolated from venous blood from healthy donors by centrifugation over a Ficoll-Paque gradient as previously described (44). In brief, EDTA-anticoagulated blood was layered over Ficoll-Paque and centrifuged at 400 × *g* for 35 min. After removal of mononuclear cells, erythrocytes were sedimented in 1% polyvinyl alcohol. Residual erythrocytes were removed by hypotonic lysis, cells were washed twice in Ca^{2+}/Mg^{2+}-free PBS, and resuspended in RPMI containing 10% FCS, at 10^7 PMN/mL. Cell purity was >97%, as quantified by flow cytometry, and cell viability was >96%, as assessed by trypan blue dye exclusion.

Purification of LTA

LTA was isolated from *S. aureus* as previously described in detail, which yields a purity of >99% (42).

Cell Lines

The A549 human lung adenocarcinoma cell line and the H69 human small cell lung cancer cell line were obtained from the American Type Culture Collection and cultured at 37°C in a humidified atmosphere (95% air and 5% CO_2). The A549 cells were kept in DMEM/F12 supplemented with 10% FCS, 2 mmol/L of L-glutamine, 10^5 units/L of penicillin, and 100 mg/L of streptomycin. The H69 cells were maintained in RPMI 1640 supplemented with 2 mmol/L of L-glutamine, 10 mmol/L of HEPES, 1 mmol/L of sodium pyruvate, 4.5 g/L of glucose, 1.5 g/L of $NaHCO_3$, and 10% FCS. Cells were grown to

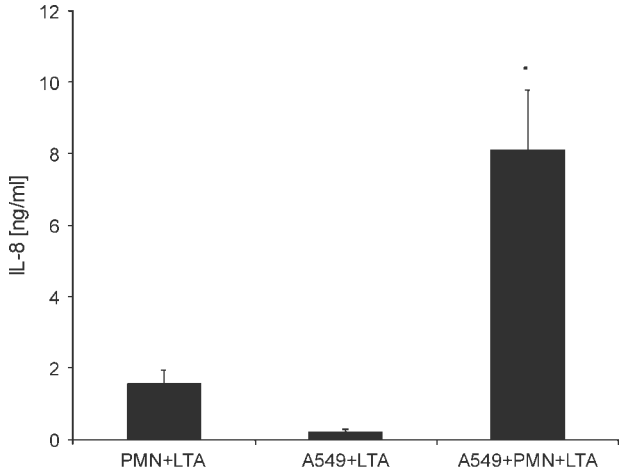

FIGURE 7. Effect of LTA on cytokine release in A549-PMN cocultures. Neutrophils (PMN, 5×10^6/mL) and A549 cells were either cultured separately and stimulated with LTA (10 μg/mL) for 24 h or both cell types were cultured together and activated with LTA (10 μg/mL, A549 + PMN + LTA). After 24 h, cell supernatants were harvested and IL-8 synthesis was analyzed by ELISA. Columns, mean of at least five independent experiments, each done in duplicate; bars, SEM. *, $P < 0.05$, significantly different values.

confluence and subcultured every 2 to 3 d, and split at a ratio of 1:10 for A549 cells and 1:4 for H69 cells.

Neutrophil-Tumor Cell Cocultures

The coculture experiments were done in 24-well cell culture plates (1 mL/well). The A549 cells were plated at a density of 10^5/mL in modified DMEM/F12 and H69 cells were resuspended at 2×10^5/mL in modified RPMI. After 24 h, medium was discarded, and cells were incubated in 1 mL of RPMI supplemented with 10% FCS. When indicated, neutrophils were directly added to the tumor cells at a final density of 5×10^6 PMN/mL. Cocultures were continuously shaken to prevent the aggregation of neutrophils. In selected experiments, neutrophils were not placed directly onto the tumor cells, but cocultured with A549 in a transwell system [700:300 μL (lower/upper) compartment; pore size, 0.4 μm]. For stimulation of cytokine synthesis, cells were activated with LPS or LTA at various concentrations and cell supernatants were harvested after 24 h of incubation, centrifuged at 13,000 × *g*, and stored at −80°C for further processing. In neutralization studies, a mouse monoclonal anti–TNF-α antibody which neutralizes TNF-α bioactivity (1 μg/mL), a nonspecific COX inhibitor, indomethacin (100 μmol/L), and the selective COX-2 inhibitor, NS-398 (10 μmol/L) were used.

Cytokine ELISAs

The release of IL-6 and IL-8 was determined in a direct sandwich ELISA. In brief, immunoassay plates were coated with mouse monoclonal anti-human IL-6 or IL-8 antibodies at a concentration of 4 μg/mL. After a blocking period, samples were added. Recombinant human IL-6 and IL-8 were used for standard titration curves. To sandwich the antigen, biotinylated antibodies were applied at 50 ng/mL of anti–IL-6 or 40 ng/mL of anti–IL-8. Plates were incubated with HRP-conjugated streptavidin followed by the addition of substrate (H_2O_2 and ABTS). Absorbance was measured at 450 nm in a microplate reader using SLT LabInstruments software to analyze the generated data. The IL-8 and IL-6 ELISA were sensitive to 15 pg/mL.

mRNA Extraction

For quantitation of cytokine mRNA, neutrophils and A549 cells were cocultured as described. In pilot experiments, assessing IL-8 and IL-6 mRNA after various time points (1, 4, 8, and 24 h), expression of cytokine mRNA plateaued after 8 h in both cell types, therefore, this incubation period was selected for further experiments. After an incubation period of 8 h, the neutrophil-containing supernatants were harvested and washed twice at 300 × *g*, whereas the A549 monolayer was washed twice, cells were digested with trypsin, washed again, and pelleted. Neutrophil contamination of A549 cells, as analyzed routinely by flow cytometry, was consistently <1%.

Aliquots of 5×10^6 neutrophils and 1×10^5 A549 cells were transferred into 1.5 mL reaction tubes. After centrifugation at 300 × *g*, the supernatant was removed and the pellet was lyzed in 350 μL of lysis buffer of the RNeasy Minikit, containing 3.5 μL of β-mercaptoethanol. After homogenization using QIAShredder columns, RNA extraction was done according to protocols. A DNase digestion of 20 min at 30°C was included. RNA was eluted with 30 μL of RNase-free water.

cDNA Synthesis and Real-time PCR

For cDNA synthesis, reagents and incubation steps were applied as described (45). An aliquot of 10 μL of each of the isolated mRNA were applied for reverse transcription. The reactions were set up with the Platinum SYBR Green qPCR SuperMix UGD, according to the protocols of the manufacturer. Using the oligonucleotide primer pairs given in Table 2, 0.5 μL of each primer (10 μmol/L) and 2 μL of cDNA were added to a final volume of 25 μL. Cycling conditions were adapted to 95°C for 6 min, followed by 45 cycles of 95°C for 5 s, 59°C for 5 s, and 72°C for 10 s. PCR products were routinely identified by 2.5% agarose gel electrophoresis.

Relative mRNA Quantitation

Relative mRNA quantitation was done by the Sequence Detection System 7900 (Applied Biosystems) and real-time PCR.

$$\frac{T_o}{R_o} = K \times (1+E)^{(CT,R-CT,T)}$$

where T_o, initial number of target gene mRNA copies; R_o, initial number of standard gene mRNA copies; E, efficiency of amplification; CT,T, threshold cycle of target gene; CT,R, threshold cycle of standard gene; and K, constant.

Based on the given equation, we used comparative quantitation (ΔC_T) normalizing IL-8 and IL-6 to two unregulated internal standard genes (45). Therefore, mRNA transcribed from the genes encoding hypoxanthine guanine phosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. In preliminary experiments, we could show that the amplification efficiencies of HPRT, GAPDH, IL-8, and IL-6 primer sets were approximately equal and amounted to 0.95 ± 0.02 (95 ± 2%). The constant, K, is assumed to be equal within a definite primer system and thus does not influence the comparison of calculated relative ratios. Due to the nonselective dsDNA binding of the SYBR Green, gel electrophoresis was done to confirm the exclusive amplification of the expected PCR product.

Table 2. Sequences and Amplicon Sizes of the Primers

Primer name	Sequence
HPRT amplicon size (94 bp)	
HPRT forward	5′-AGGAAAGCAAAGTCTGCATTGTT-3′
HPRT reverse	5′-GGCTTTGTATTTTGCTTTTCCA-3′
GAPDH amplicon size (87 bp)	
GAPDH forward	5′-CCACATCGCTCAGACACCAT-3′
GAPDH reverse	5′-AAAAGCAGCCCTGGTGACC-3′
IL-8 amplicon size (151 bp)	
IL-8 forward	5′-GCCTTCCTGATTTCTGCAGC-3′
IL-8 reverse	5′-CGCAGTGTGGTCCACTCTCA-3′
IL-6 amplicon size (104 bp)	
IL-6 forward	5′-AGCCAGAGCTGTGCAGATGAG-3′
IL-6 reverse	5′-TGGCATTTGTGGTTGGGTC-3′

NOTE: Selecting suitable intron-spanning primers, cDNA amplicon was much shorter than genomic DNA amplicon. Excluding falsification by amplification of possible pseudogene sequences, both primer sets were shown to detect no genomic DNA with cDNA sequence. The primer sets were selected to work under identical PCR cycling conditions, so that simultaneous amplification of HPRT, GAPDH, IL-6, and IL-8 was obtained in the same run.

Statistics

For statistical comparison, one-way ANOVA was done, followed by Tukey's honestly significant difference test when appropriate. $P < 0.05$ levels were considered to be significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Anlage 9

Hattar K, Franz K, Ludwig M, Sibelius U, Wilhelm J, Lohmeyer J, Savai R, Subtil FS, Dahlem G, Eul B, Seeger W, Grimminger F, Grandel U.

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ORIGINAL ARTICLE

Interactions between neutrophils and non-small cell lung cancer cells: enhancement of tumor proliferation and inflammatory mediator synthesis

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Abstract The inflammatory tumor microenvironment plays a crucial role in tumor progression. In lung cancer, both bacterial infections and neutrophilia are associated with a poor prognosis. In this study, we characterized the effect of isolated human neutrophils on proliferation of the non-small cell lung cancer (NSCLC) cell line A549 and analyzed the impact of A549–neutrophil interactions on inflammatory mediator generation in naive and lipopolysaccharide (LPS)-exposed cell cultures. Co-incubation of A549 cells with neutrophils induced proliferation of resting and LPS-exposed A549 cells in a dose-dependent manner. In transwell-experiments, this effect was demonstrated to depend on direct cell-to-cell contact. This pro-proliferative effect of neutrophils on A549 cells could be attenuated by inhibition of neutrophil elastase activity, but not by oxygen radical neutralization. Correspondingly, neutrophil elastase secretion, but not respiratory burst, was specifically enhanced in co-cultures of A549 cells and neutrophils. Moreover, interference with COX-2 activity by indomethacin or the specific COX-2 inhibitor NS-398 also blunted the increased A549 proliferation in the presence of neutrophils. In parallel, a massive amplification of COX-2-dependent prostaglandin E_2 synthesis was detected in A549–neutrophil co-cultures. These findings suggest that direct cell–cell interactions between neutrophils and tumor cells cause release of inflammatory mediators which, in turn, may enhance tumor growth in NSCLC.

Keywords Lung cancer · Neutrophils · A549 cells · Inflammation · Elastase · COX-2

Abbreviations

AA	Arachidonic acid
BSA	Bovine serum albumin
COX	Cyclooxygenase
FCS	Fetal calf serum
fMLP	*n*-Formyl-methionyl-leucyl-phenylalanine
FSC	Forward scatter
HHBSS	Hepes-buffered hanks' balanced salt solution
LPS	Lipopolysaccharide
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NSCLC	Non-small cell lung cancer
O_2^-	Superoxide anion
PE	Phycoerythrin

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PGE_2	Prostaglandin E_2
PMN	Polymorphonuclear leukocyte
SSC	Side scatter
TAN	Tumor-associated neutrophils

Introduction

Inflammatory cells and mediators of the tumor microenvironment may play a critical role in lung cancer progression. In 1863, Virchow detected leukocyte infiltration of neoplastic tissues and proposed a relationship between inflammation and cancer [1, 2]. Currently, the significance of this cancer-related inflammation has been outlined by defining inflammation as the seventh hallmark of cancer [3]. Although an established role in tumor progression has been attributed to macrophages and dendritic cells, the role of infiltrating neutrophils is less well defined [4–6].

Historically, neutrophils were exclusively regarded as part of the unspecific host defense against tumor cells. However, some recent studies gave evidence that tumor-associated neutrophils (TAN) may conversely play a decisive role in tumor progression.

Elevated systemic neutrophil counts [7] and high intratumoral leukocyte levels [8] have been identified as independent prognostic factors associated with a high relapse rate and a poor overall survival. Moreover, the intratumoral density of neutrophils has been shown to correlate with adverse prognostic factors such as elevated CRP levels [9]. In murine models of lung and pancreatic islet cell, cancer neutrophil depletion resulted in enhanced tumor growth under physiological conditions [10, 11]. Recently, it was proposed that the role of neutrophils in tumor biology is determined by their phenotypes, which may shift from an anti-tumor (N1-TAN) to a pro-tumor phenotype (N2-TAN) depending on cytokines of the tumor microenvironment [10, 12, 13]. The mechanisms leading to tumor promotion may include activation of the neutrophils` inflammatory potential such as the release of MPO and serine proteases from neutrophil granule contents [14–16]. Serine proteases may, on the one hand, induce tumor promotion by tissue degradation, thus facilitating invasion and spread of tumor cells [15]. On the other hand, in a murine model of lung adenocarcinoma, neutrophil elastase modified intracellular signaling pathways of tumor cells in a pro-tumorgenic way [16]. Beside the release of granule proteins, activated neutrophils release the reactive oxygen species superoxide anion (O_2^-) and hypochlorus acid. In lower concentrations, these substances may not be cytotoxic but genotoxic, thus promoting tumor progression [17].

In addition to releasing pre-stored secretory products, neutrophils synthesize arachidonic acid-derived lipid mediators, such as the 5-lipoxygenase-dependent leukotrienes and the cyclooxygenase (COX)-derived prostanoid prostaglandin E_2 (PGE_2). The inducible isoform of COX, COX-2, may be crucially involved in lung cancer pathogenesis: in vivo, COX-2 protein and mRNA levels are elevated and are associated with a poor outcome in lung adenocarcinoma [18, 19]. In vitro, over expression of COX-2 directly increases survival of lung adenocarcinoma cells lines [20].

Although strong evidence exists that infiltrating neutrophils play a decisive role in non-small cell lung cancer (NSCLC) progression [7–13], a direct pro-proliferative effect of isolated neutrophils on tumor cells in vitro has never been described. To mimic the interactions between neutrophils and NSCLC cells during pulmonary infection, we co-cultured freshly isolated human neutrophils with NSCLC cells of the human A549 adenocarcinoma cell line in the absence or presence of low doses of endotoxin (LPS). In essence, we found that neutrophils dose-dependently induce proliferation of unstimulated and LPS-exposed NSCLC cells, and the release of neutrophil elastase and COX-2 products were causally involved in this process.

Materials and methods

Isolation of human neutrophils

Neutrophils were isolated from venous blood of healthy donors by centrifugation over a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden). In brief, EDTA-anticoagulated blood was sedimented with 10 % dextran T 500 (Pharmacia) for 20 min. The neutrophil-containing supernatant was then layered over Ficoll-Paque and centrifuged at $400\times g$ for 20 min. After removal of the mononuclear cell band, residual erythrocytes were removed by hypotonic lysis, cells were washed twice in Ca^{++}/Mg^{++}-free Hepes-buffered Hanks' balanced salt solution (HHBSS−, no Calcium, no Magnesium, no phenol red, Gibco, Eggenstein, Germany), and finally resuspended in RPMI containing 1 % FCS at 10^7 PMN/ml for proliferation experiments or in phenol red-free HHBSS containing Ca^{++} (1.25 mM)/Mg^{++} (0.5 mM) (HHBSS++, Gibco, Eggenstein, Germany) for the assessment of respiratory burst and elastase release.

Flow cytometry

Purity of neutrophils was determined by flow cytometry analysis (BD FACSCanto, BD Biosciences, Heidelberg, Germany) using forward (FSC) and side (SSC) scatter characteristics and CD24 as neutrophil marker known to be expressed on mature neutrophils and on B lymphocytes. The cells were pelleted, resuspended in phosphate-buffered saline (PBS) containing 1 % bovine serum

albumin (BSA), and incubated with a murine anti-human CD24 antibody conjugated to phycoerythrin (PE) and FITC-conjugated murine anti-human CD14-antibodies (BD Biosciences, Heidelberg, Germany) for 15 min. As negative control, murine anti-human immunoglobulins G_1 (IgG_1)–FITC/IgG_2–PE (Simultest Control, BD, Heidelberg, Germany) were used. After the incubation period of 15 min in darkness, cells were washed again with 1 % PBS/BSA and were analyzed immediately using DIVA Software [21]. A total of 97 to >98 % of the isolated cells showed neutrophil FSC/SSC profiles and expressed CD24.

Cell staining and viability

Additionally, neutrophil purity was confirmed by performing May–Gruenwald–Giemsa staining (Merck, Darmstadt, Germany). Staining revealed a purity of 96–97 % and showed that contaminating mononuclear cells amounted to <0.5 %. Cell viability of freshly isolated as well as of neutrophils cultured for 6 h in vitro was >96 %, as assessed by trypan blue dye exclusion.

Cell culture

The A549 human lung adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured at 37 °C in a humidified atmosphere (95 % air, 5 % CO_2). A549 cells were kept in Dulbecco's modified Eagle's medium (DMEM/F12, Gibco, Eggenstein, Germany) supplemented with 10 % fetal calf serum (FCS, Greiner, Frickenhausen, Germany) 2 mM L-glutamine, 10^5 U/l penicillin, and 100 mg/l streptomycin. Cells were grown to confluence and subcultured every 2–3 days, at a split ratio of 1:10. Cell viability of A549 cells in culture was regularly assessed by trypan blue dye exclusion and was always >97 %.

Cell culture plasticware was purchased from Falcon (Mannheim, Germany).

Neutrophil/A549 co-culture for the assessment of A549 proliferation and PGE_2 release

The co-culture experiments were performed in 24-well cell culture plates (1 ml/well) at 37 °C in a humidified atmosphere (95 % air, 5 % CO_2). A549 cells were plated at a density of 10^5/ml in modified DMEM/F12. After 24 h, medium was harvested, and cells were incubated in 1 ml RPMI supplemented with 1 % FCS or in 1 ml HHBSS++ (assessment of elastase and O_2^- release). When indicated, neutrophils were directly added to the tumor cells at given densities (varying from 0.5–10 × 10^6 PMN/ml). Co-cultures were continuously shaken to prevent aggregation of neutrophils. In selected experiments, neutrophils were not placed directly onto the tumor cells, but co-cultured with A549 in a transwell system (700 µl/300 µl lower: upper compartment, pore size 0.4 µm). When indicated, LPS was simultaneously applied to neutrophil addition. In neutralization studies, the unspecific COX-inhibitor indomethacin (100 µM, Calbiochem, La Jolla, CA, USA), the selective COX-2 inhibitor NS-398 (10 µM, Calbiochem, La Jolla, CA, USA), the elastase inhibitor AAPVCK (5 µM) or the oxygen radical scavenger SOD (10 µg/ml, Sigma, Deisenhofen, Germany) were given simultaneously to neutrophil addition.

Neutrophil/A549 co-culture for the assessment of neutrophil elastase release and respiratory burst

The co-culture experiments were performed in 24-well cell culture plates (1 ml/well) at 37 °C in a humidified atmosphere (95 % air, 5 % CO_2). A549 cells were plated at a density of 10^5/ml. Cells were grown to confluence. Immediately before neutrophil addition, medium was harvested and cells were kept in HHBSS++. Freshly isolated neutrophils were directly added to A549 cells (7.5 × 10^6/ml in HHBSS++) to a total volume of 1 ml/well. Co-cultures were continuously shaken to prevent aggregation of neutrophils.

Neutrophil monocultures

Freshly isolated neutrophils (7.5 × 10^6/ml) were incubated on 24-well cell culture plates in 1 ml RPMI supplemented with 1 % FCS (for the assessment of proliferation and PGE_2 release) or in HHBSS++ at 37 °C in a humidified atmosphere (95 % air, 5 % CO_2) for the assessment of neutrophil elastase release and respiratory burst. Neutrophils were continuously shaken to prevent aggregation. To exclude that neutrophils were activated by shaking, basal release of elastase and PGE_2 was monitored compared to non-shaking conditions. Shaking did not activate neutrophils, as basal release of elastase was even lower under non-shaking conditions (0.46 vs. 0.75 U/ml, $p < 0{,}05$) and basal PGE_2 release did not differ between the two groups (105 pg/ml under non-shaking and 117 pg/ml under shaking conditions, $p = 0.54$).

A549 monoculture

The monoculture experiments were performed to assess the effect of PGE_2 and elastase on A549 growth in the absence of neutrophils. For that purpose, A549 cells were plated at a density of 10^5/ml in modified DMEM/F12 on 24-well cell culture plates (1 ml/well) at 37 °C in a humidified atmosphere (95 % air, 5 % CO_2). After 24 h, medium

was harvested, and cells were incubated in 1 ml RPMI supplemented with 1 % FCS. When indicated, cells were either sham-incubated (control) or exposed to PGE_2 (500 pg/ml, Cayman Chemical, MI, USA) or human Elastase (40 nM, Innovative Research, MI, USA) for 6 h, and MTS assay was performed as described below.

MTS assay

The MTS assay (CellTiter 96@ Aqueous One Solution Cell Proliferation Assay, Promega, Mannheim, Germany) quantifies the metabolic activity of cells. This assay is based upon the cleavage of the yellow 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) to purple formazan by metabolic active cells. The production of the colored formazan product is directly proportional to the number of viable cells in culture [22]. Based on these data, the MTS assay is widely used for the assessment of cellular proliferation. In brief, A549 cells were seeded on 24-well plated and maintained in culture for 24 h. Then, medium was exchanged to RPMI containing 1 % FCS, and neutrophils (0.5–10 × 10^6 PMN/ml) were added to a total volume of 1 ml/well. When indicated, co-cultures were stimulated with LPS (0.1 µg/ml *E. coli* LPS 0111:B4, Sigma, Deisenhofen, Germany). As negative controls, A549 cells were incubated in the absence of neutrophils with or without endotoxin stimulation (controls). After 6 h of incubation, the neutrophil containing supernatant was removed, cells were washed three times, and A549 cells were supplied with fresh medium (RPMI with 1 % FCS); 75 µl of MTS solution was added to each well to a total volume of 500 µl, and plates were again incubated for 2.5 h at 37 °C. Absorbance was read at 490 nm, background readings were subtracted from the sample wells and data were expressed as percentage of controls (A549 cells without neutrophils in the absence or presence of LPS). All samples were run in triplicates and all measurements were performed twice after 2.5 h of incubation with the MTS reagent. All data were expressed as percentage increase in MTS activity compared to unstimulated cells (controls) which were set to 100 %. In pilot experiments, monocultures of neutrophils (0.5–10 × 10^6 PMN/ml) were run in parallel to co-cultures as an additional internal control. They were incubated on 24-well plates in the absence or presence of LPS. After 6 h of incubation, the same washing procedures were performed as described above. Fresh medium containing 75 µl of MTS solution was added to each well to a total volume of 500 µl, and samples were again incubated for 2.5 h. No MTS activity of monocultured neutrophils was detected in these studies.

Superoxide anion generation

Neutrophil O_2^- generation was assessed as superoxide dismutase-inhibitable reduction of cytochrome *C* according to Cohen [23]. Monocultures of neutrophils or co-cultures of neutrophils with A549 cells were activated with the chemotactic peptide n-formyl-methionyl-leucyl-phenylalanine (fMLP, 1 µM) for 10 min in HHBSS++. Duplicate reaction mixtures containing 75 µM ferricytochrome in the presence or absence of 10 µg/ml superoxide dismutase were performed. Incubations were terminated by centrifugation at 4 °C at 1,200×*g*. O_2^- release was quantified as relative extinction at 550 nm in an Uvicon Spectrophotometer.

Release of elastase

Elastase enzyme activity was measured by monitoring the turnover of L-pyroglutamyl-L-propyl-L-valine-*p*-nitroanilide at 405 nm according to the method described by Kramps [24]. For induction of elastase release, monocultures of neutrophils or co-cultures of neutrophils with A549 cells were activated with fMLP (1 µM) for 10 min in HHBSS++. Incubations were terminated by centrifugation at 4 °C at 1,200×*g*. The cell-free supernatant was harvested and analyzed for elastase activity in an Uvicon Spectrophotometer as described above.

Release of PGE_2

PGE_2 was quantified in a commercial ELISA-system (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions and was expressed in pg/ml. For these experiments, A549 monocultures, PMN monocultures and co-cultures were activated with LPS (0.1 µg/ml) for 6 h. Culturing and stimulation of these cells was done in RPMI containing 1 % FCS in 24-well culture plates at a total volume of 1 ml. At the end of the incubation period, cell supernatants were harvested, cell debris was removed by centrifugation at 13,000×*g*, and samples were stored at −20 °C until further processing. All samples were performed as duplicates and each sample was measured twice.

Statistics

Data were analyzed by linear models using R [25]. Residuals were checked for possible deviations from normal distribution and heteroscedasticity. Results given in the text are mean and 95 % confidence (95 % CI) intervals.

For Fig. 1, several models were fitted. Dose dependency was modeled as MTS activity versus log PMN concentration for concentrations $<10^7$ ml^{-1}. In all other models, the PMN concentration was used as categorical predictor.

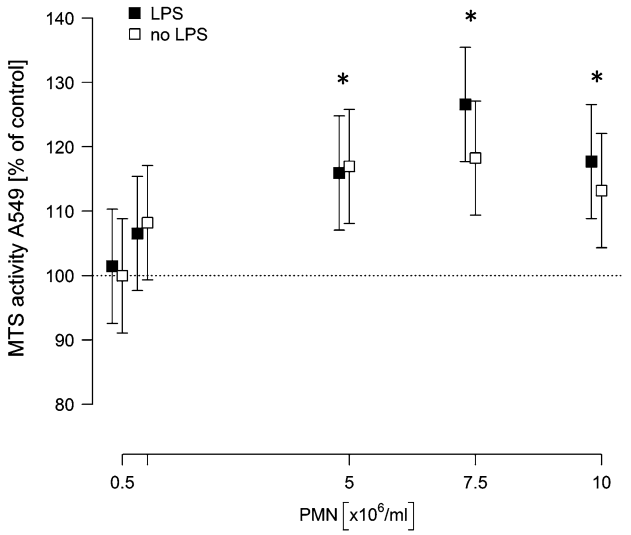

Fig. 1 Neutrophil induce a dose-dependent proliferation of A549 cells. A549 cells were co-incubated with isolated neutrophils at given concentrations in the absence or presence of LPS [0.1 μg/ml] in a total volume of 1 ml. 6 h after incubation, the neutrophil-containing supernatant was removed, cells were washed three times, and 500 μl of fresh medium were added containing 75 μl MTS solution. After 2.5 h, absorbance was read at 490 nm. Values are expressed as percentage of MTS activity of A549 cells in the absence of neutrophils, which was set to 100 %. Means and 95 % confidence intervals of at least five independent experiments, each performed in triplicates, are given. *Asterisks* indicate $p < 0.05$ for the comparisons to the respective controls

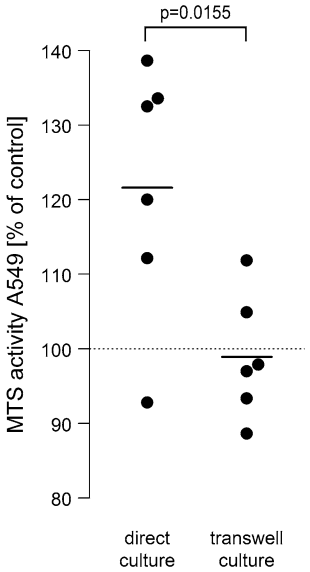

Fig. 2 Direct cell-to-cell contact is a prerequisite for neutrophile-induced A549 proliferation. A549 cells were either directly co-incubated with neutrophils (7.5×10^6/ml) or neutrophils were placed in the upper compartment of 0.4 μM pore transwells and co-cultured with A549 cells grown in the lower compartment of 24-well cell culture plates. All co-cultures were stimulated with 0.1 μg/ml LPS. After 6 h, the neutrophil-containing cell supernatant or the upper compartment were removed, A549 cells were washed three times, and again incubated for 2.5 h with 500 μl fresh medium containing 75 μl MTS solution. Absorbance was read at 490 nm. Values are expressed as percentage of MTS activity of A549 cells in the absence of neutrophils, which was set to 100 %, as indicated by the *horizontal dotted line*. *Horizontal bars* indicate averages of six independent experiments, each performed in triplicates

Results

Neutrophils induce proliferation of NSCLC cells in a dose-dependent manner

A549 monolayers were incubated with increasing concentrations of neutrophils (0–10 × 10^6/ml) in the presence or absence of 0.1 μg/ml LPS for 6 h. Both in naive and in LPS-stimulated co-cultures, neutrophils dose-dependently increased the proliferation rate of A549 cells, which is expressed as the percentage of MTS activity of A549 monocultures (Fig. 1). The pro-proliferative effect of neutrophils was observed in unstimulated as well as in LPS-stimulated co-cultures. For neutrophil concentrations of 5×10^6 PMN/ml, the activity was increased by 16 % independent of the presence of LPS. However, at PMN concentrations above 5×10^6/ml, the stimulatory effect was slightly more pronounced in the presence of LPS (27 vs. 18 % for 7.5×10^6 PMN/ml and 17 vs. 13 % for 10×10^6 PMN/ml).

Since maximum proliferation of A549 cells was induced by 7.5×10^6 PMN/ml in the presence of LPS (increase by 27 %) (95 % CI 18…35, $p < 0.001$), all further experiments were performed under these conditions.

Direct cell-to-cell contact between neutrophils and NSCLC cells is mandatory for proliferation

A549 monolayers were either directly incubated with 7.5×10^6 PMN/ml or co-cultured in a transwell system with A549 cells seeded in the lower and neutrophils in the upper compartment. All cultures were treated with LPS (0.1 μg/ml). Interestingly, the pro-proliferative effect of neutrophils was suppressed when direct cell-to-cell contact was prevented under the given experimental conditions. In direct co-culture, MTS activity increased by 21.6 % as compared to −1 % in the transwell system (Fig. 2).

Neutrophil elastase, but not oxygen radicals mediate the neutrophile-induced proliferation of A549 cells

To further elucidate the mechanisms in neutrophil-induced proliferation of A549 cells, neutrophil-derived inflammatory mediators were inhibited. In the presence of the highly specific inhibitor of neutrophil elastase AAPVCK, neutrophil-induced enhanced proliferation of A549 was prevented in the co-culture system (from 124 to 93 %). In contrast to

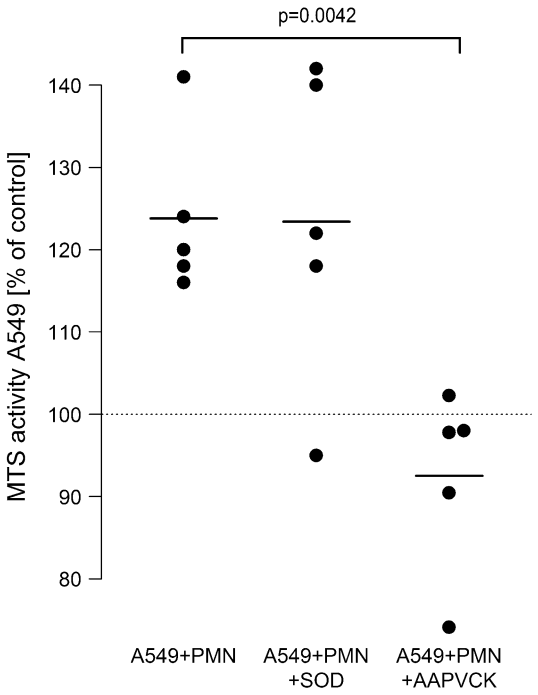

Fig. 3 Neutrophil elastase, but not oxygen radical formation, is involved in neutrophil-induced A549 proliferation. LPS-activated [0.1 µg/ml] A549 cells were co-incubated with isolated neutrophils (7.5×10^6/ml) in the absence (A549 + PMN) or presence of the oxygen radical scavenger SOD (10 µg/ml, A549 + PMN + SOD) or the elastase inhibitor AAPVCK (5 µM, A549 + PMN + AAPVCK). 6 h after incubation, the neutrophil-containing supernatant was removed, cells were washed three times, and 500 µl of fresh medium were added containing 75 µl MTS solution. After 2.5 h, absorbance was read at 490 nm. Values are expressed as percentage of MTS activity of A549 cells in the absence of neutrophils, which was set to 100 % as indicated by the *horizontal dotted line*. *Horizontal bars* indicate averages of five independent experiments, each performed in triplicates

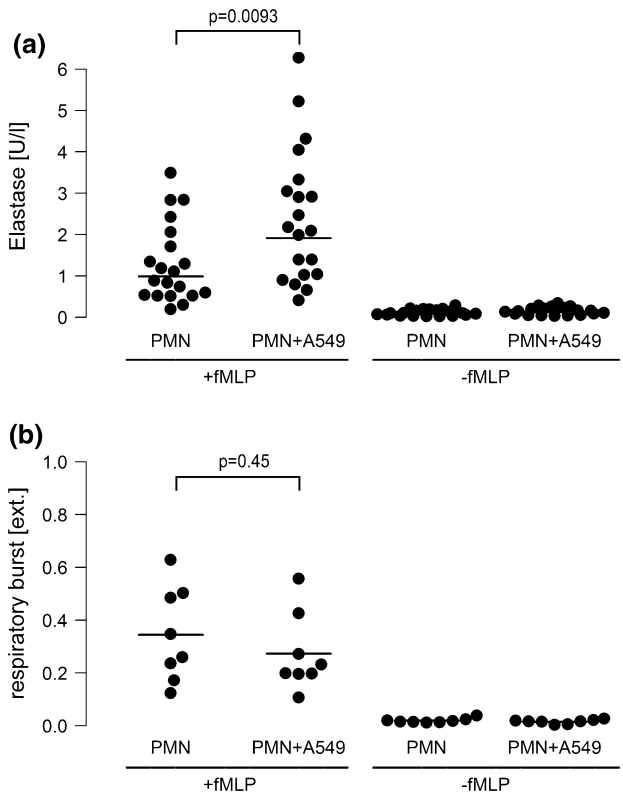

Fig. 4 Release of neutrophile elastase, but not of oxygen radicals is increased in neutrophil-A549 co-cultures. Monocultures of neutrophils (7.5×10^6/ml) or co-cultures of neutrophils (7.5×10^6/ml) and A549 cells (PMN + A549) were activated with fMLP (1 µM). Experiments were performed in the presence of LPS [0.1 µg/ml]. **a** Elastase release: 10 min after fMLP stimulation, cell supernatants were collected and centrifugation was performed at 4 °C at 1,200×*g*. The cell-free supernatant was harvested and analyzed for elastase activity in an Uvicon Spectrophotometer. Horizontal bars indicate averages of twenty independent experiments. **b** Respiratory burst: duplicate reaction mixtures containing 75 µM ferricytochrome in the presence or absence of 10 µg/ml superoxide dismutase were performed. 10 min after fMLP stimulation, incubations were terminated by centrifugation at 4 °C at 1,200×*g*. O_2^- release was quantified as relative extinction at 550 nm in an Uvicon Spectrophotometer. *Horizontal bars* indicate averages of eight independent experiments

this, neutralization of oxygen radicals by superoxide-dismutase had no detectable effect (Fig. 3). Addition of exogenous elastase (40 nM) to monocultures of A549 cells provoked an increase in proliferative activity to 115 % (95 % CI 110…121, $p < 0.001$).

Interestingly, fMLP-induced elastase secretion from neutrophils was doubled when co-cultured with A549 cells in the presence of LPS, while O_2^- release from neutrophils remained almost unchanged (Fig. 4a, b). This corresponds well to the inefficiency of oxygen radical neutralization to attenuate proliferation of A549 cells.

COX-2 activation is operative in PMN-mediated proliferation of A549 cells, and COX-2-derived PGE_2 is massively amplified in neutrophil-A549 co-cultures

To evaluate the role of COX-2 activation, the role of this isoenzyme was defined in LPS-stimulated neutrophil-A549 co-cultures. In these experiments, A549 proliferation was raised to 129 %. Unspecific COX inhibition with indomethacin as well as specific interference with COX-2 by NS-398 completely blocked the pro-proliferative effect of neutrophils on A549 cells to basline levels. Average proliferation was 91 % in indomethacin-treated and 102 % in NS-398-treated co-cultures (Fig. 5).

Moreover, when analyzing cell supernatants for COX-2-derived lipid mediators, we found a nearly tenfold amplification of PGE_2 in supernatants of co-cultured cells as compared to PGE_2 released from monocultured neutrophils or A549 cells (Fig. 6). This amplification of PGE_2 in neutrophil–A549 co-cultures was dependent on COX-2

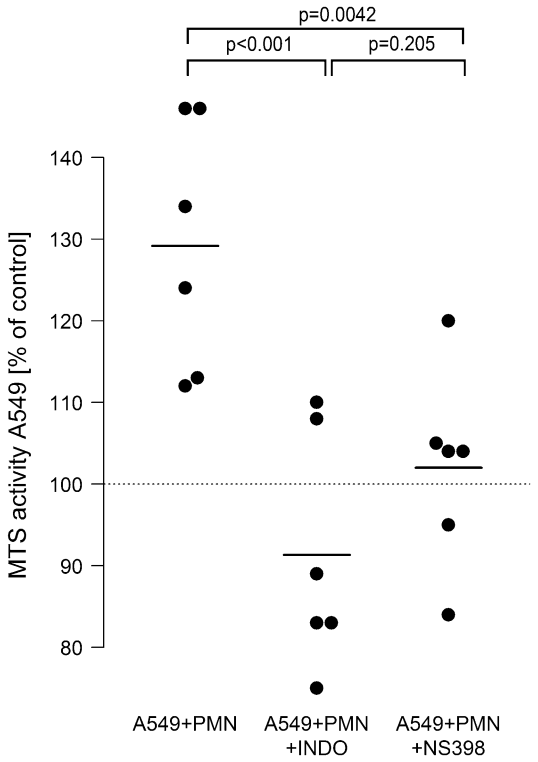

Fig. 5 COX-2 activation is involved in neutrophil-induced A549 proliferation. LPS-activated [0.1 µg/ml] A549 cells were co-incubated with isolated neutrophils (7.5×10^6/ml) in the absence (A549 + PMN) or presence of the unspecific COX-inhibitor indomethacin (100 µM) (A549 + PMN + INDO) or the selective COX-2 inhibitor NS-398 (10 µM) (A549 + PMN + NS398). 6 h after incubation, the neutrophil-containing supernatant was removed, cells were washed three times, and 500 µl of fresh medium were added containing 75 µl MTS solution. After 2.5 h, absorbance was read at 490 nm. Values are expressed as percentage of MTS activity of A549 cells in the absence of neutrophils, which was set to 100 % as indicated by the *horizontal dotted line*. *Horizontal bars* indicate averages of six independent experiments, each performed in triplicates

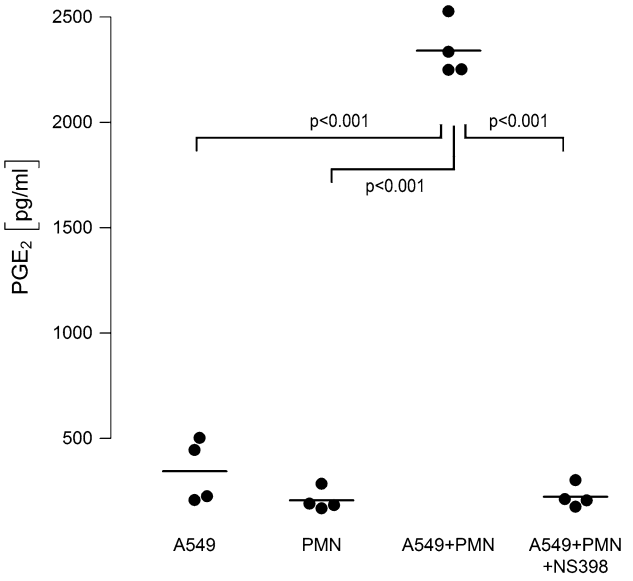

Fig. 6 COX-2-dependent PGE_2 release is amplified in A549–PMN co-cultures. Neutrophils (PMN, 7.5×10^6/ml) and A549 cells were cultured separately and stimulated with LPS [0.1 µg/ml], or both cell types were kept in co-cultures and activated with LPS. Co-cultures were performed in the absence (A549 + PMN) or presence (A549 + PMN + NS398) of the COX-2 inhibitor NS398 (10 µM). After 6 h, cell supernatants were harvested and PGE_2 synthesis was analyzed by ELISA. *Horizontal bars* indicate averages of four independent experiments, each performed in duplicates

activation, as PGE_2 was down-regulated to levels found in monocultures in the presence of the non-specific COX-inhibitor indomethacin (not shown) and the specific COX-2 inhibitor NS-398 (Fig. 6). Interestingly, addition of exogenous PGE_2 (500 pg/ml) induced an increase in proliferative activity of A549 monocultures to 121 % (95 % CI 108…134, $p = 0.007$) versus unstimulated controls.

Discussion

The inflammatory tumor microenvironment plays a crucial role in promotion and progression of tumor growth. Although neutrophilia and neutrophil tumor infiltration are frequently encountered in solid tumors like NSCLC [26, 27], the role of neutrophils in tumor biology remains unclear. In the current study, addition of neutrophils to the adenocarcinoma cell line A549 enhanced proliferation of tumor cells. Proliferation of A549 cells in co-cultures was accompanied by a release of elastase and COX-2-derived PGE_2, and inhibition of these mediators abolished tumor cell proliferation.

In our experimental setup, neutrophils dose-dependently increased the proliferation of A549 cells as quantified by an increase in MTS activity, which is directly proportional to cellular proliferation [22]. This increase in MTS activity was clearly related to A549 proliferation and not to co-incubated neutrophils. First, after 6 h of co-incubation, all neutrophils were removed by extensive washing procedures before the MTS assay was performed. Second, in pilot experiments, monocultures of neutrophils were run in parallel to co-cultures and no MTS activity was detectable in naive or LPS-stimulated PMN. Third, the increase in MTS activity in co-cultures was inhibited by antagonizing inflammatory mediators, such as COX-2-products, that are known to induce proliferation of A549 cells. And fourth, it has been shown that even when stimulated with proinflammatory agents, neutrophils have no proliferative capacity [28, 29]. Taken together, neutrophils exert a strong pro-proliferative effect on A549 cells in our experimental setup.

The effect of neutrophils on A549 proliferation was clearly dependent on the cell number of neutrophils used.

The highest proliferation rate was elicited by 7.5×10^6 PMN/ml. Greater neutrophil numbers failed to further enhance A549 proliferation. Given that proliferation of A549 was actually caused by neutrophil-derived mediators, this may, on the one hand, be explained by the fact that 7.5×10^6 PMN/ml were sufficient to induce maximal A549 cell proliferation. On the other hand, we have previously shown that activity of neutrophils is closely regulated by their cell density and is down-regulated when cell density exceeds a critical number [30]. Whether the neutrophil concentrations currently used actually mimic those found in the tumor microenvironment in vivo cannot be deducted as only few studies address the neutrophil concentration in lung tumors in vivo [8]. However, the actual PMN/A549 ratio from ~30:1 was also chosen in other studies investigating the effect of isolated neutrophils on A549 biology [31].

In our experimental setup, the maximum proliferation of A549 cells was noted when PMN–A549 co-cultures were additionally exposed to low doses of endotoxin. This may relate to the phenomenon of "neutrophil priming." Priming substances like cytokines or LPS render quiescent neutrophils more susceptible to secondary stimuli such as fMLP, bacterial exotoxins, or cell–cell contacts without activating them by themselves [32]. Such a priming effect may not only be operative in vitro, but may also be relevant in vivo. Lung cancer patients frequently suffer from gram-negative pulmonary infections, e.g., elicited by *E. coli* and *Haemophilus influenzae* [33–35], which contain LPS in their outer membrane. Therefore, the simultaneous exposure of lung cancer cells to neutrophils and LPS is very likely to occur in lung cancer patients.

Investigating the mechanism of neutrophil-induced A549 proliferation, we found that direct cell–cell contact between neutrophils and A549 cells was a prerequisite for amplified A549 proliferation in co-cultures. When A549 cells and neutrophils were cultured in transwells, which prevented direct cell–cell contact between the two cell types, enhanced proliferation of A549 cells was abrogated. We do not have an exact explanation for this phenomenon. However, neutrophils are capable to interact with A549 cells via binding of CD11/CD18 to ICAM-1 [36, 37], and ligation of ICAM-1 on A549 cells may directly activate intracellular signaling pathways (e.g., the MAP kinases ERK and JNK) with subsequent induction of cellular proliferation [38, 39]. Moreover, it has been shown that ligation of β_2-integrins on neutrophils induces activation of their proinflammatory potential, including release of elastase and lipid mediators [40, 41], which were identified as strong promotors of A549 proliferation in our co-culture model. This might offer an attractive explanation for the absence of A549 proliferation when neutrophils and A549 cells were separated by transwells.

Neutrophil mediator release was another prerequisite for tumor cell proliferation. Release of neutrophil elastase, but not oxygen radical secretion, was essential for amplified A549 proliferation in our co-culture model. First, when elastase activity was blocked by the specific inhibitor AAPVCK [42], the amplified cell proliferation was substantially reduced, while neutralizing oxygen radicals by superoxide-dismutase was ineffective. Second, elastase release from neutrophils in co-culture with A549 cells was doubled, while the respiratory burst was unchanged in the co-culture model. And third, corroborating previous investigations, addition of purified neutrophil elastase to A549 monocultures was capable to induce proliferation of A549 cells in our study [16, 43]. Taken together, these findings suggest that neutrophil elastase is a key neutrophil-derived mediator which exerts strong proliferative effects on lung cancer cells. A mechanism of elastase-induced cell proliferation may include phosphatidylinositol-3 kinase hyperactivity and subsequent interaction with autocrine growth factor systems such as the platelet-derived growth factor and its receptor as recently demonstrated by Houghton et al. [16].

Most interestingly, not only neutrophil-derived elastase but also COX-2-derived lipid mediators were crucially involved in the pro-proliferative effects in our model. In co-cultures, a remarkable amplification of PGE_2 synthesis was detected in the supernatant, which exceeded ~fivefold the sum of PGE_2 release from monocultures of A549 or neutrophils. When COX activity was blocked by the non-specific inhibitor indomethacin or the COX-2-specific inhibitor NS-398, the release of PGE_2 and the proliferation of A549 were normalized to baseline values. Thus, in LPS-stimulated PMN–A549 co-cultures, the cellular interaction leads to activation of COX-2 as indicated by amplified PGE_2 release and increased tumor cell proliferation. The cellular source of PGE_2 formation in the co-culture system remains to be elucidated. LPS is known to activate phospholipase A_2 with subsequent release of free arachidonic acid (AA) from PMN [44]. Adjacent A549 cells may internalize and metabolize AA by COX-2 to PGE_2. This transcellular eicosanoid metabolism has been detected in co-cultures of neutrophils and alveolar macrophages [45] and in knock-out mice [46]. Whether PGE_2 is the decisive COX-2 mediator in inducing cell proliferation in our model cannot be conclusively derived from our data. However, PGE_2 is the predominant COX product in lung cancer tissue [47], and addition of exogenous PGE_2 to A549 monocultures strongly promoted cellular proliferation in the current study, thus confirming previous investigations [48]. Moreover, in preliminary experiments from our laboratory, inhibition of the PGE_2 receptor EP2 effectively blocked LPS-induced A549 proliferation (Hattar et al., unpublished observations).

It is noteworthy, that both, inhibition of elastase activity and COX-2 activation equally reversed the PMN-induced tumor cell proliferation. These findings might be related to the previously described relationship between COX-2 activation and elastase release in neutrophils, with COX-2 inhibitors suppressing release of elastase [49]. Vice versa, neutrophil elastase has also been shown to stimulate PGE_2 release from bronchial epithelia by activating COX-2 [50]. Thus, elastase and COX-2 may synergize to induce A549 proliferation in the co-cultures used.

In conclusion, our study demonstrates that neutrophils are capable of inducing A549 cell growth in a co-culture model. Neutrophil elastase release as well as COX-2 activation are both amplified by neutrophil–A549 cell interactions and are proven to be key regulators of A549 proliferation. Thus, these results shed light on the significance of neutrophils and inflammatory mediators of the microenvironment of tumors.

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Conflict of interest The authors declare that they have no conflict of interest.

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