Molecular programs induced in alveolar resident and recruited mononuclear phagocytes by Toll-like receptor 2/4 agonists

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IV. ABBREVIATIONS

2-AP	2-aminopurine
AM	Alveolar macrophage
AP-1	Activator protein-1
APS	Amonium persulfate
ATP	Adenosine triphosphate
BAL(F)	Bronchoalveolar lavage (fluid)
BLAST	Basic local alignment search tool
CCL	CC Chemokine ligand
CD	Cluster of differentiation
cDNA	Complementary DNA
DAPI	4´, 6´-diamidino-2-phenylindole
DC	Dendritic cell
dNTP	Deoxynucleotide triphosphate
dsRNA	Double stranded RNA
DTT	Dithiothreitol
ERK	Extracellular signal-regulated protein kinase
FCS	Fetal calf serum
FSC	Forward side scatter
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GPI	Glycosylinositol

IκB	Inhibitor of κB
IFN	Interferon
IKK	IκB kinase
IL	Interleukin
JNK	c-Jun NH2-terminal kinase
KO	Knock-out
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MEK	MAP kinase kinase
MIP	Macrophage inflammatory protein
MP	Mononuclear phagocyte
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary-response gene 88
NF-κB	Nuclear factor κB
OD	Optical density
Pam_3CSK_4	Pam3-Cys-Ser-Lys-Lys-Lys-Lys-OH
PAMPs	Pathogen associated molecular patterns
PB	Peripheral blood
PBGD	Porphobilinogen deaminase
PCR	Polymerase chain reaction
PG	Prostaglandin

PKR	Protein kinase R
PMN	Polymorphonuclear leukocyte
Poly I:C	Polyriboinosinic:polyribocytydylic acid
PRR	Pattern recognition receptor
RANTES	Regulated upon activation normal T cell
RNAsin	Ribonuclease inhibitor
SDS	Sodium dodecyl sulphate
SPF	Specific pathogen free
SSC	Side scatter
TGF-β	Transforming growth factor beta
TIR	Toll/IL-1 receptor domain
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
WT	Wild type

V. SUMMARY

Mononuclear phagocytes play a pivotal role in lung host defence to inhaled pathogens by activation of both innate and adaptive immunity. Resident alveolar macrophages (AMs) are the primary mononuclear phagocytic cells found in the lower respiratory tract that play a central role in regulating pulmonary immune responses. Circulating mononuclear phagocytes (MPs) recruited into inflamed lungs, however are increasingly implicated as essential players in defence against a range of inhaled pathogens.

To further elucidate the function of mononuclear phagocytes in regulating pulmonary immune responses we analysed molecular programs induced by bacterial ligands that are recognized by different TLRs. First, we discovered that TLR2 ligand Pam_3CSK_4 and TLR4 ligand LPS induced in AMs the expression of PKR, previously identified as an essential component of the innate antiviral response. More important we found that both TLR2 and TLR4 agonists induced rapid phosphorylation of PKR strictly dependent on the functionality of the respective TLR. Pharmacologic inhibition of PKR activity using 2-aminopurine (2-AP) and PKR gene deletion were found to reduce the TLR2/4-induced activation of the JNK signalling pathway (MKK4/JNK/c-Jun), but did not affect p38 and ERK1/2 activation. Moreover, inhibition of PKR phosphorylation severely impaired TNF-α and IL-6 production by AMs in response to LPS and Pam_3CSK_4. Additionally, we found that PKR phosphorylation plays a major role in LPS but not Pam_3CSK_4-induced activation of the p65 subunit of NF-κB. Collectively, these results indicate that functional PKR is critically involved in inflammatory responses of primary AMs to gram-positive as well as gram-negative bacteria cell wall components.

In addition, we investigated the induction of lung inflammation and the concomitant MP recruitment after alveolar deposition of the TLR2 ligand Pam_3CSK_4. By using cell sorting, mRNA pre-amplification and whole genome oligonucleotide microarray

techniques we found that alveolar trafficking of MPs was associated with profound changes of their gene expression profiles post recruitment (~2500 genes increased). In particular, alveolar recruited MPs showed strong up-regulation for genes encoding cytokines/chemokines, PRR associated molecules and genes involved in eicosanoid metabolism. Interestingly, gene expression profiling revealed that lung recruited MPs displayed simultaneous induction of both pro- and anti-inflammatory genes. However, we observed a dynamic change of the genetic program of MPs found in BALF at different time intervals post challenge. Strong early induction of a subset of pro-inflammatory mediators such as TNF-α, CCL2 and IL-6 was found to decrease during the later resolution phase whereas increased transcript levels of central anti-inflammatory and pro-resolution mediators including IL-1RN, IRAK-M, IL-10 and BAX persisted at the same levels. Collectively, our *in vivo* study identifies for the first time the global genetic program activated in MPs at different time points during TLR2 ligand-induced recruitment to the alveolar space and thus may help to better understand how alveolar recruited MPS may contribute to the development and termination of pneumonia caused by gram-positive bacteria.

VI. ZUSAMMENFASSUNG

Mononukleäre Zellen spielen eine entscheidende Rolle bei der angeborenen und adaptiven Immunabwehr von inhalierten Pathogenen. Alveolarmakrophagen (AM) stehen dabei an vorderster Front und spielen eine zentrale Rolle bei der Regulation der pulmonalen Immunantwort. Darüber hinaus wird in der letzten Zeit zunehmend klar, dass auch Monozyten, die aus dem Gefäßbett in das entzündete Lungengewebe einwandern, eine wesentliche Rolle in der Wirtsabwehr pulmonaler Infektionen spielen.

Um die Rolle der residenten und rekrutierten mononukleären Zellpopulationen im Rahmen der pulmonalen Immunabwehr genauer zu untersuchen, analysierten wir Signaltransduktions-Kaskaden, die durch Pathogen-assoziierte Gefahrensignale über verschiedene Toll-like Rezeptoren in diesen Zellen induziert werden. Hierbei zeigte sich, dass Pam_3CSK_4 über TLR2 und LPS über TLR4 in AMs die Expression des Enzyms PKR induziert, das bislang als wichtige Komponente der angeborenen antiviralen Immunität bekannt war. Außerdem führt die Aktivierung von TLR2/4 zu einer sehr schnellen Phosphorylierung von PKR, was die Aktivierung nachgeschalteter Signaltransduktions-Kaskaden entscheidend mitbestimmt. So reduzierte die Inhibierung der PKR durch 2-Aminopurin (2-AP) oder die Gendeletion des PKR-Gens die TLR2/4-induzierte Aktivierung des JNK-Signalweges (MKK4/JNK/c-Jun). Im Gegensatz dazu erfolgte keine Inhibition der p38 und ERK1/2 Aktivierung. Darüber hinaus führte eine Inhibition der PKR-Phosphorylierung zu einer starken Hemmung der TNF-α und IL-6-Produktion der AMs nach LPS- und Pam3CSK4-Stimulation. Außerdem konnten wir zeigen, dass die PKR-Phosphorylierung eine bedeutende Rolle bei der Aktivierung der LPS-induzierten, nicht jedoch der Pam_3CSK_4-induzierten Aktivierung der p65 Untereinheit von NF-κB spielt. Zusammenfassend legen diese Ergebnisse die Vermutung nahe, dass PKR eine kritische Rolle bei der durch

Zellwandbestandteile gram-positiver und –negativer Bakterien induzierten inflammatorischen Antwort von Alveolarmakrophagen spielt.

In einem zweiten Ansatz untersuchten wir Mechanismen der Induktion einer pulmonalen Entzündungs-Reaktion und der nachfolgenden Einwanderung von Blut-Monozyten nach alveolärer Deposition des TLR2-Liganden Pam_3CSK_4. Wir kombinierten Zellsorting, mRNA-Präamplifikation und Gesamtgenom-Microarray Technologien, um Veränderungen des globalen genetischen Programms von Monozyten bei Einwanderung in den Alveolarraum zu untersuchen. Hierbei fanden sich tief greifende Veränderungen der Genexpression in den mononukleären Phagozyten nach alveolärer Rekrutierung, wobei die Expression von ca. 2500 Genen signifikant erhöht war. Im Einzelnen konnten wir eine Erhöhung der Expression von Genen der Cytokin-und Chemokin-Familien, Pattern-Recognition-Rezeptoren sowie Eiconsanoid-metabolisierenden Enzyme nachweisen. Dabei wurde interessanter Weise sowohl die Transkription pro- als auch antiinflammatorischer Gene induziert. Allerdings zeigten mononukleäre Phagozyten, die zu verschiedenen Zeitpunkten nach Pam3CSK4-induzierter Rekrutierung analysiert wurden, deutliche Unterschiede in ihrem Genexpressionsprofil: mRNA Spiegel von pro-inflammatorischen Mediatoren wie TNF-α, CCL2 und IL-6 waren zu frühen Zeitpunkten stark erhöht und sanken dann rasch ab, während die ebenfalls bereits früh erhöhte Genexpression von zentralen anti-inflammatorischen Mediatoren wie IL-1RN, IRAK-M, IL-10 und BAX auch zu späteren Zeitpunkten persistierte. Zusammenfassend erfassen diese Untersuchungen zum ersten Mal detailliert das globale genetische Programm aktivierter mononukleärer Phagozyten zu unterschiedlichen Zeitpunkten nach TLR2-Ligand-induzierter Rekrutierung in den Alveolar-Raum. Dies erlaubt einen Einblick in das Funktionsrepertoire von mononukleären Phagozyten, mit dem diese zur pulmonalen Entzündungsinduktion und –terminierung bei durch gram-positive Bakterien hervorgerufenen Pneumonien beitragen können.

1 INTRODUCTION

1.1 INNATE IMMUNITY

All multi-cellular eukaryotic organisms are constantly exposed to millions of potential pathogens daily through contact, ingestion and inhalation. To effectively detect, control and eliminate invading microbial pathogens, higher vertebrates have developed two interacting protective systems: the innate and adaptive immune systems. Innate immunity is an evolutionary conserved host defence armentarium that provides initial protection against invading micro-organisms. Remarkably, innate immunity has a higher level of specificity in its ability to distinguish distinct danger signals from different origin than was originally thought. It employs soluble factors and germline-encoded receptors called pattern recognition receptors (PRRs) to recognize specific components of microbes called pathogen-associated molecular patterns (PAMPs). PAMPs are evolutionary conserved structures that are essential for microbial physiology and survival. PAMPs include for instance viral double-stranded RNA (dsRNA), lipopolysaccharide (LPS) derived from gram-negative bacteria, un-methylated CpG di-nucleotides or lipoproteins of bacteria and fungi (1, 2). Recognition of PAMPs by PRRs activates different intracellular signalling pathways that result in the increased production of inflammatory mediators such as cytokines and chemokines, as well as recruitment of immune cells to inflamed tissues. Among PRRs, Toll-like receptors (TLRs) were highlighted as key recognition structures of the innate immune system.

1.1.1 Recognition of microbial components by pattern recognition receptors

The innate immune system utilizes a variety of PRRs that can be expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and

tissue fluids. The effectors functions of PRRs after ligand binding include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of pro-inflammatory signalling pathways, and induction of apoptosis. Functionally PRRs can be divided into three classes: secreted, endocytic, and signalling. Mannan-binding lectin (MBL), C-reactive protein (CRP), and serum amyloid protein (SAP) are secreted pattern recognition molecules, whereas macrophage mannose receptor (MMR) and macrophage scavenger receptor (MSR) represent endocytic PRRs. The TLR family is the most investigated class of signalling PRRs and appears to have a major role in the induction of immune and inflammatory responses (3).

1.1.2 Toll-like receptors

Following the discovery of the role of *Drosophila melanogaster* Toll receptor in antifungal immunity, a mammalian homologue of Toll protein termed Toll-like receptor (later named TLR4) was identified (4). The TLR family now consists of 13 mammalian members: 10 human (TLR1–10) and 12 murine (TLR1–9 and TLR11–13) receptors, of which some are homologues (Table 1.1) (5). Structurally, the intracellular domain of TLRs shows similarity to that of the interleukin-1 receptor (IL-1R) family and therefore is termed a Toll/IL-1R domain (TIR). The extracellular portion of TLR, however, is characterized by the presence of leucine-rich repeat (LRR) motifs, whereas the IL-1R family possesses extracellular immunoglobulin-like domains. LRR domains are implicated in the recognition of PAMPs (5, 6). The main PAMP ligands for TLR1 to TLR13 are summarised in table 1.1.

TLR family members are expressed on various immune cells including monocytes, macrophages, dendritic cells (DCs), B cells, and even on non-myeloid cells such as epithelial cells and fibroblasts (7). While some TLRs have been found to be expressed primarily on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11) others are found almost completely in intracellular compartments such as endosomes (TLR3, TLR7, TLR8 and TLR9) (7). The cellular expression of TLRs is

modulated rapidly in response to various stimuli such as pathogens, cytokines and environmental stresses (7, 8). Of the TLRs identified so far, TLR4 and TLR2 have been the most extensively studied.

Table 1.1 Toll like receptors and their ligands

N.D., not determined; TLR, Toll-like receptor. Modified and adapted from Akira 2004 and Takeda 2003 (7-9).

TLR	Ligand	Origin of ligand	Animal Species
TLR1	Triacyllipopeptides	Bacteria and Mycobacteria	Human, mouse
	Pam3CSK4	Synthetic compound	
TLR2	Lipoteichoic acid	Gram-positive bacteria	Human, mouse
	Peptidoglycan	Gram-positive bacteria	
	Zymosan	Fungi	
	Lipoproteins and lipopeptides	Various pathogens	
	Heat-shock protein 70	Host	
	Porins	*Neisseria*	
	Pam3CSK4	Synthetic compound	
	Atypical lipopolysaccharide	*Leptospira interrogans*	
	Lipoarabinomannam	Mycobacteria	
TLR3	Double-stranded RNA	Viruses	Human, mouse
TLR4	Lipopolysaccharide	Gram-negative bacteria	Human, mouse
	Fusion protein	Respiratory syncytial virus	
	Taxol	Plants	
	Heat-shock protein 60/70	Host	
	Hylauronic acid fragments	Host	
	Fibrinogen	Host	
TLR5	Flagellin	Bacteria	Human, mouse
TLR6	Diacyl lipopeptides	*Mycoplasma*	Human, mouse
	Zymosan	Fungi	
	Lipoteichoic acid	Gram-positive bacteria	
TLR7	Single-stranded RNA	Viruses	Human, mouse
	Imiquimod	Synthetic compound	
	Loxoribine	Synthetic compound	
	Bropirimine	Synthetic compound	
TLR8	Single-stranded RNA	Viruses	Human, mouse
	Imiquimod	Synthetic compound	
TLR9	Unmethylated CpG DNA	Bacteria, viruses, yeast	Human, mouse
	Chromatin-IgG complexes	Host	
	Hemozoin	Protozoa	
TLR10	N.D.	N.D.	Human
TLR11	N.D.	Uropathogenic bacteria	Mouse
	Profilin-like molecule	*Toxoplasma gondii*	
TLR12, TLR13	N.D.		Mouse

1.1.2.1 Toll-like receptor 4 (TLR4)

TLR4 is an essential receptor for the recognition of LPS (also referred as endotoxin), a major component of the outer membrane of gram-negative bacteria such as *Escherichia coli* or *Salmonella typhimurium*. The link between TLR4 and LPS appeared in studies showing that genetic mutation responsible for hypo-responsiveness to LPS in two naturally occurring mouse strains, C3H/HeJ and C7BL10/ScCr was within the TLR4 gene (10, 11). Similarly, mice with targeted deletion in the TLR4 gene are hypo-responsive to LPS (12). Later studies discovered that TLR4 is necessary for the signalling of a range of other pathogen components and also several endogenous host ligands (Table 1.1) (8).

TLR4 is important for host defence against different types of microbes including *Salmonella*, *Mycobacterium tuberculosis*, *Candida albicans* or *Haemophilus influenzae* (13). However, TLR4 is not the sole receptor involved in LPS recognition. LPS first interacts with a plasma protein named LPS-binding protein (LBP) to form stable LBP:LPS complexes that are recognized by the CD14 receptor on the cell surface (14). CD14 is a membrane glycoprotein anchored in the lipid bilayer by its glycosylinositol (GPI) tail and is preferentially expressed on monocyte/macrophage and neutrophil (8). CD14 can also circulate in soluble form in the plasma (15). The major role of CD14 is to concentrate LPS for effective binding to the constitutively associated TLR4/MD2 complex (16).

1.1.2.2 Toll-like receptor 2 (TLR2)

TLR2 is involved in the recognition of a variety of microbial and synthetic patterns including bacterial lipoproteins derived from gram-negative as well as gram-positive bacteria (e.g., *Staphylococcus aureus* and *Streptococcus pneumoniae*) or mycoplasma spp. (Table 1.1) (8). TLR2 has also been reported to recognize several atypical forms of LPS such as LPS derived from *Leptospira interrogans* (17).

The wide spectrum of ligand recognition by TLR2 is mostly determined by heterodimeric interaction with other TLR family members such as TLR1 or TLR6. For example, TLR2/TLR1 complex recognizes synthetic triacylated lipoproteins such as Pam_3CSK_4, whereas TLR2/TLR6 complexes recognize diacylated lipoproteins including mycoplasmal macrophage-activating lipopeptide 2 (MALP-2) (8, 18). In addition, TLR2 has been shown to cooperate with distinct types of other receptors such as dectin-1 to recognize fungal β-glucans, as well as with CD36 to recognize diacylglycerides (19). TLR2-deficient mice were found to be highly susceptible to challenge with *S. aureus* and *S. pneumoniae* (13, 20).

1.1.3 TLR-mediated signalling pathways

Binding of microbial components to their respective TLR triggers activation of signalling pathways, which all make use of adapter proteins (Figure 1.1). Four TIR domain containing adaptors, namely myeloid differentiation primary-response gene 88 (MyD88), TolI/interleukin-1 receptor domain-containing adapter protein (TIRAP/Mal), TIR-containing adaptor protein inducing interferon (IFN)-β (TRIF/TICAM1) and TRIF-related adaptor molecule (TRAM/TICAM-2) were shown to play important roles in the TLR signalling pathway. MyD88 is essential for the induction of inflammatory responses triggered by all TLRs with the exception of TLR3 (7). TIRAP is specifically involved in the MyD88-dependent pathway *via* TLR2 and TLR4, whereas TRIF is implicated in the TLR3- and TLR4-mediated MyD88-independent pathways (2, 21).

The MyD88-dependent pathway includes a number of signalling molecules such as serine/threonine kinase IL-1R-associated kinase (IRAK), tumour necrosis factor (TNF)-α receptor-associated factor 6 (TRAF6), transforming growth factor (TGF)-β-activated kinase 1 (TAK1) and TAK1 binding protein-1 (TAB1). Generally, upon stimulation with PAMPs, MyD88 recruits IRAK4 to ligated TLRs to facilitate IRAK4 mediated phosphorylation of IRAK1 and IRAK2. Activated IRAK1 associates with TRAF6 and together with TAK1 leads to the activation of two distinct pathways: the mitogen-activated protein kinases (MAPKs) and the Rel-family transcription factor

nuclear factor-κB (NF-κB). In consequence, the MyD88-dependent pathway leads to the production of inflammatory cytokines such as TNF-α, IL-6, IL-12, and IL-1β (5). However, subsequent studies have demonstrated that MyD88-independent activation of NF-κB or interferon regulatory factor (IRF)-3 transcription factors occurs in TLR downstream signalling as well. This pathway has been implicated in the expression of IFN-inducible genes and LPS-mediated maturation of DCs (5).

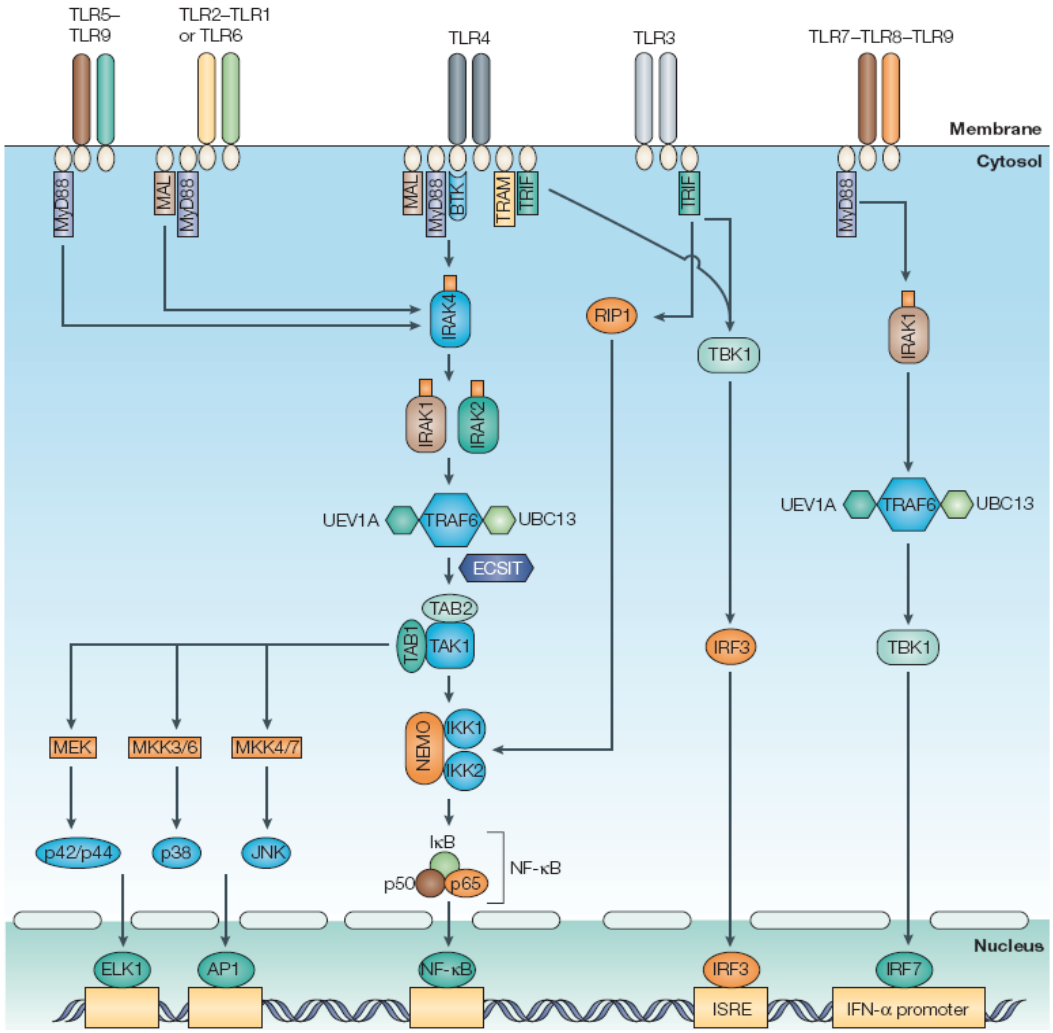

Figure 1.1 Mammalian TLR signalling

MyD88 is a Toll-like receptor (TLR) signalling adaptor protein that is used by all TLRs apart from TLR3. It interacts with the IRAK (interleukin-1 (IL-1) receptor-associated kinase) family, leading to interaction with TRAF6 (tumour-necrosis factor-receptor-associated factor 6), which ultimately leads to activation of nuclear factor-κB (NF-κB) and mitogen-activated protein (MAP) kinases such as p42/p44 MAP kinase and Jun N-terminal kinase (JNK). These pathways lead to the production of cytokines such as tumour-necrosis factor (TNF) and other pro-inflammatory proteins. MyD88 has recently been shown to have an additional role in the activation of IRF7 (interferon (IFN)-regulatory factor 7), which leads to induction of interferon-β. TLR2 and TLR4 signalling specifically recruits a second adaptor, MAL, which is supposed to act mainly as a bridging adaptor for MyD88 recruitment. TLR3 signals through TRIF (Toll/IL-1 receptor-domain containing adaptor protein inducing IFN-β), which can specifically interact with the kinase TBK1 (TRAF-family-member-associated NF-κB activator-binding kinase 2) leading to IRF3 activation. Target genes for this pathway include IFN-β. TRIF also interacts with RIP1 (receptor-interacting protein 1), which leads to activation of the IκB (inhibitor of NF-κB) kinase 1 (IKK1)–IKK2– NEMO complex. Finally, TRAM seems to be a bridging adaptor for TRIF recruitment, specifically for TLR4. UEV1A and UBC13 are ubiquitin-conjugating enzymes.

AP1, activator protein 1; BTK, Bruton's tyrosine kinase; ECSIT, evolutionarily conserved signalling intermediate in Toll pathway; ISRE, IFN-stimulated response element; MKK, MAP kinase kinase; TAB2, transforming growth factor-activated kinase (TAK)-binding protein-2. Adapted from Liew 2005 (22).

1.1.3.1 Mitogen activated protein kinases in innate immune responses

The MAP kinases are highly conserved signal transducing enzymes that are crucial for cell growth, differentiation and stress responses (23). At least three distinct MAPKs families have been described in mammalian cells: the extracellular signal-regulated protein kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 MAPK (24). MAP kinases signal through a three component protein kinase cascade: MAPK, MAPK kinase (MAPKK; MKK), and a MAPKK kinase (MAPKKK) (Figure 1.2) (23).

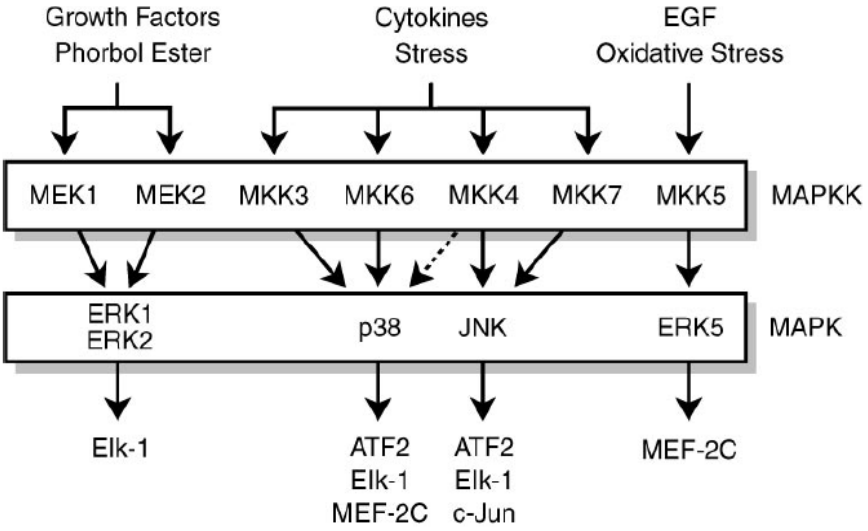

Figure 1.2 Mammalian MAP kinase pathway

Activation of MAP kinases by different stimuli first requires activation of specific MAPKK kinases (not shown) that in turn phosphorylate and activate specific MAPK kinases (MAPKK; MKK). MKK have a restricted specificity for MAP kinase substrates. MEK1 and MEK2 activate ERK MAP kinases; MKK3, MKK4 and MKK6 activate p38 MAP kinases; MKK4 and MKK7 activate the JNK pathway. Adapted and modified from Dong 2002 (24).

The classic ERK kinases include ERK1 and ERK2 which are often referred as p44/p42 MAP kinases. Upon activation, ERK1/2 translocate into the nucleus and phosphorylate numerous targets including transcription factors such as Elk-1. Many studies have demonstrated the importance of the ERK pathway in LPS signalling in monocytes and macrophages (25-27). Macrophages from Tpl2-deficient mice exhibited selective ERK deficits and reduced TNF-α production upon LPS stimulation (28). Moreover, transport of TNF-α mRNA from the nucleus to the cytoplasm was inhibited by specific ERK inhibitors (29). Inhibition of the MEK-ERK pathway in monocytes by U0126 reduced LPS induction of several inflammatory mediators including IL-1, IL-8, TNF-α and prostaglandin (PG) E2 (30). Similarly, blocking of the ERK pathway by PD98059 inhibited LPS induction of tissue factor gene expression in human monocytes (27).

The JNK protein kinases, also known as stress-activated MAP kinases (SAPKs), are encoded by three genes: *Jnk1*, *Jnk2* and *Jnk3* (31). JNK kinases are activated through phosphorylation by MKK4 and MKK7 (31). JNK activation can be strongly induced in multiple cell types by LPS or inflammatory cytokines such as TNF-α and IL-1α (24, 31). For instance, LPS stimulation of THP-1 and RAW 264.7 cells rapidly activates the JNK pathway (32). A critical role for JNK appears to be the regulation of the activator protein (AP)-1 transcription factor through phosphorylation of the N-terminal part of c-Jun, a central component of AP-1 (29, 31). Inhibition of JNK activity results in decreased levels of TNF-α in LPS-exposed macrophages (29). MKP5 is a negative regulator of JNK activity (33). Consequently, MKP5-deficient macrophages produced increased levels of inflammatory cytokines upon TLR ligand stimulation (34).

The p38 MAPK (known as p38 α) was originally described as a 38-kDa polypeptide that was rapidly tyrosine phosphorylated in response to LPS and osmotic shock (35). Subsequently, three additional isoforms of p38 have been identified: β, γ and δ. The p38 molecules are strongly activated by environmental stresses, inflammatory cytokines and bacterial ligands. Once activated, p38 MAPKs

modulate responses of different target proteins including transcription factors such as activating transcription factor (ATF)-2, signal transducer and activator of transcription (STAT)-1 and Elk-1. Lu and co-workers found that disruption of the MKK3-p38 MAP kinase pathway results in a selective defect in LPS-induced IL-12 production, whereas the production of other cytokines such as TNF-α, IL-6 and IL-1 was comparable between wild type (WT) and knock-out (KO) mice (36). In another study, defects in the MK2 gene which is a common p38 substrate resulted in reduced serum levels of TNF-α. Moreover, MK2-deficient mice displayed increased stress resistance and survival of endotoxin shock (37).

1.1.3.2 Transcription factors involved in TLR-mediated signalling pathways

Transcription factors tightly regulate the expression of inflammatory mediators. Stimulation with bacterial components induces activation of various transcription factors such as the NF-κB, AP-1, STAT-1 and IRF family of transcription factors (38-40).

NF-κB represents a master transcription factor required for maximal expression of many genes including cytokines, growth factors, adhesion molecules and acute phase proteins (41). NF-κB belongs to the Rel family of proteins that includes p50, p105, p52, p65, Rel B and c-Rel, which function as homo- and heterodimers (42, 43). Under physiological conditions NF-κB dimers are kept in the cytoplasm in an inactive state by inhibitors of κB (IκB). Upon stimulation, IκB proteins are phosphorylated by IκB kinases (IKKs) and NF-κB proteins are released and translocated to the nucleus where they can bind to the specific gene regions (44). The most ubiquitous activated form of NF-κB involved in LPS signalling is a heterodimer consisting of p50 and p65 subunits (29). Generally, regulation of NF-κB activity can be divided into four steps: nuclear translocation, phosphorylation, interaction with the basal transcription complex and redox regulation (45).

The involvement of NF-κB in the innate immune response has been studied broadly. Muller *et al.* have demonstrated for the first time that LPS can activate

NF-κB in monocytic cells (46). In later studies NF-κB activation has been linked to the production of TNF-α, IL-1, IL-6, cyclooxygenase (COX)-2 and CC chemokine ligand 2 (CCL2, also referred to as monocyte chemoattractant protein (MCP)-1) in response to LPS (41). Studies using KO mice have shown that each member of the family plays an important role in LPS signalling. For instance, B cells from mice deficient in p50, p65, c-Rel, or Rel B displayed an impaired LPS response (43). In addition, mice deficient in functional subunits of NF-κB were very susceptible to microbial infections (47-49).

AP-1 represents another important family of transcription factors involved in TLR downstream signalling (29). This family is composed of homo- and heterodimers of the c-Jun and c-Fos family (50, 51). Activity of AP-1 is regulated through phosphorylation by JNK and ERK MAP kinases (52). Stimuli such as LPS, peptidoglycan and dsRNA enhance the transcriptional activity of AP-1 (29).

1.2 INNATE IMMUNITY IN THE LUNGS

The lungs serve as a major interface between the host and the external environment. Each day, a human being breathes in and out more than 7000 L of air (29). Consequently, the respiratory tract is continuously exposed to numerous microbes, toxic gases and particles which are potentially harmful to the mammalian organism. Therefore, complex defence mechanisms of the respiratory tract from nostrils down to alveoli have been developed to protect the lung. The innate immune system of the lung consists of several physical, humoral and cellular components that work in concert in order to clear invading pathogens, preserve gas exchange, regulate inflammation and alert the adaptive immune system (Table 1.2).

Table 1.2 Components of the innate immune system of the lung
Adapted and modified from Beutler 2004 and Martin 2005 (53, 54).

Physical component	
	nasopharyngeal anatomic barriers, cough, mucociliary apparatus
Humoral component	
	cytokines, chemokines, antimicrobial peptides, lipases, lactoferrins, lyzozyme,mannose-binding lectin, defensins, complement, acute phase proteins, immunoglobulins
Cellular component	
	alveolar macrophages, lung epithelial cells, monocytes, polymorphonuclear leukocytes, dendritic cells

Mechanisms such as coughing, sneezing and the mucociliary system in most cases effectively remove larger particulates from the upper airways. However, smaller particles, bacteria and viruses can escape the defence line of the upper airways and reach the alveolar space where they interact with soluble factors, pulmonary epithelium and mononuclear phagocytes (MPs).

1.2.1 Mononuclear phagocytes in lung host defence

The MP system consists of bone marrow (BM) progenitor cells, monocytes, DCs and tissue macrophages. The major MPs subsets in airways and lung parenchyma are alveolar macrophages (AMs), with a $CD11c^+$ $CD11b^-$ $CX3CR1^-$ surface phenotype, and $CD11c^+$ $CD11b^+$ $CX3CR1^+$ lung DCs. (55, 56). Because of their phagocytic activity, antigen presentation capacity and secretion of soluble mediators MPs play a crucial role in lung innate and adaptive immune responses to pathogens.

1.2.1.1 Resident alveolar macrophages

AMs are specialized mononuclear phagocytic cells that function as a first host defence line against inhaled particles and pathogens in the lower respiratory tract (57). These cells are broadly distributed at the air-tissue interface in the alveolar space in close proximity to alveolar epithelial cells (AECs) and are separated by a distance of only 0.2–0.5 µm from interstitial DCs (58). AMs originate primarily from BM derived blood monocytes or may undergo limited local proliferation (59). Normally, AMs account for approximately 95% of airspace leukocytes of human lungs and 100% of the pathogen-free mice (54).

AMs have ability to ingest all types of inhaled particulates that reach the alveolar space. Remarkably, AMs are generally poor initiators of lung immune responses, thereby keeping the airspace quiet and preserving the ability of the lung to perform gas exchange. They can remove and digest relatively inert particulates without triggering inflammatory responses that may damage the structural integrity of alveolar tissue (54, 58, 60). However, when pathogen exposures are sufficiently large or when faced with highly virulent pathogens, AMs initiate a localized inflammatory response by secretion of a wide array of mediators. These include cytokines (e.g. TNF-α and IL-6), chemokines (e.g. CCL2 and RANTES) and lipid mediators (arachidonic metabolites) thereby promoting neutrophil, activated monocyte and lymphocyte sequestration at sites of inflammation in the lungs

(Table 1.3). To avoid uncontrolled AM activation, several regulatory mechanisms have been evolved. One possible inhibitory mechanism involves surfactant proteins A and D, which bind AM receptors and suppress inflammatory activity in uninfected lungs. Recently, Snelgrove *et al.* demonstrated a new mechanism by which AECs suppress AM activity under baseline conditions involving the negative regulator CD200 receptor (CD200R) on macrophages and its ligand CD200 on airway epithelium (60, 61).

The ability of AMs to interact with microbes is mediated by surface receptors capable to bind specific ligands. The major receptors found on AMs include scavenger receptors, Fc receptors, G protein-coupled receptors (GPCRs), cytokines and chemokines receptors and TLRs (29). Among TLRs, function of TLR2 and TLR4 has been the most extensively studied. Both TLR2 and TLR4 receptors are expressed and functionally active on AMs (62, 63). In AMs, recognition of LPS by TLR4 results in rapid activation of signalling pathways including all three major MAP kinases, p38, ERK1/2 and JNK *in vitro* and *in vivo* (29, 64). Likewise, activation of NF-κB is an early event after LPS binding (as early as 30 min). It has been demonstrated that depletion of AMs attenuated NF-κB activation in whole lung tissues and decreased the pro-inflammatory content in BALF. In addition to the initiation and regulation of inflammation, AMs actively participate in the resolution of inflammatory infiltrates and tissue repair by phagocytosis of apoptotic cells and elaboration of enzymes, anti-inflammatory cytokines (e.g. IL-1RN and IL-10) and growth factors such TGF-β1 (Table 1.3).

AMs have an additional role in the regulation of acquired immune responses. They can effectively suppress adaptive immunity by down-regulation of T- and B-cell activation and antigen presentation activities of DCs (65, 66). It has been demonstrated that *in vivo* depletion of AMs using clodronate-filled liposomes leads to significant increase in the pulmonary inflammatory response to harmless particulate and soluble antigens (67).

Table 1.3 Secretory products of alveolar macrophages
IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; MCP, monocyte chemoattractant protein; RANTES, regulated upon activation normal T cell; MIP, macrophage inflammatory protein; GM-CSF, granulocyte macrophage colony stimulating factor; G-CSF, granulocyte macrophage colony stimulating factor; M-CSF, macrophage colony stimulating factor; TGF, transforming growth factor; PDGF, platelet-derived growth factor; LTB, leukotriene B4; HETE, monohydroxyeicosatetraenoic acid; TIMP, tissue inhibitor of metalloproteinases. Adapted and modified from Nikod 1999 (68).

Cytokines	
	IL-α/β, IL-1RN, TNF-α, IFN-β, IL-6, IL-10, IL-12, IL-23
Chemokines	
	IL-8, RANTES, MCP1/5, MIP-1α/β
Growth factors	
	GM-CSF, G-CSF, M-CSF, TGF-β, PDGF
Lipid mediators	
	LTB4, 5-HETE
Reactive gases	
	Nitric oxide, Superoxide radical
Defensins and lizozyme	
Complement	
	C3b, C5, C1q
Enzymes	
	Metalloelastases, Collagenase, TIMPs Metalloproteases, Arginase I

1.2.1.2 Peripheral blood and alveolar recruited monocytes

Peripheral blood (PB) monocytes are a heterogeneous cell population of circulating MPs that represent about 5–10% of PB leukocytes in humans and mice. Under steady-state condition, half of the circulating monocytes leave the bloodstream each day and following recruitment to the peripheral organs, they can differentiate into tissue macrophages or DCs. Bacterial infections or stimulation with PAMPs, however, heavily increase trafficking of monocytes to the site of inflammation, where they can contribute directly to immune defences against microbial pathogens and/or differentiate into resident MP (55, 59).

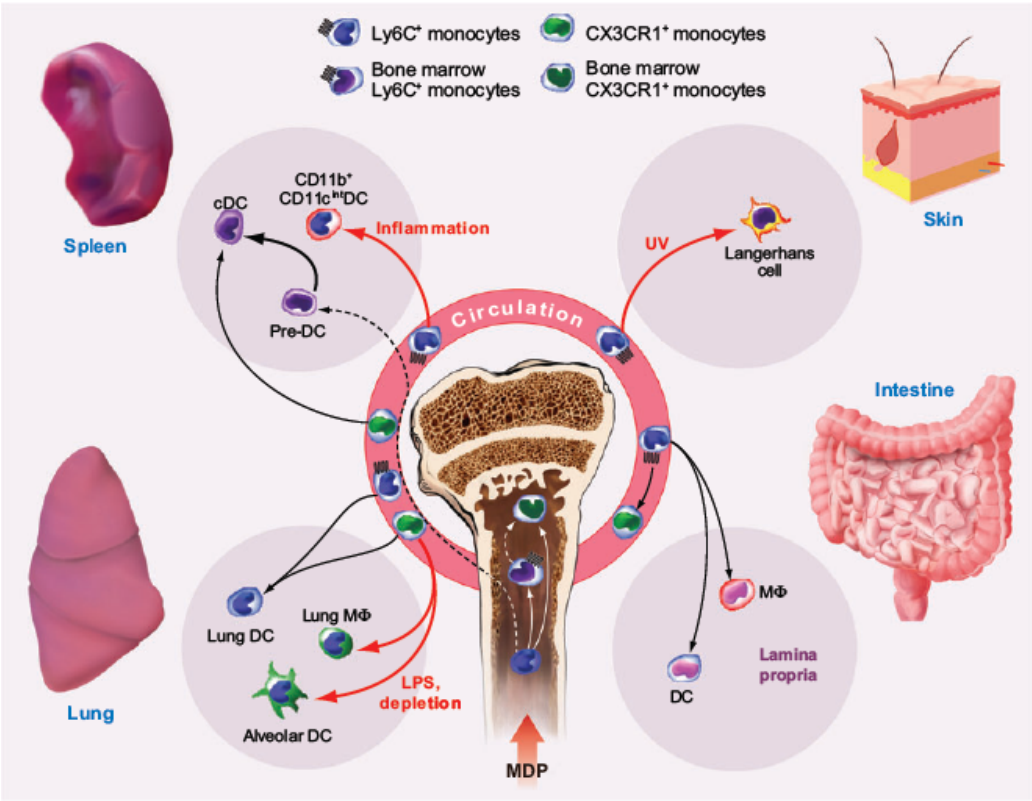

Figure 1.3 Monocyte differentiation into DCs and tissue macrophages
Macrophage-DC progenitors (MDPs) give rise to Ly6C$^+$ bone marrow monocytes, which exit the bone marrow, in part guided by CCR2-dependent signals. Ly6C$^+$ monocytes convert into CX3CR1$^+$ monocytes, although the location of this event, in the circulation or bone marrow, remains incompletely understood. Black arrows indicate differentiation steps into tissue DCs and macrophages that occur under homeostatic conditions. Red arrows indicate differentiation steps that occur under inflammatory conditions (UV-induced skin injury, intratracheal LPS administration, or depletion of autologous CD11c$^+$ cells). Dashed arrows represent steps that remain uncertain. In the case of splenic cDCs, splenic pre-DCs are the most significant upstream precursor in numeric terms (*bold arrow*), although MDPs and CX3CR1$^+$ monocytes may contribute as well. Adapted from Serbina 2007 (55).

Mouse blood monocytes are identified primarily by their expression of receptor for monocyte colony stimulating factor (CSFR1, also referred to as CD115), CD11b and low levels of F4/80. They are derived from haematopoietic stem cell precursors that initiate myeloid differentiation and give rise to multipotent precursors (Lin$^-$, Sca1$^+$ and CD117$^+$ positive cells). Recently myeloid lineage macrophage-DC progenitors (MDPs) have been isolated from BM of CX3CR1^{GFP} knock-in mice. When introduced into BM, MDPs gave rise to Ly6C$^+$

and CXCR1^{+} BM monocytes from which subsequently the two principal circulating subsets are derived (Figure 1.3). One population (referred to herein as Ly6C^{+}) expresses low levels of CX3CR1 (CX3CR1^{low}), high levels of Ly6C (Ly6C^{high}) and high levels of CCR2 (CCR2^{high}). It has been demonstrated that Ly6C^{+} monocytes home to peripheral tissues in response to inflammatory stimuli, prompting their designation as inflammatory monocytes. This population corresponds to the main human monocyte population, which is CD14^{+} CD16^{-} (so called CD14^{+}). The second subset of mouse monocytes is similar to human CD14^{+} CD16^{+} monocytes and is defined by high levels of CX3CR1 (CX3CR1^{high}), low levels of Ly6C (Ly6C^{low}) and CCR2 (CCR2^{low}) (referred to herein as CX3CR1^{+}). In contrast to Ly6C^{+}, CX3CR1^{+} monocytes traffic into peripheral tissues under non-inflammatory conditions and therefore are referred to as non-inflammatory monocytes (55, 59). A more detailed description of the surface antigen expression on human and mouse monocyte subsets is summarized in the table 1.4.

Table 1.4 Surface antigen expression on murine and human monocyte subsets
Adapted and modified from Serbina 2007 (55).

	Murine Ly6C+ monocytes	Murine CX3CR1+ monocytes	Human CD14+ monocytes	Human CD16+ monocytes
Monocyte markers				
CD11b	+	+	+	+
CD14	ND	ND	++	+/−
CD16 (FcγRIII)	ND	ND	−	+
CD115	+	+	ND	ND
F4/80	+	+	ND	ND
Gr-1 (Ly6C/Ly6G)	++	+/−	ND	ND
Ly6C	++	+/−	ND	ND
Ly6G	−	−	ND	ND
Chemokine receptors				
CCR1	ND	ND	+	−
CCR2	+	−	+	−
CCR4	ND	ND	+/−	−
CCR5	ND	ND	+/−	+/−
CCR7	ND	ND	+/−	−
CXCR1	ND	ND	+/−	−
CXCR2	ND	ND	+	−
CXCR4	ND	ND	+/−	+
CX3CR1	+/−	++	+/−	++
Other receptors and lineage markers				
7/4	+	−	ND	ND
CD4	ND	ND	+	+
CD11a	+	++	ND	ND
CD11c	−	+	+	+
CD31	+	+	+	+
CD32 (FcγRII)	ND	ND	+	++
CD33	ND	ND	++	+
CD43	−	+	++	+
CD49b	+	−	ND	ND
CD62L (L-selectin)	+	−	+	−
CD64 (FcγRI)	ND	ND	−	+
CD86	ND	ND	+	++
MHC class II	Inducible[b]	Inducible[b]	+	++
NK1.1	−	−	ND	ND
Scavenger receptor	ND	ND	+/−	+

Surface expression levels have been arbitrarily designated as undetectable (−), marginal (+/−), positive (+), and high (++) based on flow cytometric analysis; ND, not determined.
[b] MHC class II levels are marginal under homeostatic conditions but increase rapidly with infectious or inflammatory stimulation.

Regardless of the pulmonary MPs, adoptively transferred CX3CR1$^+$ monocytes traffic to the lungs of recipient mice, and these cells, along with Ly6C$^+$ monocytes, give rise to pulmonary DCs. Innate immune activation, by infection or tissue injury, however results in increased recruitment of monocytes to inflamed tissues and under this conditions only CX3CR1$^+$ monocytes give rise to lung macrophages and alveolar DCs as well (Figure 1.3) (55, 56). Subsequently macrophages and DCs regulate inflammatory response through elaboration of cytokines/chemokines, phagocytosis or antigen presentation. It is also known that before differentiation, recruited inflammatory monocytes may modulate local host defence responses. Besides their increased cytokine production and phagocytosis, monocytes are also able to kill bacteria by producing reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs) and through the action of phagolysosomal enzymes (55). Recently, MPs recruited to the alveolar space under acute inflammatory conditions were found to display a prominent pro-inflammatory phenotype manifested by increased levels of mRNA for TNF-α, macrophage inflammatory protein (MIP)-2, KC, IFN-inducible protein (IP)-10 and various lysosomal proteases (69). Therefore freshly recruited inflammatory MPs may promote acute lung injury (ALI). Consequently monocytes were found to regulate neutrophilic influx during ALI (70). In addition, monocytes actively contribute to the both acute and chronic inflammatory disease such as acute respiratory distress syndrome (ARDS), bronchiolitis obliterans and idiopathic pneumonia syndrome (70-72).

1.2.2 Lung inflammatory response

The lung inflammatory response to microbial challenge is a complex process involving coordinated interaction of many inflammatory mediators, primarily cytokines and chemokines, various cell adhesion molecules (including P and E selectins) and different cell populations (including macrophages, epithelial cells and neutrophils) (73, 74).

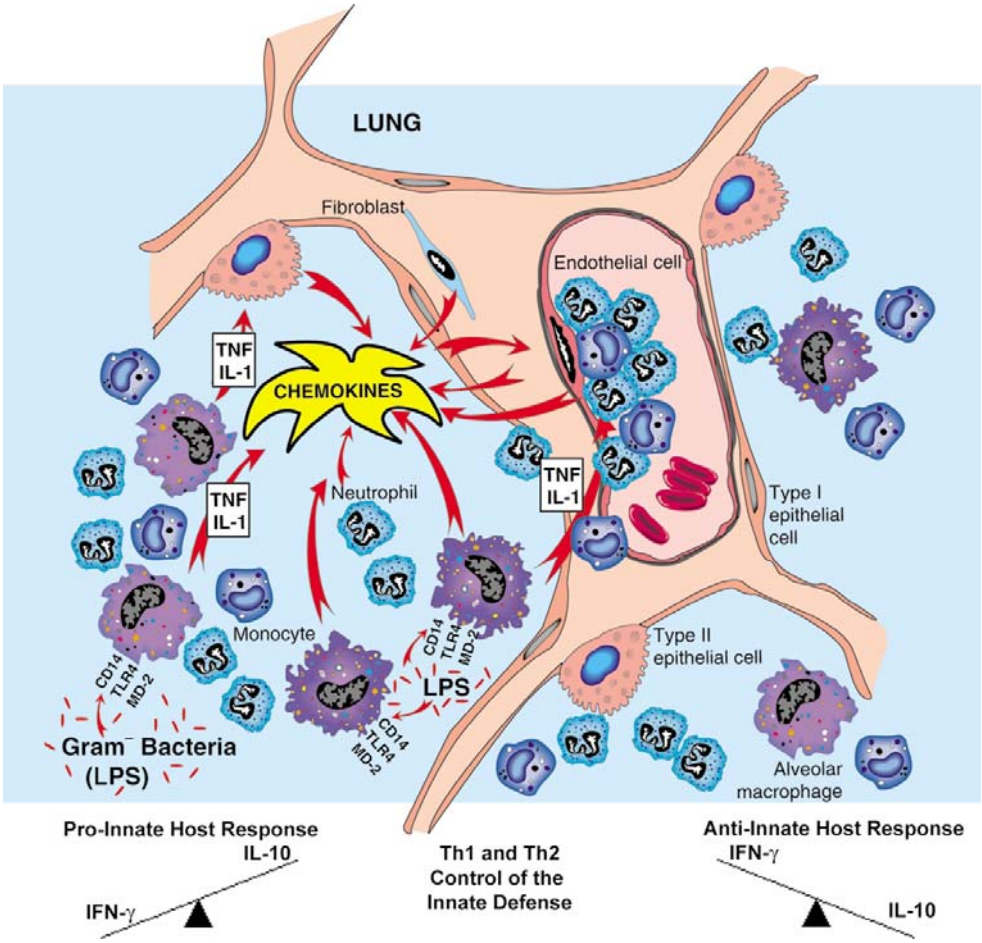

Figure 1.4 Lung inflammatory response to microbial challenge
TLR, Toll-like receptor; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; Th, T helper. Adapted from Strieter (74).

Lung inflammatory response can be induced by alveolar deposition of bacterial components such as LPS. In the initial stages of inflammation bacterial ligands activate resident cells in lung interstitium and alveolus, primarily macrophages, to secrete pro-inflammatory cytokines such as TNF-α and IL-1 and chemokines including RANTES, MIP-2 and KC (45, 54, 73) (Figure 1.4). These mediators act in concert to promote polymorphonuclear leukocyte (PMN) accumulation in lung microvasculature by activating endothelial cell adhesion molecule expression and induce migration of PMNs into lung interstitium and alveolar space. Within hours,

PMNs propagate inflammation through secretion of reactive oxygen species (e.g., hypochlorite), antimicrobial proteins (e.g., bactericidal permeability-inducing protein and lactoferrin), and proteolytic enzymes. However, reactive substances secreted by PMNs also affect host cells and can damage host tissues contributing to the lung pathology in pneumonia. Lung inflammatory response is further characterized by delayed recruitment of MPs, especially monocytes that can actively contribute to the pathogenesis of both acute and chronic lung inflammatory diseases. To control inflammatory lung responses, numerous strategies have been evolved such as elaboration of anti-inflammatory mediators like IL-10 and TGF-β1 (74, 75). For instance IL-10 blocks expression of IL-2, IFN-γ, IL-1, TNF-α, IL-12 and CXC and CC chemokines (74).

2 AIM OF THE STUDY

Resident alveolar macrophages are the major mononuclear phagocytic cells found in the lower respiratory tract. They act as sentinels for pathogens and their components such as LPS and bacterial lipoproteins sensed by TLR4 and TLR2, respectively. The signalling pathways activated in alveolar macrophages by different TLR agonists have not yet to been fully elucidated. Therefore, in the present study we aimed to further explore the molecular programs activated by bacterial TLR ligands. In particular, we sought to identify and functionally characterize novel mechanisms that regulate alveolar macrophage responses induced by TLR4 ligand LPS and TLR2 ligand Pam_3CSK_4.

In addition to activation of lung resident cells, alveolar recognition of inhaled pathogens promotes increased recruitment of circulating mononuclear phagocytes to inflamed tissue. Experimental data on their migration pathways, traffic related cell differentiation and functional role in inflamed lungs *in vivo* are scarce. Moreover, compared to the TLR4 ligand LPS a limited number of studies have addressed the induction of lung inflammation and the concomitant mononuclear phagocyte recruitment in response to TLR2 agonists. Therefore, this study was undertaken to analyse the global gene expression profile of mononuclear phagocytes recruited from the circulation into TLR2 agonist Pam_3CSK_4-treated inflamed lungs.

Perspectively, comprehensive insights into the molecular programs induced by microbial challenge in pulmonary mononuclear phagocytes may help to identify new molecular target for selective therapeutic intervention.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Animals

BALB/c and C57BL/6 WT mice were purchased from Charles River (Sulzfeld, Germany). CC3TLR/J BALB/c (TLR4-mutated) mice were purchased from Jackson Laboratories (Bar Harbour, USA) and TLR2-KO C57BL/6 mice from Oriental Yeast Company (Tagata Shizouka, Japan). PKR-KO C57BL/6 mice were a kind gift of Jovan Pavlovic (University of Zurich, Switzerland). CX3CR1$^{GFP/GFP}$ mice were generated on a mixed C57BL/6 x 129/Ola background by targeted disruption of CX3CR1 gene. Parent CX3CR1$^{GFP/GFP}$ and CX3CR1$^{+/+}$ mice were bred to yield heterozygous CX3CR1$^{+/GFP}$ offspring (76). All animals were kept under specific pathogen-free (SPF) conditions and used throughout the study between 8 and 12 weeks of age. Experimental protocols involving animals were approved by institutional and local government comities.

3.1.2 Equipment

Abbocath	Abbott, Germany
ABI PRISM 2400 PCR-thermocycler	Applied Biosystems, USA
ABI PRISM 7900HT Sequence detector	Applied Biosystems, USA
Agilent Bioanalyser 2100	Agilent Tech., Germany
BioDocAnalyse video system	Whatman – Biometra, Germany
Cell culture incubator	Heraeus, Germany
Cytospin Cytocentrifuge	Thermo Scientific, Germany
Developing machine, Curix 60	Agfa, Germany

Electrophoresis apparatus	Keutz, Germany
ELISA reader	Molecular Devices, Germany
Eppendorf tubes (1.5ml/2 ml)	Eppendorf, Germany
Film cassette	Cronex, Germany
Filter tip	Greiner bio-one, Germany
Filter units	Millipore, USA
Fluorescence 8 chamber glass slides	BD, Germany
Fluorescence Activated Cell Sorter	BD Biosciences, Germany
Fluorescence microscope	Leica, Germany
GenePix 4100A scanner	Axon Instruments, USA
Mini Protean 3 cell	Bio-Rad, USA
Mini spin centrifuge	Heraeus, Germany
Mini Trans Blot	Bio-Rad, USA
Mouse Whole Genome	Agilent Tech., Germany
Multifuge centrifuge, 1S-R	Heraeus, Germany
NanoDrop ND-1000	Nano Technologies, USA
PCR tubes (0.2 ml)	Applied Biosystems, USA
Pipetmans: P10, P20, P100, P200, P1000	Gilson, France
Pipette tip	BD, Germany
Power supply	Biometra, Germany
Serological pipette: 5, 10, 25, 50 ml	Falcon, USA
Stereomicroscope	Leica, Germany
Test tubes:15, 50 ml	Greiner Bio-One, Germany
Test tube thermostat	Roth, Germany
Tissue culture plates: 24, 48, 96 well	Greiner Bio-One, Germany

3.1.3 Kits

BD SMART Probe amplification kit	Clontech, Germany
DC protein assay	Bio-Rad, Germany
ELISA kits	R&D Systems, USA
Platinum® SYBR® Green I qPCR SuperMix-UDG	Invitrogen, Germany
RNeasy Micro kit	Qiagen, Germany
RNeasy Mini kit	Qiagen, Germany
TransAM p65 NF-κB assay kit	Active Motif, Belgium

3.1.4 Reagents

2-AP	Sigma-Aldrich, Germany
5 X first strand buffer	Invitrogen, Germany
Acetic Acid	Merck, Germany
Acetone	Sigma-Aldrich, Germany
Acrylamide solution, Rotiphorese Gel 30	Roth, Germany
Agarose	Peqlab, Germany
Ammonium persulfate (APS)	Promega, Germany
β-mercaptoethanol	Sigma-Aldrich, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Germany
Bromophenol blue	Sigma-Aldrich, Germany
Calibrate beads	BD Biosciences, Germany
DAPI	Sigma-Aldrich, Germany
DifcoTM Skim Milk	BD, Germany
Dithiothreitol (DTT)	Invitrogen, Germany
dNTPs	Applied Biosystems, USA

Dublecco' s phosphate buffered saline (PBS)	PAA Laboratories, Austria
ECL Plus Western Blotting Detection System	Amersham Biosciences, UK
E. coli lipopolysaccharide (0111:B4)	Calbiochem, Germany
Ethanol	Carl Roth, Germany
Ethidium bromide solution	Carl Roth, Germany
Ethylendinitrilo-*N, N, N´, N´,* -tetra-acetic acid (EDTA)	Promega, USA
Ethylene glycol-bis (2-amino-ethylether)-N,N,*N',N'* –tetra-acetic-acid (EGTA)	Sigma-Aldrich, Germany
Fetal calf serum (FCS)	PAA laboratories, Austria
Glycine	Sigma-Aldrich, Germany
Hydrochloric acid (HCl)	Merck, Germany
Igepal CA-630	Sigma-Aldrich, Germany
Isoflurane (1-chloro-2,2,2-trifluoroethly difluoromethyl ether)	Abbott, Germany
Ketavet (Ketamine hydrochloride)	Pharmacia & Upjohn, Germany
Methanol	Fluka, Germany
M-MLV reverse transcriptase	Promega, USA
Mounting medium	Dako, Germany
N,*N*,*N'*,*N'*-tetramethyl-ethane-1.2-diamine (TEMED)	Sigma-Aldrich, Germany
Pam3-Cys-Ser-Lys-Lys-Lys-Lys-OH (Pam_3CSK_4)	EMC Microcollections, Germany
Paraformaldehyde	Sigma-Aldrich, Germany
Penicillin-streptomycin	PAA Laboratories, Austria
Platinum Taq DNA polymerase	Invitrogen, Germany
Polyriboinosinic:polyribocytydylic acid (Poly I:C)	Alexis Biochemicals, Germany
Polyvinylidene difluoride (PVDF) membranes	Micron Separations, USA

Precision Plus Protein™ Standards	Bio-Rad, USA
Protease inhibitor cocktail	Roche, Germany
Random hexamers	Boehringer, Germany
RNase away	Molecular Bioproducts, USA
RNase inhibitor	Promega, USA
Rompun (Xylazine hydrochloride)	Bayer, Germany
RPMI 1640 medium	PAA laboratories, Austria
Sodium chloride	Braun, Germany
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, Germany
Sodium ortho vanadate	Sigma-Aldrich, Germany
Tris	Carl Roth, Germany
Tween 20	Sigma-Aldrich, Germany

3.1.5 Software

BioConductor	http://www.bioconductor.org
BLAST	http://www.ncbi.nlm.nih.gov
FACS DiVa software	BD Biosciences, Germany
GeneBank Analyzer	Developed by Dr. Jochen Wilhelm (Giessen University)
GENECODIS	www.genecodis.com
GenePix Pro 5.0	Axon, USA
MicroWin 3.0	Microtek, Germany
Primer Express 2.0	Applied Biosystems, USA
R software	http://www.cran.r-project.org

3.2 Methods

3.2.1 Treatment of animals

3.2.1.1 Mouse model of TLR2/4 ligands-induced lung inflammation

In order to study lung inflammatory responses *in vivo*, WT or $CX_3CR1^{+/GFP}$ mice received intratracheal (i.t.) application of TLR ligands. Pam_3CSK_4 was used as a synthetic TLR2 agonist recognized by the TLR1/TLR2 complex, whereas LPS served as specific TLR4 ligand. Briefly, mice were anesthetized with tetrazoline hydrochloride (2.5 mg/Kg) and ketamine (50 mg/Kg) and the trachea was exposed. Subsequently, a 26-gauge catheter was inserted into trachea and Pam_3CSK_4 (10 µg or 50 µg/mouse dissolved in a total volume of 60 µl) or LPS (1 µg or 10 µg/mouse dissolved in a total volume of 60 µl) was installed under stereomicroscopic control. After installation, the catheter was removed, wounds were closed and mice were allowed to recover with free access to food and water.

3.2.1.2 Collection and analysis of blood and bronchoalveolar lavage (BAL) fluid

Mice were sacrificed with an overdose of isoflurane and blood and BAL fluid (BALF) collection was done as described previously (69). Briefly, blood was collected from the *vena cava inferior* using 23-gauge cannula connected to a 1-ml insulin syringe that was filled with 100 µl of NaCl-EDTA as an anticoagulant. Lysis of red blood cells was performed using ammonium chloride solution.

For BAL, the trachea was exposed and a small incision was made to insert a shortened 21-gauge cannula connected to a 1-ml insulin syringe, followed by repeated i.t. instillations of 0.5 ml aliquots of PBS (pH 7,2, supplemented with 2 mM EDTA). BAL was performed until a total volume of 5 ml was recovered. BALF

was spun for 10 min at 1400 rpm at 4 °C and cells were resuspended in 1 ml of RPMI 1640 medium containing 10 % FCS and L-glutamine.

To study the inflammatory BALF profile, the cells were counted with a hemocytometer and quantitation of AMs and neutrophils was done on differential cell counts of Pappenheim-stained cytocentrifuge preparations as recently described (77).

3.2.1.3 Fluorescence activated cell sorting (FACS)

Recovered BALF and blood cells were pooled from 10-12 $CX_3CR1^{GFP/+}$ mice to obtain sufficient cell numbers for flow sorting. Prior to cell sorting, blood and BALF samples were filtered through a 40 µm cell strainer. The GFP-positive MPs were counted and sorted using a high-speed FACSVantage SE flow cytometer (BD Biosciences) as described previously (69). In short, the sorting algorithm for GFP-positive cells employed (1) forward scatter area (FSC-A) *versus* side scatter area (SSC-A), (2) FSC-A *versus* FL1 (F525 ± 15 nm; FITC/GFP) and (3) FL1 *versus* FL2 characteristics (F575 ± 25 nm). These settings allowed for the exclusion of lymphocytes, AMs and neutrophils in analyzed samples. The purity of sorted GFP-positive cells was ≥ 98 %. In addition, Pappenheim staining verified monocytic cell morphology of isolated GFP-positive cells. Dot plot profiles were obtained from 10^4 cells for each sample.

3.2.2 Cell culture and stimulation

3.2.2.1 Culture of murine primary alveolar macrophages

AMs were harvested by BAL from WT BALB/c, CC3TLR/J, TLR2-KO and PKR-KO mice as described in section 3.2.1.2. Cells were cultured in RPMI 1640 medium supplemented with 10 % FCS, L-glutamine and penicillin/streptomycin at 37 °C in a humidified 5 % CO_2 environment. After two hours, cells were washed with RPMI 1640 to remove non-adherent cells. Macrophages were seeded at 2.5-3 x 10^5

cells/well in 24-well tissue culture plates for western blotting and TransAM assay, at 8 x 10^4 cells/well in 96-well tissue culture plates for ELISA or at 6 x 10^4 cells/well on glass slides for immunofluorescence microscopy.

AMs were left untreated or stimulated with Pam_3CSK_4 (2 µg/ml), poly I:C (50 µg/ml) or LPS (200 ng/ml) for different periods of time as specified. Were indicated, AMs were pre-incubated with 4 mM 2-AP for 30 min.

3.2.3 RNA analysis

3.2.3.1 Isolation of total RNA

Total cellular RNA isolation from primary AMs was performed using RNeasy Mini Kit. Total cellular RNA from freshly sorted GFP-positive MPs was performed using the RNeasy Micro kit. In both the cases, RNA was isolated following the instructions of the manufacturer and was quantified spectrophotometrically using Nanodrop ND-100. For microarray experiments the RNA quality was assessed by the 18S/28S-rRNA bands in capillary electrophoresis.

3.2.3.2 Microarray analysis

The microarray experiments were performed in the Department of Pathology (University of Giessen) by Dr. Jochen Wilhelm and Marlene Stein. Purified total RNA was amplified and Cy-labeled using the SMART™ (switching mechanism at the 5' end of RNA templates of reverse transcriptase) kit following the kit instructions. Briefly, this method creates full-length complementary DNA (cDNA) from all oligo-A RNA with introduced specific primer binding sites at either end. One priming site is introduced by the oligo-dT primer and the other one is linked by template-switching of the MMLV RT: the RT adds a stretch of Gs to the end of the cDNA when the end of the RNA template is reached. A C-rich oligodesoxyribonucleotide containing the PCR-primer sequence binds to this stretch; the RT then switches the template and produces a sense cDNA strand that

now contains the binding site for the PCR primer at either side. These full-length cDNAs are subsequently amplified by PCR. In preliminary experiments, the number of amplification cycles was determined so that the PCR is not run until the plateau. The samples were finally amplified for 24 cycles, followed by 4 cycles with aminoallylated dCTP. After purification, monoreactive Cy-dyes were coupled to the incorporated amino groups. Cy3- and Cy5-labeled cDNAs were hybridized overnight to 44K 60mer oligonucleotide spotted microarray slides. Hybridization and subsequent washing and drying of the slides were performed following the Agilent hybridization protocol.

Dried slides were scanned using the GenePix 4100A scanner. Image analysis was performed with GenePix Pro 5.0 software, and calculated values for all spots were saved as GenePix results files. Stored data were evaluated using the R software (http://www.R-project.org) and the limma package from BioConductor (78). The spots were weighted for subsequent analyses according to the spot intensity, homogeneity, and saturation. The spot intensities were corrected for the local background using the method of Edwards with an offset of 64 to stabilize the variance of low-intensity spots (79). The M/A data were LOESS normalized before averaging (80). Genes were ranked for differential expression using a moderated t-statistic (81). Candidate lists were created by adjusting the false-discovery rate (FDR) to 10 %.

Raw data was submitted to NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hpgntswwcowiifq&acc=GSE11978).

3.2.3.3 Reverse transcription reaction

In order to measure messenger RNA (mRNA), reverse transcriptase (RT) was used to convert mRNA into cDNA which is then amplified by real-time PCR. To perform synthesis of cDNA, 50 ng to 500 ng of total RNA was mixed with random

hexamers (10 pmol) in PCR tube, heated at 70 ^{0}C for 5 min, placed on ice and the following RT mix reaction components were added.

RT mix

5x first strand buffer	5 µl
dNTPs	10 mM each
DTT	250 mM
Ribonuclease inhibitor	20 U
MMLV	200 U

Subsequently this mixture was incubated for 1 h at 42 ^{0}C, heated to 94 ^{0}C for 10 min to inactivate the enzyme and used for real-time PCR amplification immediately or stored at -20 ^{0}C till further use.

3.2.3.4 Quantitative real-time PCR

The transcriptional regulation of selected genes was analyzed using real-time quantitative PCR. Real-time PCR is a method based on the detection and quantification of a fluorescent reporter signal that increases in direct proportion to the amount of the PCR product in reaction (82, 83). Quantitative real-time PCR was performed using SYBR green, a fluorogenic dye that emits a strong fluorescent signal upon binding to the minor groove of double-stranded DNA product. PCR reactions were performed in 25 µl reactions using the following master mix.

Real time master mix

cDNA	5 µl
Forward primer	10 pmol
Reverse primer	10 pmol
SYBR® Green I	12 µl
ROX reference dye	0.5 µl
$MgCl_2$ (50 X)	1 µl

Cycling conditions were as following: 1 cycle of 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 sec, 60 °C for 30 s and 72 °C for 10 sec. After the cycling process, melting curve analysis and agarose gel electrophoresis (in selected cases) was performed to confirm the exclusive amplification of the expected PCR products. Relative gene expression is expressed as Delta Ct (ΔCT) with mouse porphobilinogen deaminase (PBGD) serving as reference gene: ΔCT = Ct [PBGD] – Ct [target gene] (higher values indicate higer mRNA levels). Differential gene expression between conditions is expressed as Delta Delta Ct (ΔΔCT) what corresponds to the log2 fold-difference in mRNA levels between the conditions compared (84). The mRNA sequences of the genes of interest were obtained from NCBI http://www.ncbi.nlm.nih.gov/sites/entrez. The oligonucleotide primer pairs were designed using Primer Express 2.0. Oligonucleotide primers are listed in *Appendix* (Table 6.1.)

3.2.4 Protein analysis

3.2.4.1 Cell extracts and western blotting

After removing the medium, cells were washed twice with cold 1 x PBS and ice-cold cell-lysis buffer was applied. The cell lysates were kept on ice for 30 min, followed by centrifugation for 15 min at 13000 rpm at 4 °C. Resulting supernatant was used as a crude extract.

Cell-lysis buffer

20 mM	Tris (pH 7.5)
150 mM	NaCl
1 mM	EDTA
1 mM	EGTA
0.5 %	Igepal CA-630
2 mM	Na_3VO_4
1 X	Protease inhibitor mix

Protein concentration was determined using Bio-Rad *DC* (detergent compatible) protein assay. This method is a colorimetric reaction similar to the well-documented Lowry assay (85). Subsequently, proteins were separated according to their size by SDS polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and denaturating conditions in 1 x SDS-running buffer (80 V and 40 mA for 2 h). Gels were composed of a 5 % stacking and a gel 10 % separating gel. Before samples were loaded into gel, proteins were mixed with loading buffer and heated at 95 °C for 5 min.

10 x Loading buffer

50 mM	Tris-HCL
100 mM	DTT
2% (w/v)	SDS
10% (v/v)	Glycerol
0,1% (w/v)	Bromophenol Blue

1 x SDS running buffer

25 mM	Tris base
250 mM	Glycine
0.1% (v/v)	SDS

Stacking gel 5 %

125 mM	Tris (pH 6.8)
5 %	Acrylamid
0.1% (w/v)	SDS
0.1%(v/v)	Temed
0.1% (w/v)	APS

Separating gel 10 %

375 mM	Tris (pH 8.8)
10 %	Acrylamid
0.1% (w/v)	SDS
0.1%(v/v)	Temed
0.1% (w/v)	APS

After gel electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a Bio-Rad transfer chamber containing transfer buffer. Transfer was performed at 120 V and 250 mA. After one hour, membranes were incubated in blocking buffer at room temperature for 1 h. Subsequently primary antibodies were added and membranes were incubated overnight at 4 °C, afterwards washed three times and incubated with horse radish peroxidase (HRP)-conjugated secondary antibody for 1 h. Enhanced Chemiluminescence (ECL) system was used to visualize immune complexes. Signals were detected using radiographic films. For reprobing, membranes were incubated at room temperature for 1 h in stripping buffer. Primary and secondary antibodies used for western blotting are listed in *Appendix* (Table 6.2).

Transfer buffer pH 7.4

25 mM	Tris base
192 mM	Glycine
10 % (v/v)	Methanol

Washing buffer (1 L)

100 ml	10 X PBS
900 ml	dH_2O
0.05 %	Tween 20

Blocking Buffer

5 % (w/v)	non-fat dry milk
1 X	PBS
0.05 %	Tween 20

Stripping Buffer (100 ml)

10 ml	Glycine (1M)
89 ml	dH_2O
1 ml	37 % HCl

3.2.4.2 Enzyme-linked immunosorbent assay (ELISA)

The quantification of murine TNF-α and IL-6 proteins in AM culture supernatants was performed by commercially available ELISA kits following the instructions of the manufacturer.

3.2.4.3 Immunofluorescence microscopy

Cells were washed twice with 1 x PBS and fixed with cold (-20 °C) methanol:acethone mixture (1:1) for 10 min. After washing, cells were blocked overnight with 10 % BSA in PBS, washed twice and then incubated overnight with rabbit anti-p65 NF-κB antibody (1:50) diluted in 1 % BSA at 4 °C. Immune complexes were detected with Alexa Fluor 488 goat anti-rabbit secondary antibody diluted in 1 % BSA (1:1000) at room temperature for 1 h in darkness. After washing, cells were fixed in 4 % paraformaldehyde for 10 min. Nuclei were stained with DAPI (1:100) diluted in 1 % BSA. The coverslips were mounted on slides in mounting medium. Cells were imaged by conventional fluorescence microscopy. Primary and secondary antibodies used for immunofluorescence microscopy are listed in *Appendix* (Table 6.2).

3.2.4.4 TransAM p65 NF-κB assay

Whole cell extract (5 µg) was used to monitor activation of the p65 subunit of NF-κB by the commercially available TransAM p65 NF-κB assay kit purchased from Active Motif following the instructions of manufacturer.

3.2.5 Statistical analysis

The data for BALF cell profile are presented as mean ± SD. Differences between treatments groups were analysed by one-way ANOVA followed by Dunnett *post hoc* test. A value of $p \leq 0.05$ was considered as significant. ΔCt and ΔΔCt values are given as mean ± SD or SEM of at least three independent experiments.

Differences in mean ΔCt values were tested by two-tailed (paired or unpaired) t-tests. A value of $p \leq 0.05$ was considered significant.

4 RESULTS

4.1 Gene expression of TLR associated proteins induced by inflammatory stimuli in AMs

Initial experiments aimed to analyse the transcriptional regulation of genes involved in TLRs signalling in response to bacterial components that are differentially recognized by distinct TLR family members. We used synthetic lipoprotein Pam_3CSK_4 that is specifically recognized by the TLR1/2 complex, whereas LPS derived from *Escherichia coli* was used as specific ligand for TLR4 (86). Genes selected for evaluation belonged to different functional categories: PRRs (CD14, CD36, TLR2 and TLR4), cytokine regulators (SOCS 1/3), inflammatory cytokines (TNF-α, IL-6), antiviral defence and negative regulators of TLRs (IRAK-M, ST2 and SIGIRR). The full names of genes are presented in table 4.1.

AMs isolated from BALF of WT BALB/c mice were left either untreated or stimulated with the TLR2 ligand Pam_3CSK_4 or the TLR4 ligand LPS. Using quantitative real-time PCR, we found that both ligands induced comparable changes in the expression profile of TLR molecules after 6 h and 24 h *in vitro* (Table 4.1). Transcription of two PRRs, TLR2 and CD14 was significantly increased, whereas expression of TLR4 and CD36 mRNA remained unchanged after treatment with both ligands. Most drastic changes in mRNA levels were noted for the pro-inflammatory cytokines IL-6 and TNF-α and for SOCS proteins. Interestingly, transcription of dsRNA-activated protein kinase R (PKR), the antiviral defence molecule, was significantly increased in response to Pam_3CSK_4 and LPS ligands. Among negative regulators, IRAK-M mRNA was significantly up-regulated after treatment with both TLR ligands, whereas ST2 was induced only by the TLR2 ligand after 6 h.

Table 4.1 mRNA expression of TLR associated genes in cultured AMs

Primary AMs isolated from WT mice were stimulated with LPS or Pam_3CSK_4 for 6 h or 24 h. After RNA isolation, the relative amount of each mRNA gene transcript was determined using real-time PCR. Relative gene expression was calculated using the ΔΔCT method as described in *Materials and Methods*. Positive values indicate up-regulation of that particular gene, whereas negative values indicate the down-regulation of a given gene. The results are given as mean ± SD from three independent experiments. * indicates *P* at least < 0.05.

Gene symbol	Gene description	ΔΔCT±SD			
		LPS		Pam3CSK4	
		6h	24h	6h	24h
CD14	CD 14 antigen	*2.7±0.6	*3.3±0.6	*2.7±0.4	*2.9±0.4
CD36	CD 36 antigen	0.2±0.4	-1.3±0.3	0.2±0.3	-0,8±0.3
TLR2	Toll-like receptor 2	*2.4±0.2	*3.5±0.2	*1.6±0.1	1.7±0.5
TLR4	Toll-like receptor 4	0.1±0.5	*0.6±0.1	0.3±0.4	0.0±0.2
SOCS-1	Suppresor of cytokine signalling 1	*4.6±1.1	*4.1±0.8	*3.3±0.2	*4.7±0.1
SOCS-3	Suppresor of cytokine signalling 3	*4.7±0.2	*3.8±0.2	*4.5±0.1	*3.7±0.3
TNF-α	Tumor necrosis factor alpha	*3.4±0.4	-0.6±0.4	*2.7±0.4	0.4±0.5
IL-6	Interleukin 6	*10.0±2.1	*7.8±2.1	*9.8±0.2	*9.1±0.5
PKR	Protein kinase R	*3.0±0.5	*2.2±0.2	*1.7±0.3	*1.6±0.3
IRAK-M	Interleukin-1 receptor-associated kinase M	*1.8±0.1	*2.4±0.2	*2.1±0.4	*2.5±0.3
ST2	Interleukin 1 receptor-like 1	0.4±0.7	–0.2±0.5	*1.4±0.5	0.2±0.8
SIGGIR	Single immunoglobulin domain IL-1R-related	–1.7±0.3	0.5±0.4	-1.5±0.3	-0.5±0.6

To study regulation of TLRs and associated signalling molecules in the alveolar space under *in vivo* conditions, WT BALB/c mice were i.t. administrated with Pam_3CSK_4 (10 µg/mouse) or LPS (1 µg/mouse). Subsequently, AMs were separated by high-speed FACSDiva flow cytometry as described in *Materials and Methods* (Figure 4.1). After sorting, total RNA was isolated and subjected to quantitative real-time PCR. We observed that gene expression patterns from *in vitro* experiments largely matched with *in vivo* data (Table 4.2). Interestingly, AMs expressed significantly more PKR mRNA after LPS administration, whereas there was no increase of PKR mRNA after i.t. Pam_3CSK_4 administration. Based on these results and data from the literature we decided to analyze in detail the role of PKR in AMs in response to bacterial TLR ligands.

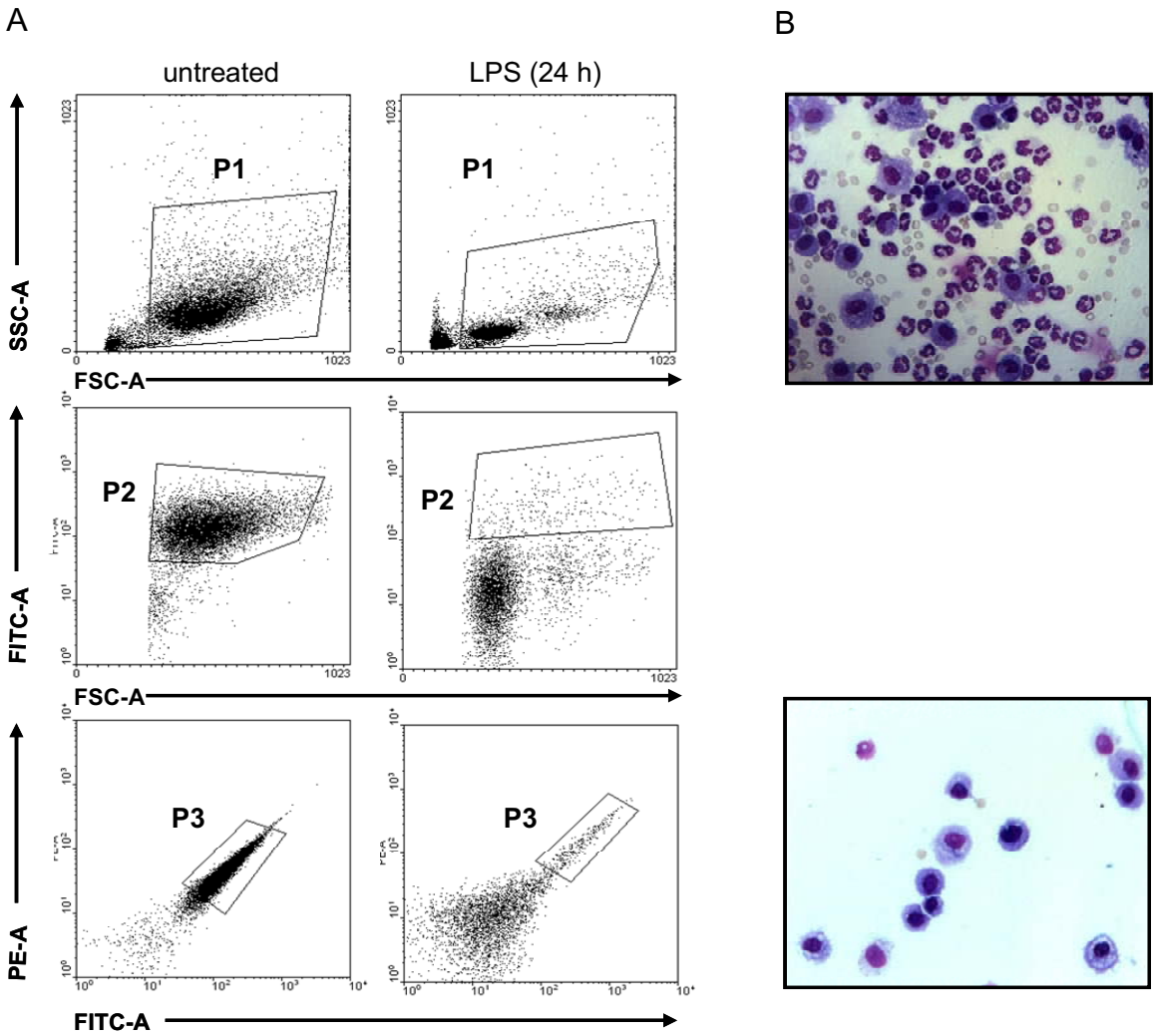

Figure 4.1 Flow cytometric identification and separation of AMs of untreated and LPS treated WT mice

(A) A representative FACS profile of BALF cells collected from WT mice either left untreated (*left column*) or intratracheally administrated with LPS (1 µg/mouse) (*right column*). AMs were gated according to the forward scatter area (FSC-A) vs. side scatter area (SSC-A) (P1), FSC-A vs. fluorescence 1 area (FL1-A, FITC-A) (gate P2) and FL1-A vs. fluorescence 2 area (FL2-A, PE) (gate P3, green high auto-fluorescent AMs). (B) Pappenheim-stained cytospins of BALF cells isolated from LPS-treated mice prior to sorting (*upper photomicrograph*) and after sorting (*bottom photomicrograph*).

Table 4.2 mRNA expression of TLR associated genes in flow-sorted AMs

Gene expression profiles of selected genes in flow-sorted AMs collected from WT mice intratracheally administrated with LPS or Pam_3CSK_4 for 6 h or 24 h. After RNA isolation, the relative expression of each mRNA gene transcript was determined using real-time PCR. Relative gene expression was calculated using the ΔΔCT method as described in *Materials and Methods*. Positive values indicate up-regulation of that particular gene, whereas negative values indicate the down-regulation of a given gene. The results are given as mean ± SD from three independent experiments. * indicates *P* at least < 0.05.

Gene symbol	Gene description	ΔΔCT±SD			
		LPS		Pam3CSK4	
		6h	24h	6h	24h
CD14	CD 14 antigen	4.2±1.5	2.1±0.8	2.4±1.4	1.8±0.5
CD36	CD 36 antigen	0.5±0.4	0.5±0.5	1.6±0.7	0.6±0.8
TLR2	Toll-like receptor 2	*2.8±0.2	0.7±0.2	*2.2±0.1	0.6±0.7
TLR4	Toll-like receptor 4	−0.4±0.3	−0.5±0.5	−0.4±0.8	−0.5±0.6
SOCS-1	Suppresor of cytokine signalling 1	*3.6±1.6	*1.9±0.6	1.8±0.9	*2.0±0.6
SOCS-3	Suppresor of cytokine signalling 3	*5.9±0.8	*3.4±0.9	*4.7±1.1	3.2±1.1
TNF-α	Tumor necrosis factor alpha	*5.6±1.2	1.8±0.8	*4.3±1.8	2.4±0.8
PKR	Protein kinase R	*3.0±0.9	0.0±0.2	0.0±0.5	0.0±0.4
IRAK-M	Interleukin-1 receptor-associated kinase M	*3.2±0.8	1.3±0.5	2.5±0.6	1.9±0.5

4.2 Role of PKR in TLR2/4 signalling in murine AMs

4.2.1 PKR protein expression after stimulation with TLR2/4 ligands

To determine the regulation of PKR protein expression after PAMP stimulation we used western blotting. In agreement with the observations on mRNA expression levels, PKR protein expression was increased after both Pam_3CSK_4 and LPS stimulation *in vitro* (Figure 4.2).

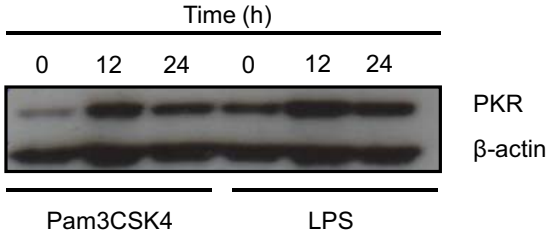

Figure 4.2 PKR protein accumulation after stimulation with TLR ligands
WT AMs were stimulated with either Pam_3CSK_4 (2 µg/ml) or LPS (200 ng/ml) for the indicated time intervals. Cells lysates were resolved on SDS-PAGE and probed with anti-PKR antibody as described in *Materials and Methods*. Anit-β-actin antibody served as loading control. The data are representative of two independent experiments.

4.2.2 Induction of PKR phosphorylation by TLR2/4 ligands

Since PKR phosphorylation is necessary for its activation, we examined whether PKR is phosphorylated in murine primary AMs after stimulation with TLR ligands *in vitro*. Isolated AMs were left untreated or stimulated with Pam_3CSK_4 or LPS for indicated time intervals and PKR phosphorylation was evaluated by western blotting. PKR was found to be activated by both TLR ligands within 15 min, reaching maximum phosphorylation levels at 30 min after stimulation (Figure 4.3).

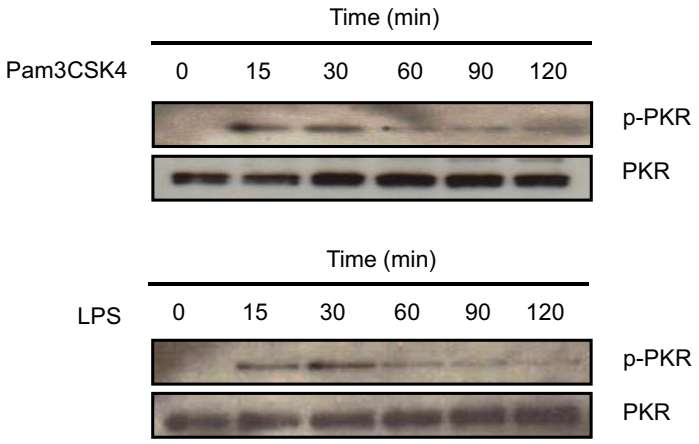

Figure 4.3 Time-course analysis of PKR phosphorylation
WT AMs were stimulated with Pam_3CSK_4 (2 µg/ml) or LPS (200 ng/ml) for the indicated time intervals. Cells lysates were resolved on SDS-PAGE and probed with anti-phospho PKR and anti-PKR antibodies as described in *Materials and Methods*. The data are representative of three independent experiments.

To elucidate whether Pam_3CSK_4 and LPS-induced activation of PKR was dependent on specific binding to the respective TLR, we isolated AMs from TLR2-deficient (TLR2-KO) and TLR4 mutated mice (CC3TLR/J). Cells were stimulated with LPS, Pam_3CSK_4 or poly I:C (synthetic dsRNA) for 30 min and PKR phosphorylation was assessed by western blotting (Figure 4.4). No activation of PKR was observed after stimulation with LPS and Pam_3CSK_4 in TLR4 mutated and TLR2-KO cells, respectively. As expected, poly I:C triggered PKR activation in WT, TLR2-KO and TLR4-mutated macrophages. Taken together, these results demonstrate that not only TLR4 but also TLR2 ligands are capable to induce PKR phosphorylation in AMs. Moreover this activation was strictly dependent on the functionality of the respective TLR.

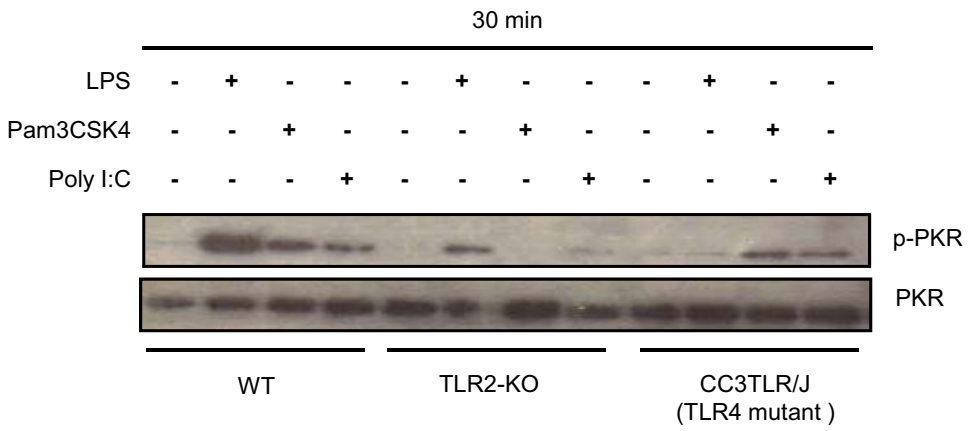

Figure 4.4 Activation of PKR by PAMPs is TLR dependent
AMs isolated from WT, TLR2-KO and CC3TLR/J mice were left untreated or stimulated with LPS (200 ng/ml), Pam_3CSK_4 (2 µg/ml) or Poly I:C (50 µg/ml) for 30 min. Cells lysates were resolved on SDS-PAGE and probed with anti-phospho PKR and anti-PKR antibodies as described in *Materials and Methods*. The data are representative of two independent experiments.

To investigate further the role of PKR kinase activity in TLR2/4 signalling, we applied 2-aminopurine (2-AP), a widely used specific inhibitor for PKR phosphorylation. 2-AP is acting as a competitive inhibitor of adenosine triphosphate (ATP) during the autophosphorylation of activated PKR (87). Pre-treatment of AMs with the PKR inhibitor 2-AP (4 mM) for 30 min prior to stimulation with the respective TLR agonist, drastically reduced PKR phosphorylation but also resulted in down-regulation of PKR mRNA levels (Figure 4.5).

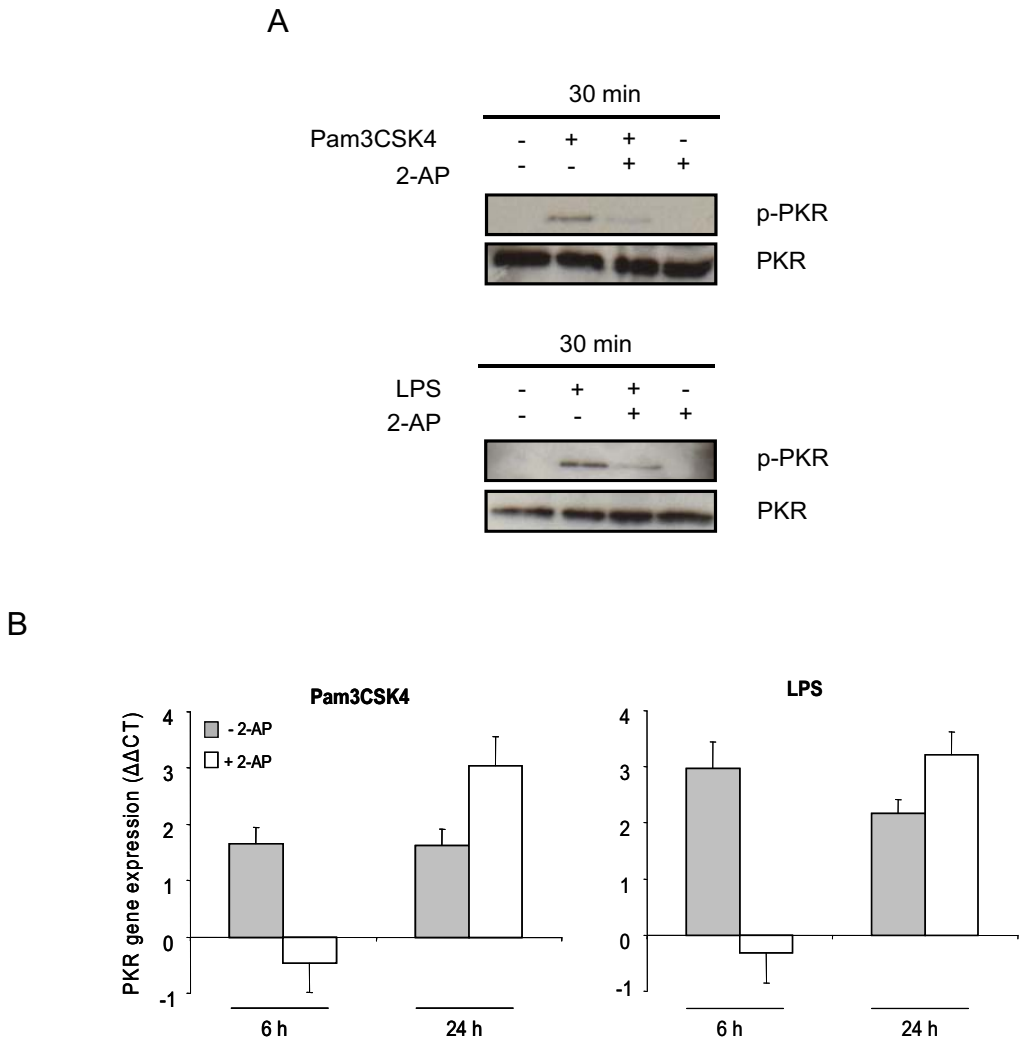

Figure 4.5 Pharmacological inhibition of PKR phosphorylation by 2-AP

WT AMs were stimulated with Pam_3CSK_4 (2 µg/ml) or LPS (200 ng/ml) for the indicated time intervals and the effect of 2-AP on PKR activation and expression was evaluated. (A) Cells lysates were resolved on SDS-PAGE and probed with anti-phospho PKR and anti-PKR antibodies as described in *Materials and Methods*. The data are representative of three independent experiments. (B) 2-AP effect on PKR mRNA was analysed by real-time quantitative PCR as described in *Materials and Methods*. Data are represented as mean ± SD of three independent experiments.

4.2.3 Involvement of PKR in PAMP-induced release of inflammatory cytokines

Induction of TLR signalling by PAMP binding to the respective receptor ultimately leads to pro-inflammatory cytokine secretion. To evaluate whether PKR activation is required for TLR2 and TLR4 mediated cytokine release, WT AMs in the presence or absence of the PKR inhibitor 2-AP and PKR-KO AMs were stimulated with LPS or Pam_3CSK_4 for 6 h (Figure 4.6).

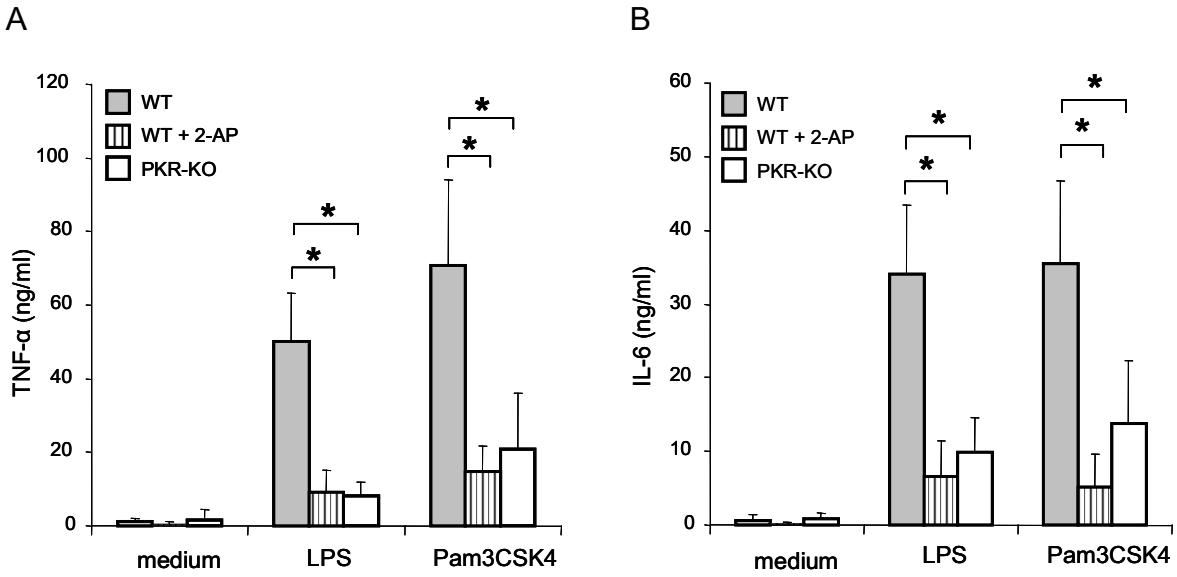

Figure 4.6 PAMP-induced inflammatory cytokine secretion requires PKR phosphorylation
AMs from WT or PKR-KO mice were incubated with medium alone or with 2-AP (4 mM) for 30 min followed by stimulation with LPS (200 ng/ml) or Pam_3CSK_4 (2 µg/ml) for 6 h. Collected supernatants were assayed for TNF-α (A) and IL-6 (B) concentrations by ELISA as described in *Materials and Methods*. Data are represented as mean ± SD of at least three independent experiments. * indicates *P* at least < 0.01.

WT cells pre-treated with 2-AP as well as PKR-KO cells displayed a significantly reduced PAMP-induced release of TNF-α and IL-6 as assessed by ELISA in the supernatant. These data indicate that PKR phosphorylation is critically involved in TLR2 and TLR4 mediated cytokine secretion by AMs.

4.2.4 Role of PKR in NF-κB activation

PAMP-induced production of TNF-α and IL-6 is dependent on the activation of transcription factors such as NF-κB. To assess whether the activation of NF-κB following TLR2 or TLR4 signalling depends on PKR phosphorylation, WT AMs in the presence or absence of 2-AP and PKR-KO AMs were stimulated with LPS or Pam_3CSK_4. Activation of the p65 subunit of NF-κB was determined with a NF-κB p65 ELISA-based assay. p65 activation induced by LPS was found to be significantly reduced after pre-treatment with 2-AP. Correspondingly, LPS induced p65 NF-κB signalling was found to be significantly attenuated in PKR-KO AMs (Figure 4.7 A). In contrast, p65 activation elicited by the TLR2 agonist Pam_3CSK_4 stimulation was only slightly impaired by pre-treatment of WT AMs with 2-AP or in PKR-KO cells (Figure 4.7 B).

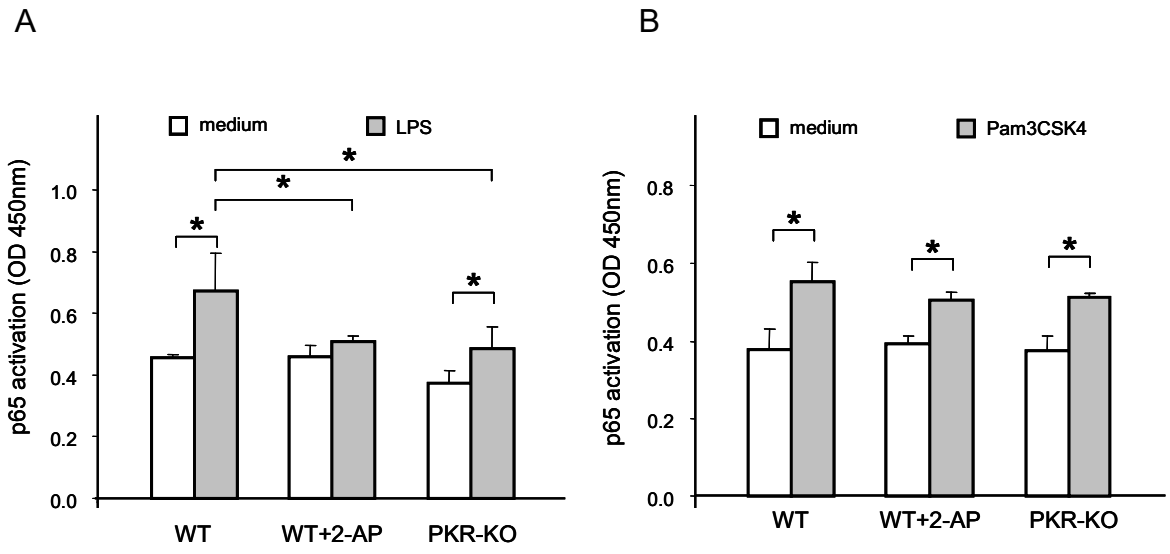

Figure 4.7 Pam_3CSK_4-induced activation of NF-κB is PKR-independent
AMs from WT or PKR-KO mice were incubated with medium alone or with 2-AP for 30 min followed by stimulation with LPS (200 ng/ml) for 1 h (A) or with Pam_3CSK_4 (2 µg/ml) for 2 h (B). Activation of p65 subunit of NF-κB was monitored in whole cell extracts (5 µg) using the ELISA-based TransAM NF-κB kit assay as described in *Materials and Methods*. Results are presented as mean ± SD of at least three different experiments. * indicates *P* at least < 0.05. TransAM assay specificity was monitored using nuclear extracts isolated from Jurkat cells as described in *Appendix* (Figure 6.2).

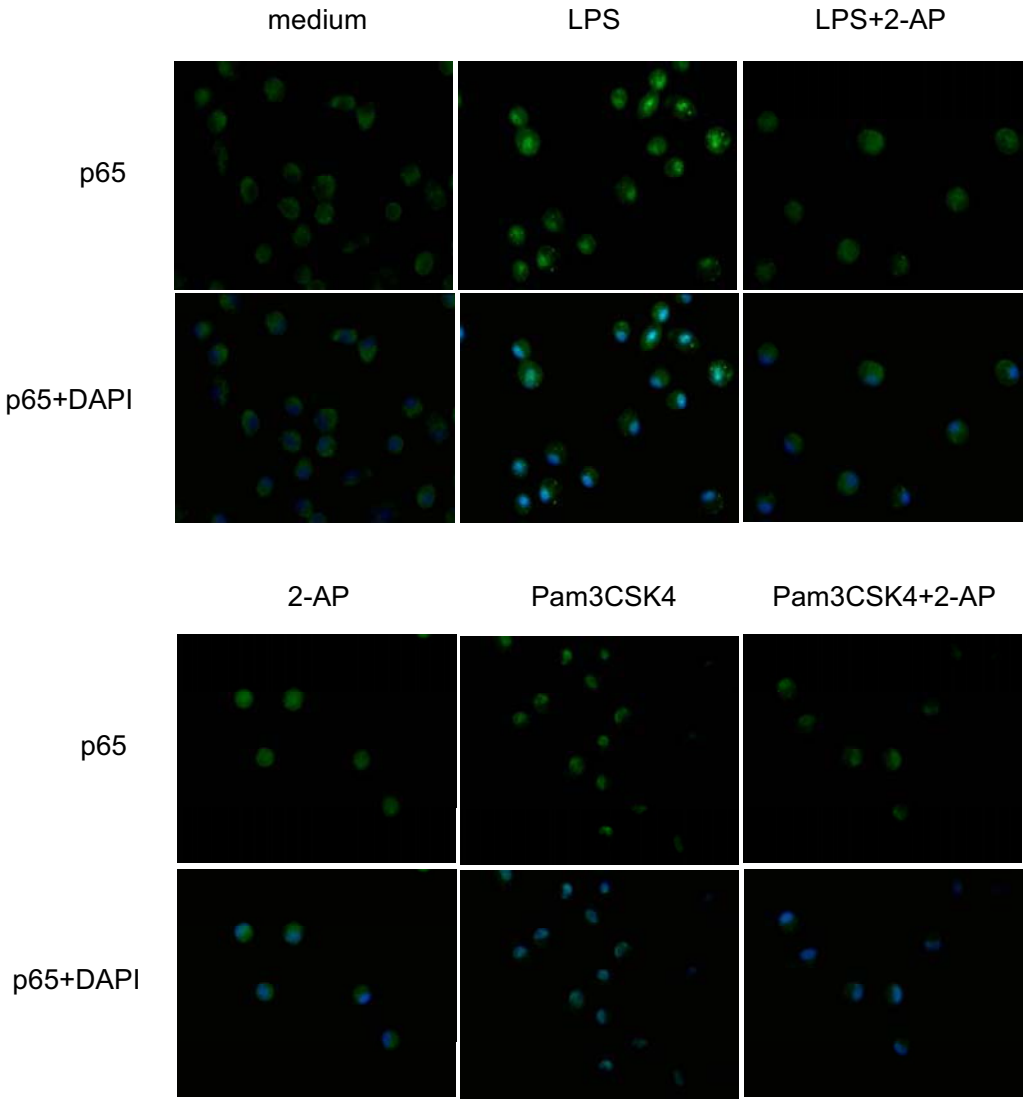

Figure 4.8 Immunofluorescence microscopy of p65 nuclear translocation

AMs from WT mice were incubated with medium alone or with 2-AP for 30 min followed by stimulation with LPS (200 ng/ml) for 1 h or with Pam_3CSK_4 (2 µg/ml) for 2 h. After washing, cells were stained with primary rabbit antibody against p65, followed by incubation with a secondary Alexa fluor 488 labeled goat anti-rabbit Ig antibody (green) as described in *Materials and Methods*. Nuclei were counterstained with DAPI (blue). The cells were imaged by conventional microscopy. Images are representative of three independent experiments. Secondary antibody control is presented in *Appendix* (Figure 6.3).

To confirm this differential effect of PKR phosphorylation inhibition on TLR2 vs. TLR4 mediated NF-κB activation, we examined PAMP-induced nuclear translocation of p65 by immunofluorescence microscopy (Figure 4.8). In untreated AMs, p65 was localized exclusively in the cytoplasm, following stimulation with LPS or Pam_3CSK_4, p65 translocated to the nucleus. 2-AP pre-treatment caused a significant reduction of p65 nuclear translocation after LPS but not after Pam_3CSK_4 stimulation. Together, these results suggest that PKR phosphorylation plays a major role in LPS but not Pam_3CSK_4-induced activation of the p65 NF-κB subunit.

4.2.5 The role of PKR in MAPKs signalling pathway

Previous studies have demonstrated that PKR mediates activation of MAPKs in response to inflammatory stimuli in various cell types (88-90). Therefore we aimed to delineate the role of PKR in this process in AMs. PKR inhibitor 2-AP and AMs deficient in functional PKR were used to describe the role of PKR in MAPKs activation. We found that only JNK phosphorylation was affected by pharmacological suppression of PKR activity and in PKR-KO AMs (Figure 4.9).

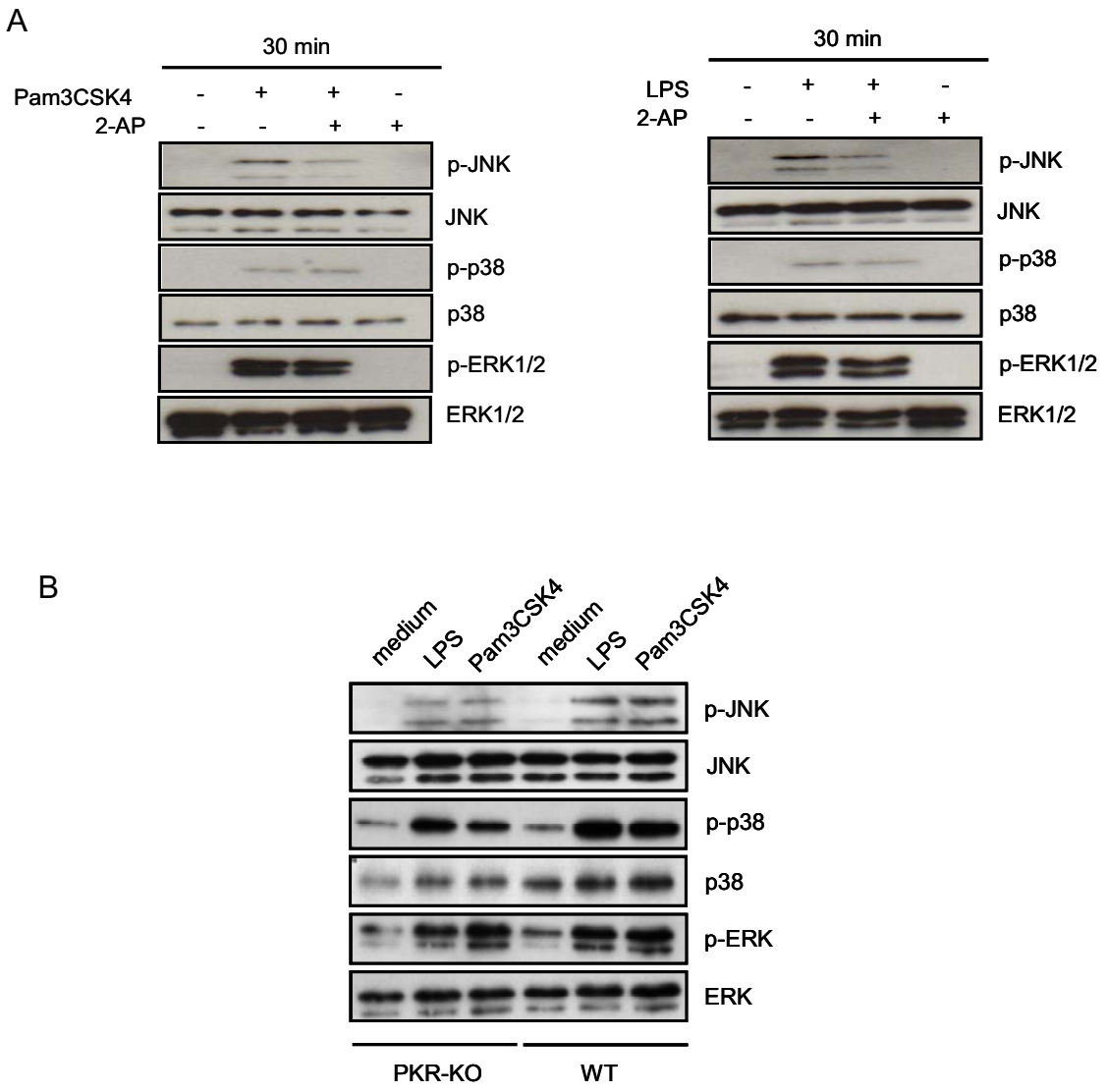

Figure 4.9 PKR regulates the TLR2/4 ligand-induced JNK signalling pathway

MAP kinases activation was evaluated in WT AMs incubated with medium alone or with 2-AP for 30 min (A) or in AMs derived from PKR-KO mice (B). Cells were stimulated with Pam_3CSK_4 (2 µg/ml) or LPS (200 ng/ml) for 30 min. Subsequently, cells lysates were resolved on SDS-PAGE and analyzed for MAP kinases phosphorylation using antibodies against phospho-JNK, phospho-p38, phospho-ERK1/2, total JNK, total 38 and total ERK as described in *Materials and Methods*.

To further confirm the role of PKR in the JNK signalling pathway, we tested the effect of 2-AP on the regulation of MKK4, a kinase upstream of JNK, and on the downstream JNK target c-Jun. Inhibition of PKR markedly reduced phosphorylation of MKK4 and strongly decreased c-Jun phosphorylation as assessed by mobility shift (Figure 4.10). Collectively, our data indicate that PKR

regulates the JNK signalling pathway (PKR-MKK4-JNK-c-Jun) in AMs activated by TLR2 or TLR4 ligands.

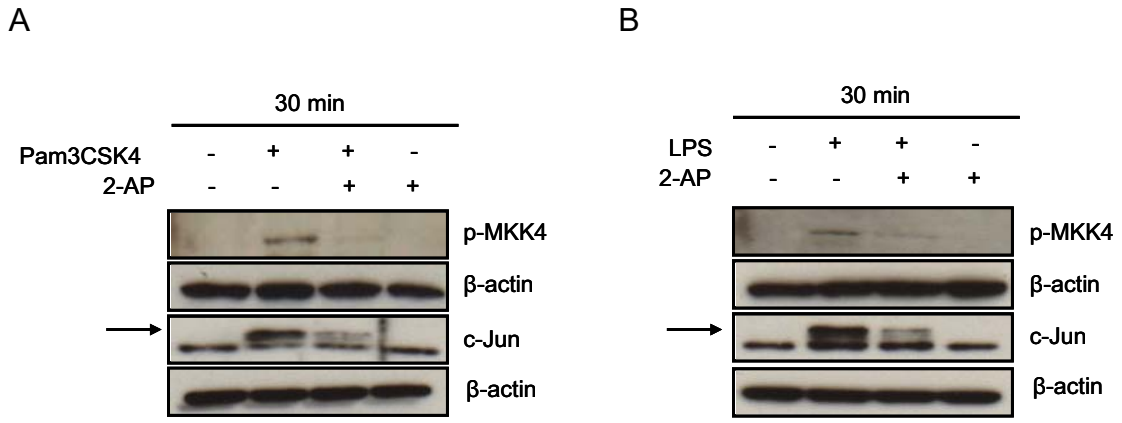

Figure 4.10 PKR regulates JNK signalling pathway *via* MKK4

WT AMs were incubated with medium alone or with 2-AP for 30 min, followed by stimulation with Pam_3CSK_4 (2 µg/ml) (A) or LPS (200 ng/ml) (B) for 30 min. Cells lysates were resolved on SDS-PAGE and analyzed for JNK pathway activation using antibodies against phospho-MKK4 and total c-Jun as described in *Materials and Methods*. Anti-β-actin antibody served as loading control. Arrow indicates the mobility shift of phosphorylated c-Jun. Data are representative of three independent experiments.

4.3 Genome-wide transcriptional profiling of MPs recruited to mouse lungs in response to alveolar challenge with the TLR2 agonist Pam_3CSK_4

4.3.1 Induction of lung inflammation and alveolar trafficking of circulating MPs by intratracheal deposition of Pam_3CSK_4

To determine the capacity of the synthetic TLR2 agonist Pam_3CSK_4 to induce lung inflammation *in vivo*, WT C57Bl/6 mice received i.t. application of Pam_3CSK_4 (50 µg/mouse) and BALF cellular constituents were monitored at various time points post challenge. Pulmonary administration of Pam_3CSK_4 provoked rapid recruitment of neutrophils into the alveolar compartment that was detectable as early as at 6 h, peaked at approximately 24 h after challenge and significantly declined within the next 24 h. The number of BALF macrophages was found to be slightly increased at 24 h post challenge without reaching statistical significance (Figure 4.11).

To further assess alveolar trafficking of MPs we employed transgenic $CX3CR1^{+/GFP}$ mice expressing green fluorescence protein (GFP) under control of the CX3CR1 promoter as described before (69, 76). This approach allowed identifying and further high purity sorting of GFP-positive MPs from the peripheral blood and of freshly recruited MPs from BALF as described in *Materials and Methods*. Heterozygous $CX3CR1^{+/GFP}$ mice were either left untreated or received i.t. application of the TLR2 ligand Pam_3CSK_4 (50 µg/mouse) and recruited MPs numbers were assessed in BALF after 24 h or 48 h. Very few GFP-positive cells (0.3 %-0.5 %) were detected in BALF collected from untreated mice (Figure. 4.12). In contrast, at 24 h and even more pronounced at 48 h after Pam_3CSK_4 i.t. application we observed significantly increased proportions of GFP-positive cells in BALF samples (1.4-1.7 % and 2.5-3.5 %, respectively). These data demonstrate that i.t. application of the TLR2 ligand Pam_3CSK_4 induced enhanced trafficking of circulating MPs into the alveolar space.

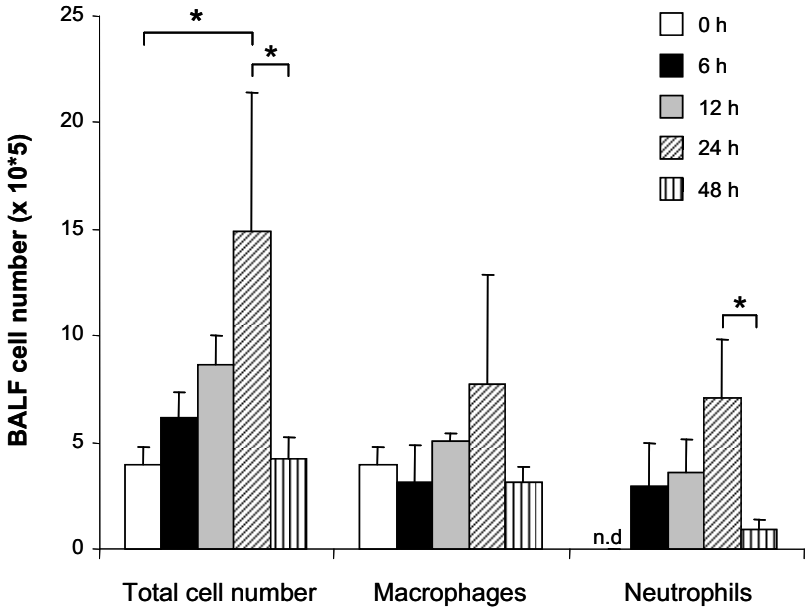

Figure 4.11 Inflammatory cell accumulation in the bronchoalveolar compartment in response to alveolar deposition of Pam_3CSK_4

C57BL/6 WT mice were either left untreated (0 h) or were challenged intratracheally with Pam_3CSK_4 (50 µg/mouse) for 6h, 12h, 24h, or 48 h. After indicated times, BALF cells were isolated and microscopic differentiation and quantification of macrophages and alveolar recruited neutrophils were performed as described in *Materials and Methods*. Means of different time points (five mice per treatment group) were compared using the ANOVA test as described in *Materials and Methods*. * indicates *P* at least < 0.01.

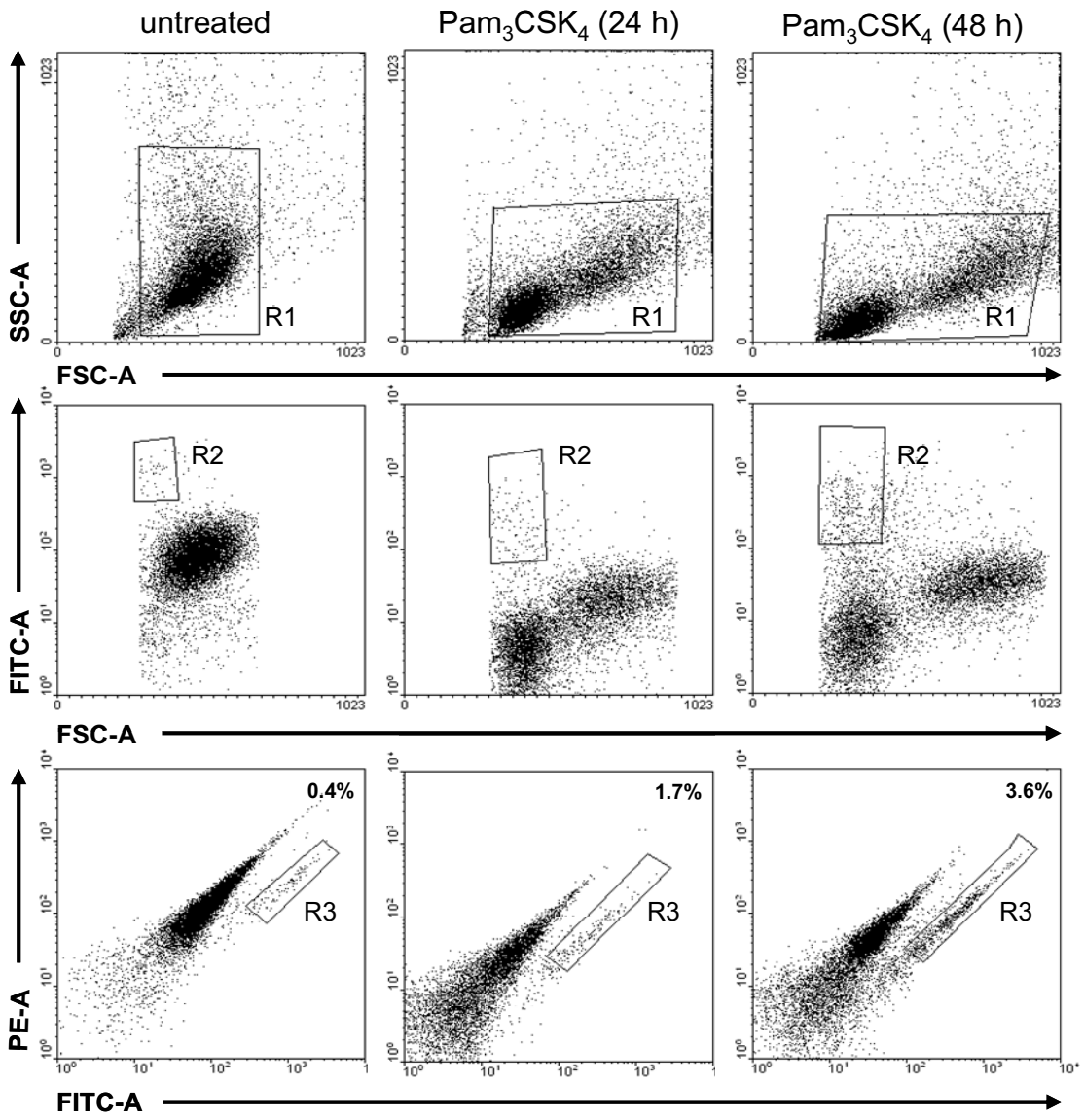

Figure 4.12 Lung recruitment of MPs in the absence or presence of the Pam_3CSK_4
A representative FACS profile of BALF cells collected from $CX3CR1^{+/GFP}$ mice either left untreated (left column) or i.t. administrated with Pam_3CSK_4 (50 µg/mouse) for 24 h (middle column) or 48 h (right column). At the indicated time points, mice were sacrificed and BALF and blood (not shown) were collected followed by fluorescence activated cell sorting of peripheral blood and alveolar MPs as described in *Materials and Methods*. Briefly, alveolar MPs were gated according to forward scatter area (FSC-A) vs. side scatter area (SSC-A) (R1, living cells), FSC-A vs. fluorescence 1 area (FL1-A, FITC-A) (gate R2, GFP positive MPs) and FL1-A vs. fluorescence 2 area (FL2-A, PE-A) (gate R3, GFP-positive MPs among R2). Data show one representative of four independent experiments.

4.3.2 Genome-wide transcriptional profiling of MPs recruited to the alveolar space after pulmonary challenge with Pam_3CSK_4

To investigate global changes in the gene expression profiles of MPs recruited to Pam_3CSK_4 exposed lungs during maximal inflammation (24 h), 10 to 12 heterozygous $CX_3CR1^{+/GFP}$ mice received i.t. application of the TLR2 ligand Pam_3CSK_4 (50 µg/mouse). Twenty four hours post challenge, GFP-expressing MPs were isolated in high purity from both PB and BALF and comparative global gene expression profiles of PB and alveolar recruited MPs were determined using SMART amplification method and whole mouse genome oligonucleotide arrays.

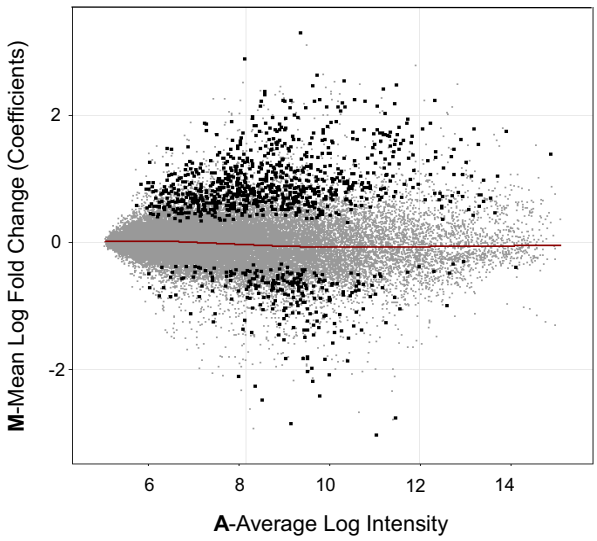

Figure 4.13 Global gene expression differences of alveolar recruited vs. PB MPs after i.t. application of Pam_3CSK_4

The plot shows the average log_2 signal ratio (M values) plotted against the average log_2 of the spot intensity (A values). Positive (negative) M values indicate an up (down)-regulation upon alveolar recruitment. The black dots mark candidate genes identified as differentially regulated by a moderated t-test using a false-discovery rate of 10 %.

We found that the global transcriptome of MPs recruited to the alveolar compartment at 24 h in response to Pam_3CSK_4 was dramatically changed in comparison to circulating MPs obtained at the same time point (Figure 4.13). Approximately 3700 genes differentially expressed between PB and alveolar recruited MPs were identified (10 % FDR) and the majority of which (~2500) were up-regulated in the alveolar MP cell population. This group contains a broad panel of genes encoding pro-inflammatory cytokines previously described to be robustly up-regulated by different microbial components in various cell types such as interleukin IL-1α, TNF-α, IL-6, and IL-12A (Table 4.3). We additionally observed up-regulated mRNA levels of chemokines such as KC, CXCL11, CXCL12, CXCL13, CXCL16, CCL2 and CCL7, whereas the gene expression of the neutrophil and monocyte chemoattractant CXCL7 was significantly down-regulated in alveolar mononuclear phagocytes.

Table 4.3 Differential gene expression between alveolar recruited and PB MPs

Gene symbol	Accesion No.	Gene description	Coefficients	SEM	p-value
Anti-inflammatory genes					
IL1RN	NM_031167	Interleukin 1 receptor antagonist	2,5	0,44	0,003
FPR1	NM_013521	Formyl peptide receptor 1	1,7	0,20	<0,001
ADORA2B	NM_007413	Adenosine A2b receptor	1,6	0,14	<0,001
IRAKM	NM_028679	Interleukin-1 receptor-associated kinase M	1,6	0,05	<0,001
ANXA1	NM_010730	Annexin 1	1,1	0,13	<0,001
BAX	NM_007527	Bcl2-associated X protein	1,1	0,13	<0,001
Il18BP	NM_010531	Interleukin 18 binding protein	0,7	0,08	<0,001
IL10	NM_010548	Interleukin 10	0,7	0,15	0,014
Eicosanoids metabolism					
PTGES	NM_022415	Prostaglandin E synthase	2,0	0,36	0,003
LTC4S	NM_008521	Leukotriene C4 synthase	1,6	0,33	0,006
LTB4DH	NM_025968	Leukotriene B4 12-hydroxydehydrogenase	0,9	0,11	<0,001
COX2	NM_011198	Cyclooxygenase 2	0,7	0,12	0,002
HPGD	NM_008278	Hydroxyprostaglandin dehydrogenase 15	-1,5	0,30	0,005
ALOX12	NM_007440	Arachidonate 12-lipoxygenase	-1,7	0,24	0,003
Cytokines and chemokines					
IL1A	NM_010554	Interleukin 1 alpha	2,9	0,36	<0,001
CXCL16	NM_023158	Chemokine (C-X-C motif) ligand 16	2,8	0,40	0,002
TNFα	NM_013693	Tumor necrosis factor	2,3	0,30	<0,001
IL12A	NM_008351	Interleukin 12a	1,8	0,20	<0,001
IGF1	NM_010512	Insulin-like growth factor 1, transcript variant 1	1,6	0,22	<0,001
CCL3	NM_011337	Chemokine (C-C motif) ligand 3	1,6	0,07	<0,001
CCL2	NM_011333	Chemokine (C-C motif) ligand 2	1,0	0,15	0,004
IL6	NM_031168	Interleukin 6	0,9	0,13	<0,001
IL12B	NM_008352	Interleukin 12b	0,8	0,18	0,006
IL1B	NM_008361	Interleukin 1 beta	0,6	0,14	0,006
CXCL7	NM_023785	Chemokine (C-X-C motif) ligand 7	-2,1	0,30	<0,001
Anti-oxidative					
PRDX1	NM_011034	Peroxiredoxin 1	1,4	0,29	0,006
PRDX6	NM_007453	Peroxiredoxin 6	1,2	0,11	<0,001
GSTM1	NM_010358	Glutathione S-transferase, mu 1	1,2	0,23	0,004
GSTT3	NM_133994	Glutathione S-transferase, theta 3	1,2	0,14	<0,001
GSTT2	NM_010361	Glutathione S-transferase, theta 2	1,2	0,12	<0,001
SOD3	NM_011435	Superoxide dismutase 3, extracellular	1,1	0,11	<0,001
GSTM5	NM_010360	Glutathione S-transferase, mu 5	0,8	0,12	<0,001
GCLC	NM_010295	Glutamate-cysteine ligase, catalytic subunit	0,4	0,05	<0,001
Migration					
ITGA5	NM_010577	Integrin alpha 5	1,6	0,18	<0,001
AQP9	NM_022026	Aquaporin 9	0,9	0,20	0,006
Membrane receptors					
CD200R1	NM_021325	CD200 receptor 1	1,7	0,244	0,001
GPR31C	NM_001013832	G protein-coupled receptor 31	1,4	0,24	0,002
GPR137B	NM_031999	G protein-coupled receptor 137B	1,3	0,13	<0,001
IL11RA	NM_010549	Interleukin 11 receptor, alpha chain 1	1,2	0,14	<0,001
GPR84	NM_030720	G protein-coupled receptor 84	1,2	0,16	0,002
GPR178	NM_001033178	G protein-coupled receptor 178	1,0	0,12	<0,001
GIT1	NM_001004144	G protein-coupled receptor kinase-interactor 1	0,5	0,08	0,002
GPR89	NM_026229	G protein-coupled receptor 89	0,5	0,05	<0,001
GPR137	NM_207220	G protein-coupled receptor 137	-0,3	0,07	0,008
GPR34	NM_011823	G protein-coupled receptor 34	-0,4	0,07	0,002
M-CSFR (CD115)	NM_007779	Colony stimulating factor 1 receptor	-0,6	0,09	0,002

Gene symbol	Accesion No.	Gene description	Coefficients	SEM	p-value
Il10RA	NM_008348	Interleukin 10 receptor, alpha	-0,9	0,13	<0,001
Anti-microbial					
LCN2	NM_008491	Lipocalin 2	1,9	0,20	<0,001
Proteases					
CTSL	NM_009984	Cathepsin L	1,8	0,11	<0,001
CTSZ	NM_022325	Cathepsin Z	1,5	0,30	0,005
CTSC	NM_009982	Cathepsin C	1,5	0,19	<0,001
CTSB	NM_007798	Cathepsin B	0,7	0,10	<0,001
CTSS	NM_021281	Cathepsin S	0,6	0,13	0,005
Apoptosis					
P2RX4	NM_011026	Purinergic receptor P2X, ligand-gated ion channel 4	2,5	0,09	<0,001
CFLAR	NM_009805	CASP8 and FADD-like apoptosis regulator	1,7	0,17	<0,001
BCL2L11	NM_207680	BCL2-like 11	1,4	0,17	<0,001
FAS	NM_007987	TNF receptor superfamily, member 6	1,1	0,22	0,006
CD40	NM_170701	CD40 antigen, transcript variant 3	0,7	0,08	<0,001
Pattern recognition receptors					
CLEC4N	NM_020001	C-type lectin domain family 4, member n	2,4	0,32	<0,001
SR-A	NM_031195	Macrophage scavenger receptor SR-AI/II	2,1	0,24	<0,001
CLEC4E	NM_019948	C-type lectin domain family 4, member e	1,8	0,26	<0,001
CD14	NM_009841	CD 14 antigen	1,7	0,28	0,002
CLEC4D	NM_010819	C-type lectin domain family 4, member d	1,6	0,21	<0,001
CLEC5A	NM_001038604	C-type lectin domain family 5, member a	1,5	0,16	<0,001
STAB1	NM_138672	Stabilin 1	1,5	0,29	0,004
TLR2	NM_011905	Toll-like receptor 2	1,3	0,15	<0,001
NOD1	NM_009251	nucleotide-binding oligomerization domain containing 1	-0,8	0,09	0,002
CLEC2I	NM_020257	C-type lectin domain family 2, member i	-1,0	0,10	0,001
CLEC1B	NM_019985	C-type lectin domain family 1, member b	-1,3	0,14	<0,001
Complement					
CFB	NM_008198	Complement factor B	2,1	0,20	<0,001
C3AR1	NM_009779	Complement component 3 receptor 1	2,1	2,06	<0,001
C5AR1	NM_007577	Complement component 5 receptor 1	1,3	0,13	<0,001
C1QB	NM_009777	Complement component 1, q subcomponent	1,0	1,40	0,002
C3	NM_009778	Complement component 3	0,6	0,67	0,007
Activated macrophages					
SPP1	NM_009263	Secreted phosphoprotein 1	2,3	0,37	0,002
FN1	NM_010233	Fibronectin	1,8	0,12	<0,001
ARG1	NM_007482	Arginase 1	1,7	0,22	<0,001
TGFβI	NM_009369	Transforming growth factor, beta induced	1,3	0,14	<0,001
Transporters and channels					
SLC9A4	NM_177084	Solute carrier family 9, member 4	2,5	0,55	0,007
ATP6V1C1	NM_025494	ATPase, H+ transporting, lysosomal V1 subunit C1	2,2	0,16	<0,001
SLC25A4	NM_007450	Solute carrier family 25, member 4	0,9	0,08	<0,001
SLC7A11	NM_011990	Solute carrier family 7, member 11	0,9	0,07	<0,001
DNA-binding					
ATF3	NM_007498	Activating transcription factor 3	1,7	0,24	<0,001
BATF	NM_016767	Basic leucine zipper transcription factor, ATF-like	1,1	0,15	<0,001
ZFP238	NM_013915	zinc finger protein 238	0,8	0,06	<0,001
ZFHX1B	NM_015753	zinc finger homeobox 1b	0,7	0,09	0,002

Gene symbol	Accesion No.	Gene description	Coefficients	SEM	p-value
ATF5	NM_030693	Activating transcription factor 5	0,6	0,09	0,003
IRF5	NM_012057	Interferon regulatory factor 5	-0,7	0,08	<0,001
GATA1	NM_008089	GATA binding protein 1	-1,1	0,09	<0,001
Phagocytosis					
FCGR2B	NM_010187	Fc receptor, IgG, low affinity IIb	1,7	0,16	<0,001
FCGR3	NM_010188	Fc receptor, IgG, low affinity III	1,2	0,16	<0,001
FCGR1	NM_010186	Fc receptor, IgG, high affinity I	0,8	0,19	0,009
FCER1G	NM_010185	Fc receptor, IgE, high affinity I, gamma polypeptide	0,4	0,07	0,002
CLEC7A	NM_020008	C-type lectin domain family 7, member a (Clec7a)	-0,9	0,19	0,006
Annexins					
ANXA4	NM_013471	Annexin 4	2,2	0,26	<0,001
ANXA5	NM_009673	Annexin 5	1,5	0,33	0,007
ANXA8	NM_013473	Annexin 8	1,4	0,17	0,002
ANXA2	NM_007585	Annexin 2	0,9	0,15	0,003
ANXA7	NM_009674	Annexin 7	0,7	0,10	0,001

The table illustrates selected genes with differential gene expression between alveolar recruited and PB MPs according to the biological interest. The coefficients correspond to the log2 fold changes. *P* values are adjusted for multiple testing by the method of Benjamini and Hochberg. Positive coefficients values in the columns indicate mean fold up-regulation of that particular gene in alveolar MPs in comparison to PB MPs, whereas negative values indicate mean fold down-regulation of the respective gene in alveolar MPs as compared to PB MPs.

Notably, this Pam_3CSK_4-elicited pro-inflammatory activation in freshly alveolar recruited MPs coincided with pronounced induction of anti-inflammatory mediators such as IL-1RN, IRAK-M and IL-10. Other up-regulated genes related to limitation and resolution of inflammation were formyl peptide receptor 1 (FPR-1), IL-18 binding protein (IL-18BP), adenosine A2b receptor (ADORA2B), annexin 1 (ANXA-1) and Bcl2-associated X protein (BAX). Moreover, alveolar MPs recruited after i.t. Pam_3CSK_4 application contained elevated mRNA levels of genes associated with an alternative activated macrophage phenotype such as arginase-1 (ARG-1), TGFβ induced (TGFβI) and fibronectin-1 (FN-1). In addition, alveolar trafficking of MPs in response to Pam_3CSK_4 elicited marked induction of genes encoding enzymes involved in PG and leukotriene (LT) metabolism such as COX-2, prostaglandin E synthase (PTGES) as well as LT C4 synthase (LTC4S) and LT B4 12-hydroxydehydrogenase (LTB4DH). Opposite down regulation was

observed for arachidonate 12-lipoxygenase (ALOX12) and hydroxyprostaglandin dehydrogenase 15 (HPDG) transcripts.

Finally, a large number of genes encoding surface receptors were differentially regulated in MPs recruited to the lung by alveolar exposure to Pam_3CSK_4. Thus we observed elevated transcript levels in alveolar MPs for PRR genes such as CD14, TLR2, CLEC4N and scavenger receptor (SR)-A and reduced levels for IL-10 receptor and M-CSFR (known as CD115). Moreover, alveolar trafficking of mononuclear phagocytes was found to be associated with marked up-regulation of some of G-protein coupled receptors (GPCRs) such as GPR31C, GPR137B, GPR84 and GPR178.

To determine which gene ontology (GO) categories were statistically over-represented among the genes found to be the differentially regulated in alveolar recruited vs. circulating MPs we used the web-based tool GENECODIS (91). When grouped according to the biological processes, GO analysis highlighted a significant over-representation of molecules associated with inflammatory/immune responses including genes involved in chemotaxis (e.g. C3AR1 and FPR-1), apoptosis (e.g. FAS and CD40) and phagocytosis (such as CD23 and CD64). In addition, genes related to protein and ATP biosynthesis and lipid storage were significantly over-represented (Table 4.4).

Table 4.4 Gene ontology (GO) biological processes classification of differentially regulated genes

GO analysis was performed online using GENECODIS web-based tool.

GO annotations	Biological process	*P*
GO:0006412,GO:0006418	protein biosynthesis, tRNA aminoacylation for protein translation	0.006
GO:0006915,GO:0006955	apoptosis, immune response	0.008
GO:0006911,GO:0007166, GO:0050766	phagocytosis, engulfment, cell surface receptor linked signal transduction, positive regulation of phagocytosis	0.009
GO:0006935,GO:0007165, GO:0007600	chemotaxis, signal transduction, sensory perception	0.012
GO:0006754,GO:0006810, GO:0006811,GO:0015986, GO:0015992	ATP biosynthesis, transport, ion transport, ATP synthesis coupled proton transport, proton transport	0.012
GO:0007166,GO:0042535	cell surface receptor linked signal transduction, positive regulation of tumor necrosis factor-alpha biosynthesis	0.02
GO:0019915	lipid storage	0.02
GO:0006754,GO:0006810, GO:0006811,GO:0015992	ATP biosynthesis, transport, ion transport, proton transport	0.02
GO:0007166,GO:0050776	cell surface receptor linked signal transduction, regulation of immune response	0.02
GO:0000074,GO:0006955	regulation of cell cycle, immune response	0.02

4.3.3 Real time PCR validation of microarray results

To verify the microarray findings, we performed quantitative real-time PCR for selected genes without SMART pre-amplification step. Genes chosen for validation were preferentially related to inflammatory processes. Heterozygous CX3CR1$^{+/GFP}$ mice received i.t. application of Pam_3CSK_4 (50 µg/mouse) for 24 h. The results obtained by real-time PCR largely matched with the microarray based gene expression profiles (Figure 4.14). However, real-time PCR data did not provide any evidence for differential expression of PTGES, FN1 and ANXA-1. The mRNA levels measured by real-time PCR tended to be higher than gene expression levels determined by arrays, what is expected and due to the pre-amplification and the lower dynamic range of the microarray signals. The mRNA levels of IL-1RN and IL-10 were below the detection limit in PB MPs but could be consistently detected in alveolar MPs in four independent experiments, indicating the up-regulation of these genes upon recruitment. *Vice versa*, ALOX12 mRNA levels found to be below the detection limit in alveolar MPs in four separate experiments were consistently detectable in circulating MPs indicating ALOX12 down-regulation upon recruitment.

In our model of TLR2 ligand Pam_3CSK_4-initiated lung inflammation we observed a significant decrease of recruited neutrophils together with an increase of MP infiltrates at 48 h compared to 24 h post challenge (Figure 4.11 and 4.12). Since this shift is considered to be a key histological hallmark of inflammatory infiltrate resolution we decided to determine whether MPs recruited until 48 h post challenge differentially regulate inflammatory mediators compared to corresponding cells recruited until 24 h. Therefore we monitored changes in the gene expression profiles of molecules related to inflammation of recruited MPs recovered from BALF after 48 h compared to 24 h after i.t. application of Pam_3CSK_4. Interestingly, we noticed that mRNA levels of pro-inflammatory molecules such as TNF-α, IL-6, CCL2 or IL-12A were significantly lower in recruited MPs isolated from BALF at 48 h compared to 24 h, whereas the data did

not provide evidence for differential expression of gene with key anti-inflammatory and resolution function such as IRAK-M, IL-10, IL1-RN, BAX and ANXA-1 (Figure 4.15).

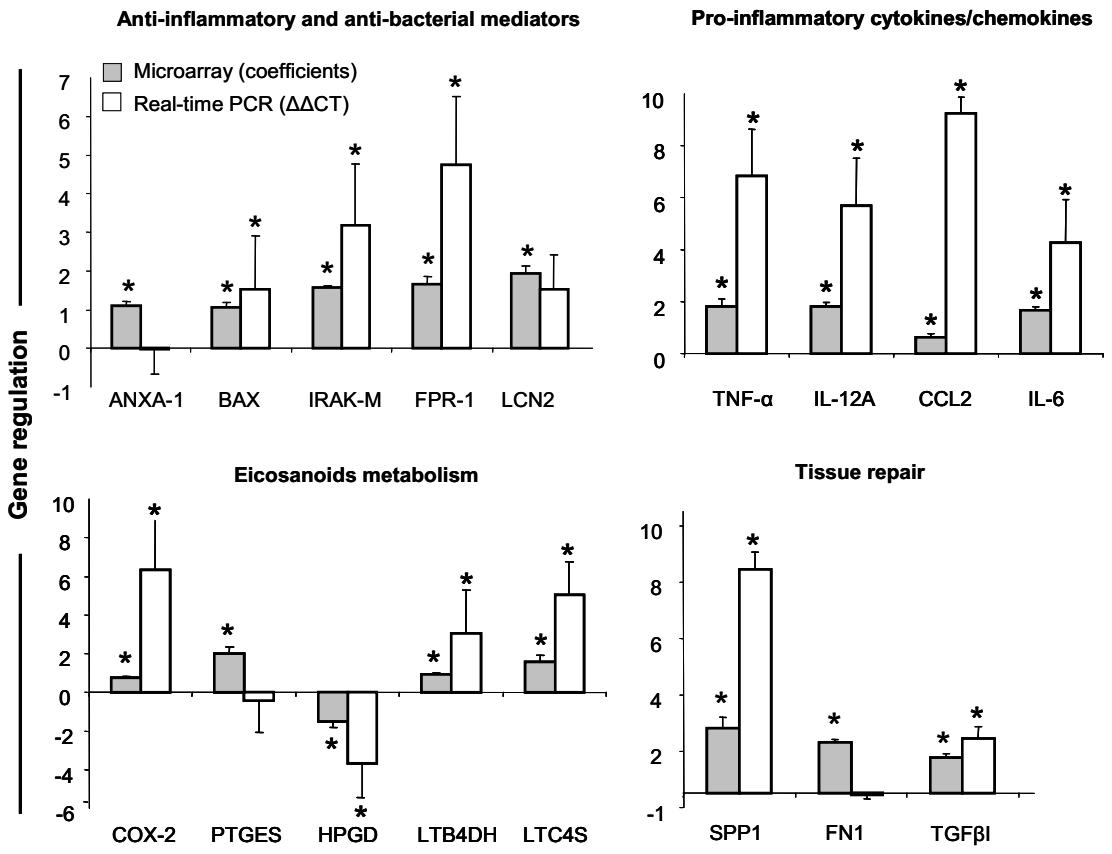

Figure 4.14 Real-time quantitative PCR validation of microarray data
CX3CR1 $^{+/GFP}$ mice were either left untreated or i.t. administrated with Pam_3CSK_4 (50 µg/mouse) for 24 h. After cell sorting, RNA was isolated and the relative mRNA level of each gene was determined using real-time PCR as described in *Materials and Methods*. The coefficients values from microarray analysis (grey bars) were compared with ΔΔCT values from real-time PCR (white bars). Data are given as mean ± SEM. Mean ΔCT of MPs isolated from blood and alveolar compartment were compared using t-test. * indicates *P* at least < 0.05.

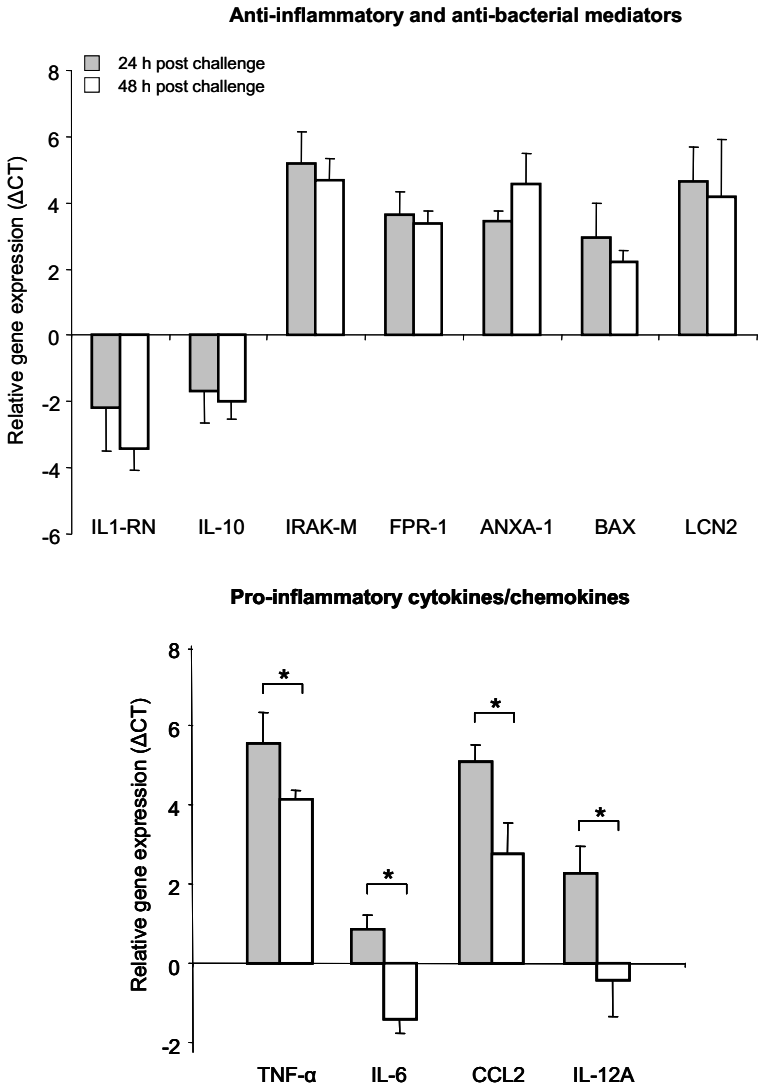

Figure 4.15 Time-dependent changes in the gene expression profile of alveolar recruited MPs

CX3CR1 $^{+/GFP}$ mice were either left untreated or i.t. administrated with Pam_3CSK_4 (50 µg/mouse) for 24 h or 48 h. After cell sorting, RNA was isolated and the relative mRNA level of each gene was determined using real-time PCR as described in *Materials and Methods*. Relative gene expression was calculated using the ΔΔCT method. The results are given as mean ± SEM from three independent experiments. Mean ΔCT of samples collected at different time points post-treatment were compared using t-test. * indicates *P* at least < 0.05.

5 DISCUSSION

The aim of the current study was to analyse the molecular programs activated by bacterial ligands in alveolar resident as well as alveolar recruited MPs. We have found that key antiviral defence molecule named PKR is critically involved in an inflammatory response of AMs to gram-positive as well as gram-negative bacteria cell wall components *in vitro*. We have also analyzed, by microarray technology, the whole genome response of MPs recruited from circulation to the alveolar space following pulmonary challenge with the TLR2 ligand Pam_3CSK_4. We found that lung recruited MPs displayed simultaneous induction of both pro- and anti-inflammatory genes. In addition, we observed a dynamic change of the genetic program in MPs obtained from BALF at different time points post alveolar TLR2 ligand deposition.

5.1 Requirement for PKR in TLR2/4-medaited signalling in murine AMs

To identify novel molecular pathways activated in resident AMs by the *E. coli* LPS (TLR4 agonist) and synthetic lipoprotein Pam_3CSK_4 (TLR2 agonist) we performed a gene expression screen of selected TLR associated molecules. We found that both TLR2 and TLR4 ligand induced a similar expression pattern of TLR molecules *in vitro* and in a murine model of lung inflammation. The screen confirmed that TLR ligands induced activation of previously reported genes such as TNF-α, IL-6, SOCS proteins and IRAK-M. Interestingly, our gene expression study identified an antiviral defence gene named PKR that was significantly up-regulated by bacterial ligands *in vitro* and in murine model of lung inflammation. It has been previously reported that LPS induced PKR mRNA in peritoneal and ANA-1 macrophages, but the regulation of PKR gene expression in AMs was unknown (92). Furthermore, our experiments demonstrated for the first time that also TLR2 ligand (synthetic lipoprotein Pam_3CSK_4) is a potent inducer of PKR mRNA and protein *in vitro*.

In contrast to the *in vitro* observations, we noticed that agonists for distinct TLRs differentially regulated PKR transcription *in vivo*. The expression of PKR in AMs was significantly increased after alveolar deposition of LPS at 6 h post challenge and decreased at later time points (24 h post challenge). In contrast, alveolar administration of Pam_3CSK_4 did not affect PKR mRNA levels at any of the analysed time points. Altogether, our findings suggest that PKR could be involved in TLR downstream signalling in AM during pulmonary bacterial infections.

PKR is a serine/threonine protein kinase ubiquitously expressed in the cytoplasm that can be induced by IFN and dsRNA. This enzyme was initially characterized as translational inhibitor in IFN-induced antiviral response (93). It contains two functionally distinct domains: an N-terminal ds-RNA binding domain (DBD) and a C-terminal kinase domain (KD). Binding of dsRNA induces PKR dimerization, trans-autophosphorylation and subsequent phosphorylation of substrate targets. The most intensively characterised cellular substrate of PKR kinase activity is the alpha subunit of the eukaryotic translational initiation factor 2 (eIF-2), a heterotrimeric protein (composed of: α, β and γ subunits), that binds guanosine triphosphate (GTP) and the initiator $tRNA_i^{met}$ to form a ternary complex. This ternary complex eIF2–GTP–Met-$tRNA_i^{met}$ binds to the 40S ribosomal subunit to form a 43S preinitiation structure, which binds mRNA and 60S ribosomal subunit with concomitant hydrolysis of GTP. To promote another round of translation initiation, guanosine diphosphate (GDP) associated with free eIF-2 must be exchanged for GTP by the activity of eIF-2B complex. Phosphorylation of eIF-2α on Ser51 leads to the inhibition of protein synthesis by preventing the recycling of eIF-2. The phosphorylated form of eIF-2 cannot form a ternary complex with the initiator $tRNA^{met}$ and the 40S ribosomal subunit. As a consequence, phosphorylation of eIF-2 by activated PKR leads to the shutdown of protein synthesis, resulting in a block of virus replication and selective apoptosis of the infected cells. Translation is highly sensitive to inhibition by activated PKR and phosphorylation of only 10-20% of eIF-2α suffices to block translation. In addition to its role in antiviral defence, PKR is involved in many aspects of cellular function

including, protein synthesis, cell growth, differentiation, tumor suppression and apoptosis.

Recently, a growing number of evidence implicated PKR in several signal transduction pathways activated by a wide range of pro-inflammatory factors including LPS, dsRNA, TNF-α and IL-1 (93). Cytokines and bacterial products activate indirectly PKR *via* upstream activators that could be kinases or other protein activators (Figure 5.1) (93). PKR has been shown to mediate activation of important transcription factors such as NF-kB (by poly I:C) (94), ATF-2 (by poly I:C and TNF-α), STAT1 (by IFN-γ and LPS) (95) or IRF-1 (by dsRNA) (96). It has been also suggested that PKR is involved in TLR signalling in various cell types such as glial cells, fibroblasts or mast cells. However, there are no reports addressing PKR function in lung innate immunity in response to bacterial PAMPs.

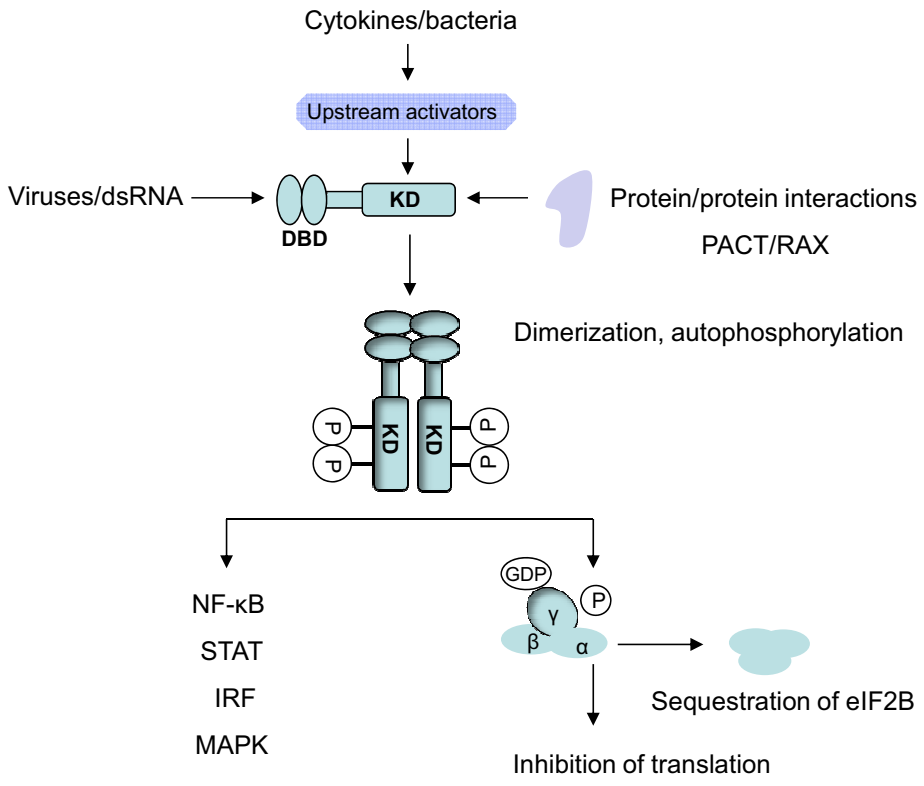

Figure 5.1 Functions of PKR in signal transduction and translation
dsRNA produced as a result of virus infection, cytokines and bacteria activate PKR, inducing dimerization and autophosporylation and allowing the PKR kinase to phosphorylate substrate targets. For instance, by phosphorylation of α subunit of EIF2, activated PKR inhibits translation. In addition, proteins such as PACT/RAX have been reported to directly associate with PKR, similarly stimulating autophosphorylation. PKR has additionally been reported to be involved in regulating a number of signalling pathways involving TNF-α, NF-kB, although the mechanisms of action remain to be determined. Adapted and modified from Barber 2005 (97).

5.1.1 TLR2 and TLR4 agonist induced phosphorylation of PKR

Autophosphorylation is the first step in converting PKR to its active form (98). Our experiments have demonstrated for the first time that in AMs not only LPS induced TLR4 signalling but also Pam_3CSK_4 induced TLR2 signalling stimulated PKR phosphorylation within minutes. In addition, using TLR deficient and mutated mice we could show that activation of PKR by LPS and Pam_3CSK_4 was strictly

dependent on the cellular expression of the respective PRR molecules. These data indicate that PKR can act as an early step in the TLR2/4 induced inflammatory response. However, the mechanism of PKR activation by TLR ligands remains an important issue to be explored. The properties of dsRNA as a negatively charged polysaccharide resemble LPS, but it is rather unlikely that LPS and Pam_3CSK_4 can directly bind to intracellular located PKR. Recently, Horng and colleges demonstrated that PKR is a downstream target of the TIRAP/Mal signalling pathway that is essential for TLR2 and TLR4 (99). Besides TIRAP, PKR has been shown to physically interact with other proteins such as MKK6 or PACT (100, 101). Thus, activation of PKR by TLR ligands is likely to require complex but rapid signalling pathways involving protein-protein interactions.

To delineate the role of PKR in TLR2/4-mediated activation of AMs, we applied nucleoside analogue 2-AP. Previously, 2-AP has been shown to posses a relative specificity for the inhibition of PKR phosphorylation by competing with ATP (92). In earlier experiments, 2-AP selectively blocked the expression of various genes in response to dsRNA, IFNs and virus infection (102, 103). In our *in vitro* assays, we observed a strong inhibition of PKR phosphorylation by 2-AP, most probably by competitive inhibition of ATP at the PKR-ATP binding site. However, 2-AP also blocked PKR induction by TLR ligands, thereby suggesting that inhibitor may act on the kinase activity and gene expression of PKR. Our results are in contradiction to previous study demonstrating that 2-AP affects only phosphorylation, but not gene expression of PKR (54, 92). The reasons for such differences in the 2-AP effect on PKR gene expression may be a result of (a) cell type (ANA-1 macrophages vs. primary AMs); (b) different concentrations of LPS and 2-AP used in the studies; and finally (c) the method and time point for evaluation of PKR gene-expression. It is also possible that 2-AP blocked the expression of genes directly involved in the regulation of PKR transcription but on the other hand PKR may regulate the transcription of it is own mRNA. Nevertheless, in our experimental system, we used 2-AP to specifically blocked PKR phosphorylation.

5.1.2 PKR-dependent secretion of TNF-α and IL-6 cytokines

Pro-inflammatory cytokines are known to be important in the host response to pulmonary bacterial infections. They contribute to the recognition of pathogens, recruitment of immune cells from bloodstream to inflamed tissues and the removal of the invading pathogens (54, 104). TNF-α is a typical pro-inflammatory cytokine primarily produced by MPs. Production of TNF-α is increased in patients with pneumonia and in animal models of pulmonary infection caused by *S. pneumoniae*, *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* (54). One consequence of elevated levels of TNF-α is increased IL-6 production. IL-6 induces production of acute phase response proteins, reduces albumin and transferrin levels and directs differentiation of B cells to immunoglobulin-producing plasma cells (75).

AMs play a primary role in inflammation, principally through elaboration of variety of cytokines including TNF-α and IL-6. To delineate the role of PKR in AM cytokine secretion, 2-AP and PKR KO mice were used. Our data clearly demonstrated that PKR is closely involved in inflammatory cytokine secretion. In addition, using PKR-KO AMs we provided data that confirm the specificity of 2-AP as a valuable PKR inhibitor in our system. The profound decrease of PAMP-induced TNF-α and IL-6 production after PKR inhibition suggests that PKR activity may also determine the extent of alveolar inflammation *in vivo*. However, inhibition of PKR did not completely abolish the cytokine production. It will be interesting to evaluate whether remaining cytokines are still potent inducers of inflammatory response. Our results support the pro-inflammatory properties and immune defence activities of PKR, proposed in various cell types. Goh and co-workers demonstrated that PKR-deficient mouse embryonic fibroblasts (MEFs) were unable to cytokine production in response to bacterial LPS. Moreover, IL-6 and IL-12 p40 serum levels after intra peritoneal (i.p.) LPS challenge were diminished in PKR-KO mice, suggesting requirement of PKR in TLR4-mediated inflammatory responses (88). Therefore, the requirement for functional PKR in animal model of lung inflammation/infection needs to be evaluated. PKR was also found to play a crucial

role in the regulation of inflammatory cytokine expression in human blood monocytes stimulated with bacillus Calmette-Guerin (BCG) (89).

5.1.3 Requirement of PKR for LPS induced activation of NF-κB

Binding of bacterial PAMPs to their respective TLRs leads to increased production of inflammatory cytokines and chemokines. Expression of pro-inflammatory genes is regulated by transcriptional mechanisms. NF-κB represents one of the major transcription factors linked to the production of variety of inflammatory mediators including TNF-α, IL-6, IL-12 and CCL2 (45). The main regulatory mechanism of NF-κB activation is mediated by interaction with the IκB proteins that retain NF-κB proteins in the cytoplasm in an inactive state. On exposure of the cell to activation signals such as LPS, IκB proteins are phosphorylated by IκB kinases (IKKs). The phospho-IκB proteins become targets for ubiquitination and degradation, whereas NF-κB proteins are translocated to the nucleus to activate the transcription of cognate genes. There have been a number of proteins identified as possible IKKs (42, 44, 45).

The NF-κB link to PKR was suggested by a series of experiments showing that dsRNA could activate NF-κB in various cell lines, however the mechanism still remained controversial (105). PKR has been proposed to be a component of the IKK complex and directly phosphorylates IκB proteins (106). For example, induction of E-selectin, an important for leukocyte rolling adhesion molecule, by TNF-α and poly I:C is impaired in PKR-KO endothelial cells due to the defective activation of NF-κB (107). Similarly Gon and colleges observed that pre-treatment of cells with 2-AP attenuated LPS-induced NF-κB activation in RBL-2H3 mast cells (90). Other groups, however, reported that activation of NF-κB by inflammatory stimuli did not require PKR kinase activity (108). Der and co-workers showed that PKR gene deletion had no effect on TNF-α or LPS-induced NF-κB activation (96). Our work supports requirement for PKR phosphorylation in NF-κB activation in response to LPS. Using both pharmacological inhibition and gene KO approaches we have demonstrated that LPS-induced activation and translocation of p65

depends on PKR phosphorylation. However, we could observe that NF-κB remains still active in PKR-KO AMs, suggesting that PKR-independent activation of NF-κB exists. Our results differ with those of Hsu and colleges, who found that PKR-KO BM derived macrophages (BMDMs) exhibited normal IKK activation and normal induction of numerous NF-κB target genes in response to LPS (109). These different observations may be due to distinct mechanisms of NF-κB activation in different macrophages subsets in mice.

In contrast to LPS, TLR2-mediated activation of NF-κB was found to be largely PKR phosphorylation-independent. This finding suggests that the molecular steps regulating NF-κB activity differ in TLR2 and TLR4 mediated signalling pathways of AMs.

5.1.4 PKR dependent regulation of the JNK signalling pathway

The MAP kinases are a family of evolutionary conserved enzymes that connect cell surface receptors to regulatory targets and coordinate activation of gene transcription, protein synthesis, cell cycle machinery, cell death, and differentiation (23, 24). They represent the second major signalling pathway activated by TLRs in AMs involved in cytokine production (29). The connection between MAPK and PKR has been recognized just recently, through only a few studies. Goh and co-workers demonstrated for the first time that PKR-deficient MEFs were unable to activate p38 and JNK MAP kinases in response to bacterial LPS or poly I:C (88). Moreover, authors observed that activation of the specific MAPK kinases, MKK3 and MKK6 activating p38 MAPK and MKK4 activating JNK MAPK, were correspondingly reduced in PKR-deficient MEFs after stimulation with LPS or poly I:C. Interestingly, JNK activation was no longer dependent on PKR activity in immortalized fibroblast cell lines generated from the MEFs (88). Similarly, Cheung and colleagues revealed that the early signal transduction events of mycobacterial activation of human monocytes requires PKR dependent ERK phosphorylation (89). In our study, we observed phosphorylation of three major MAP kinases, p38, JNK and ERK1/2 in response to Pam_3CSK_4 and LPS in AMs. Inhibition of PKR

phosphorylation by 2-AP and gene deletion in PKR-KO AMs however abolished PAMP-induced JNK but not ERK1/2 MAPK and p38 MAPK activation. Our results are in line with those of Goh and colleagues, who found that PKR was required for efficient activation of JNK by LPS in primary low-passage MEFs (88). However, our results differ from those of Hsu and colleagues, who found that BMDMs derived from PKR-KO exhibited normal p38, JNK and ERK MAP kinases activation in response to LPS (109).

JNK have been reported to be an important mediator of the production of inflammatory cytokines induced by LPS. A critical role for JNK appears to be the regulation of the AP-1 transcription factor through phosphorylation of the N-terminal part of c-Jun, a central component of AP-1 (29, 31). Correspondingly, we observed reduced phosphorylation of c-Jun/AP-1 when PKR was inhibited. In addition, phosphorylation of MKK4, a direct activator of JNK, was found to be dependent on PKR activity. Collectively, these data indicate that PKR specifically regulates the JNK signalling pathway (PKR-MKK4-JNK-c-Jun) in AMs activated by TLR2 and TLR4 agonists. Our results suggest that there are multiple pathways for JNK, p38 and ERK1/2 MAPK activation in AMs. Moreover our observations support the idea that PKR can be one of the MAPKKKs (93).

In summary, our study shows that the secretion of TNF-α and IL-6 by primary AMs in response to cell wall components of gram-positive as well as gram-negative bacteria *in vitro* is critically dependent on PKR phosphorylation. Activated PKR was found to act upstream of the Pam_3CSK_4 and LPS-activated JNK pathway. PKR also regulates TLR4- but not TLR2-induced activation of NF-κB in AMs. These data identify PKR phosphorylation as important signalling step in the TLR2/4-mediated inflammatory response of AMs to bacterial PAMPs (Figure 5.2).

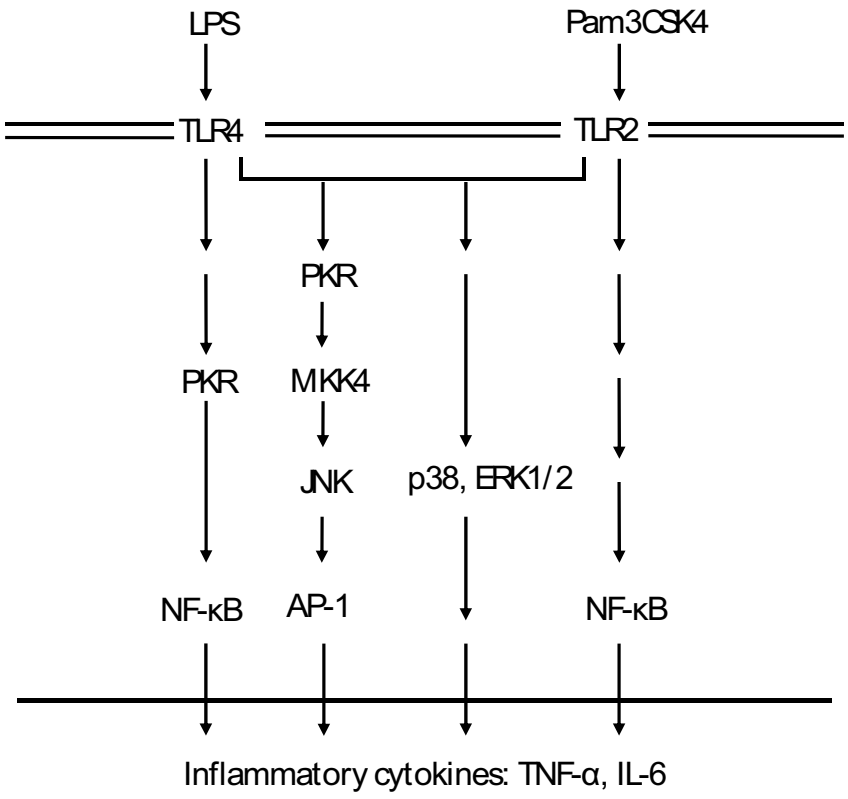

Figure 5.2 Model of PKR transducer function in TLR2/4-mediated signalling

5.2 Transcriptional profiling of MPs recruited to mouse lungs in response to alveolar challenge with the TLR2 agonist Pam_3CSK_4

5.2.1 Alveolar trafficking of PB MPs is associated with global changes in their gene expression profile

MPs represent a central part of both the innate and adaptive immune response to pathogens. Under steady-state condition, half of the circulating MPs leave the bloodstream each day to enter peripheral tissues (59). Inflammatory stimuli such as invading pathogens or TLR ligands, however, elicit increased MP traffic to the sites of inflammation where MPs can modulate local host responses by e.g. secreting inflammatory mediators such as cytokines, chemokines and lipids (55). Compared to the TLR4 ligand LPS restricted to gram-negative bacteria, a limited number of studies have addressed the induction of lung inflammation and the concomitant MP recruitment in response to TLR2 ligands. Consequently, *in vivo* experimental data on migration and function of MPs recruited to the lungs in response to TLR2 agonists are rare.

This study is the first report showing that selective TLR2 ligand stimulation within the alveolar compartment likewise promoted lung inflammation in mice and induced the migration of circulatory immune cells including MPs into the inflamed alveolar space. This model seems of considerable interest because most cases of community acquired pneumonia (CAP) are caused by gram-positive bacteria lacking LPS but expressing TLR2 ligands. Our study also represents the first global expression profiling of MPs recruited from the circulation to the alveolar compartment in response to the TLR2 agonist Pam_3CSK_4. By combination of FACS cell sorting, mRNA amplification methods and whole genome microarrays we were able to profile MP transcriptome from small amounts of BALF and blood of Pam_3CSK_4-treated mice. We found that expression of approximately 2500

genes was significantly changed in alveolar recruited MPs compared to circulating MPs. In particular, trafficking of MPs was associated with drastic up-regulation of a broad array of pro-inflammatory molecules including TNF-α, IL-1α/β, IL-6, CXCL16, KC or CCL2 indicating that inflammatory elicited MPs bear the potential to promote lung inflammation by increasing alveolar levels of cytokines and chemotactic mediators for neutrophils, monocytes and lymphocytes. This observation supports previous reports in which MPs were identified as active contributors to the development of acute lung inflammation in patients with ALI and were shown to promote neutrophil recruitment in response to alveolar challenge with LPS in mice (70, 110). Interestingly our transcriptome analysis revealed that Pam_3CSK_4 challenge induced not only pro-inflammatory but also pronounced anti-inflammatory MP responses. Thus we noticed strong up-regulation of IL-1RN, IRAK-M, IL-10, ANXA-1, BAX, IL-18BP and FPR-1 genes in alveolar recruited MPs.

IL-1RN acts as specific inhibitor of IL-1α/β signalling by competitive binding to the IL-1 receptor. Previous reports already demonstrated that IL-1RN is mainly produced by monocytes/macrophages in response to TLR4 ligands such as LPS (75). Thus, the strong increase of alveolar MP IL-1RN transcripts observed after Pam_3CSK_4 administration suggests a potential role for IL-1RN in blocking the action of IL-1α/β and in limiting of TLR2 driven lung inflammatory responses. IRAK-M represents another host strategy evolved to dampen inflammatory TLR signalling. IRAK-M negatively regulates MyD88 dependent TLR2/4/9 signalling by inhibiting the dissociation of IRAK1 and IRAK4 from the TLR complex (22). *In vivo,* IRAK-M-deficient mice exhibited increased inflammatory response during bacterial infections (111). Moreover, it has been demonstrated that lung macrophages from IRAK-M–deficient septic mice produced higher levels of pro-inflammatory cytokines such as IL-12 and TNF-α when stimulated with LPS *ex vivo* (112). After Pam_3CSK_4 challenge augmented IRAK-M transcript levels were found in alveolar recruited MPs both at 24 and 48 h. These data suggest that activation of IRAK-M may represent a potential mechanism to limit TLR2-medaited pulmonary

inflammation. Our study revealed that Pam_3CSK_4 induced high up-regulation of IL-10 mRNA levels in alveolar MPs. IL-10 is an important immunomodulatory cytokine with known anti-inflammatory and immunosuppressive properties (75). It has been shown that LPS-induced secretion of IL-10 limits production of pro-inflammatory cytokines including TNF-α, IL-1 and IL-12 thereby promoting the termination of inflammation (113). Consequently increase of anti-inflammatory and pro-resolving transcripts observed after Pam_3CSK_4 administration suggests a potential role for alveolar recruited MPs in limiting TLR2 driven lung inflammatory responses.

Lipids such as arachidonic acid derivatives are potent inflammatory mediators (114). Our gene array data revealed that inflammatory trafficking of MPs was associated with profound regulation of genes involved in PG and LT metabolism such as COX-2 and PTGES. COX-2 catalyzes PG synthesis including PGE2 commonly considered as central pro-inflammatory lipid mediator involved in the pathogenesis of several inflammatory diseases such as periodontitis and rheumatoid arthritis (115, 116). In lungs, however, it has been reported to display anti-inflammatory and pro-resolution activity as well (117). For example PGE2 has been shown to down-regulate the production of pro-inflammatory cytokines such as IL-12 or CCL2 thus inhibiting inflammatory cell recruitment (118). In addition, the expression of the genes encoding the enzymes LTB4DH involved in deactivation of the PMN chemoattractant LTB4 and HPGD promoting the deactivation of PGs and lipoxins were found to be up-regulated in MPs recruited to the alveolar air space. Thus by alveolar recruitment, MPs may acquire the potential to actively contribute to lowering the local levels of lung lipid inflammatory mediators.

In vivo studies indicated the potential of circulating MPs to differentiate into lung macrophages under both inflammatory and non-inflammatory conditions (56). Recently macrophages have been assigned to two groups: classically activated (pro-inflammatory activities) and alternatively activated macrophages (anti-inflammatory activities) (119). In our study we found that i.t. administration of

Pam_3CSK_4 elevated mRNA levels of genes such as SPP-1, ARG-1, TGFβI, FN-1 and IL-10 that are preferentially associated with an alternatively activated macrophages phenotype. Alternatively activated macrophages are known to play a major role in tissue repair during the healing phase by secretion of extracellular matrix (ECM) components and release of anti-inflammatory mediators (119). Based on these results freshly recruited MPs may represent important factors for the resolution of pulmonary inflammation.

The present work also revealed differential expression of GPCRs with unknown function such as GPR31C, GPR137B.and GPR178 in lung recruited compared to circulating MPs. Since different stages of mononuclear phagocyte differentiation are characterized by different repertoires of GPCR, these differentially regulated receptors may reflect MP changes during migration to the lungs. However, the potential role of these molecules during lung inflammatory processes remains to be further elucidated in detailed functional studies.

5.2.2 Attenuation of pro-inflammatory gene expression levels in alveolar MPs during late inflammatory response phases

As unbalanced production of pro-inflammatory mediators may lead to inflammatory damage of the highly sensitive respiratory structures, the activation of host defence responses in the lung must be tightly regulated and later terminated (120). Numerous control strategies have been evolved including inhibition of pro-inflammatory mediators and secretion of anti-inflammatory and pro-resolving mediators. In our model of TLR2 ligand Pam_3CSK_4-initiated lung inflammation, intense inflammatory injury peaks at 24 h post challenge and then slowly recedes. At the cellular level, these events are characterized by increased trafficking of MPs and reduced neutrophil accumulation within the lung. This observation is consistent with the current concept of cellular inflammation resolution. Elucidation of molecular programs activated in MPs during this late phase may provide

important insights into how MPs contribute to the termination of TLR2 mediated lung inflammatory diseases.

In this respect, we monitored temporal changes in the gene expression of pro- and anti-inflammatory mediators of alveolar recruited MPs at 24 h and 48 h post Pam_3CSK_4 treatment. We found that mRNA levels of pro-inflammatory molecules such as TNF-α, IL-6, CCL2 and IL-12A were significantly lower in BALF MPs at 48 h compared to BALF MPs at 24 h, whereas gene expression of molecules with key anti-inflammatory and pro-resolution function such as IRAK-M, IL-10, IL1-RN, BAX and ANXA-1 persisted at the same level at 48 h and 24 h. Thus MPs recruited during later phase of inflammation, activate regulatory mechanisms to switch off the pro-inflammatory phenotype overrepresented during maximal inflammation. Moreover, by persistent high induction of IL-10 and IL1-RN genes MPs may actively contribute to the termination of inflammation.

Recruitment of MPs from the circulation to the alveolar space is a multistep process involving multiple cellular interactions. In addition, after trafficking to the airspace MPs become exposed the specialized alveolar microenvironment stimulated by PAMP induced inflammation with the potential to further modulate the phenotype of the recruited cell population. Our global gene profiling approach does not allow to assign the observed changes in the genetic program of lung recruited MPs to any of these migratory steps. We also cannot discriminate whether the changes in gene activation found in alveolar recruited MPs at 48 vs. 24 h are due to phenotype changes occurring in MPs within the alveolar compartment or reflect the recruitment of functionally different MP during later stages that have acquired altered functional programs either within the vascular compartment or during the trafficking process. The fact, however, that the gene expression profiles of circulating MPs obtained at 48 vs. 24 h also display a shifted profile suggests that indeed the extravasation of functionally modified MPs may contribute to the altered gene expression profile of BALF MPs at later time points after inflammatory challenge.

In conclusion, our data demonstrates the feasibility to evaluate cell specific transcriptome profiles in highly purified flow sorted MPs from both PB and BALF in a mouse model of TLR2 ligand induced lung inflammation. We identified more than 3500 genes differentially expressed, mostly up-regulated in alveolar recruited compared to PB MPs demonstrating that inflammatory MP trafficking into lung tissue was associated with large scale genetic program activation. Notably, the MP gene clusters undergoing activation in response to i.t. Pam_3CSK_4 application encoded both pro- and anti-inflammatory activities, however with a profound profile change over time. MPs recruited within 24 h post challenge showed simultaneous induction of both pro- and anti-inflammatory genes, whereas in MPs isolated from BALF after 48 h post challenge mainly genes encoding anti-inflammatory mediators were found to persist in an up-regulated. Collectively, our results provide important new insights into how MPs contribute to the promotion and termination/resolution of TLR2 mediated inflammation.

5.3 Future perspectives

Cell specific profiles of the molecular programs induced by gram-positive as well as gram-negative bacteria ligands in pulmonary MPs provide important insights into the host defence molecular network in infectious lung diseases such as pneumonia and ARDS. In the present study we aimed to analyse the molecular programs activated by TLR2/4 ligands in alveolar resident as well as alveolar recruited MPs to further delineate their role in pulmonary defence. This knowledge may help to identify new molecular targets for selective therapeutic intervention in pneumonia.

We discovered that key antiviral defence molecule PKR was activated and critically involved in inflammatory response induced by bacterial TLR agonists in AMs *in vitro*. Our data clearly demonstrate that treatment with specific pharmacological inhibitor 2-AP and PKR gene deletion reduced acute inflammatory cytokine response. Therefore modulation of PKR activity may have a therapeutic potential in treatment of patients during pulmonary bacterial infections.

Since *S. pneumoniae* and *K. pneumoniae* are frequently isolated causative pathogens of pneumonia it is reasonable to determine the role of PKR in host defence in gram-positive and gram-negative pneumonia in mice. Nevertheless, studies with human samples are required to clarify the role of PKR during bacterial lung infections in patients. Since several cancer drugs have been shown to modulate PKR activity; their potential in the treatment bacterial lung infections should be evaluated.

In addition to activation of lung resident cells, alveolar recognition of inhaled pathogens promotes increased recruitment of circulating MPs to inflamed tissue. Therefore we examined the global gene expression profiles of MPs recruited from peripheral blood to the alveolar space following alveolar deposition of the TLR2 ligand Pam_3CSK_4. We found that alveolar trafficking of MPs was associated with profound changes of their gene expression profiles post recruitment. Importantly, whereas the induced mRNA levels of selected pro-inflammatory mediators were found to decrease during later inflammation phases, the increased transcript levels of central anti-inflammatory mediators persisted. To further study the role of MPs in TLR2-driven inflammatory response it would be interesting to evaluate whether the transcriptome level changes are also reflected at the proteome and metabolome level. These studies should be also complemented by whole genome expression profiling of MPs recruited to the lungs in response to infection with TLR2 ligands bearing gram positive bacteria such as *S. pneumoniae*. It is known that MPs are not a homogenous population. In our study using $CX3CR1^{+/GFP}$ mice we were not able to specifically monitor MP subset specific changes in gene expression. In addition, we could not discriminate whether the changes in gene expression found in alveolar recruited MPs at 48 h vs. 24 h are due to phenotype changes occurring during migratory processes. Therefore it will be of interest to isolate distinct alveolar and vascular populations of recruited MPs and perform gene expression analysis. In addition using specific KO mouse models or gene silencing strategies would be helpful to further define the role of alveolar recruited MPs and their relative contribution to the lung inflammatory response.

6 APPENDIX

Table 6.1 List of primers used for quantitative real-time PCR

Target gene	Genebank accesion	Sequence (5'→3') forward	revere
ANXA1	NM_010730	CGTGAAGTGTGCCACCAGC	CGAACGGGAGACCATAATCCT
BAX	NM_007527	TCAAGGCCCTGTGCACTAAAG	ATCCAGACAAGCAGCCGCT
CCL2	NM_011333	AGCATCCACGTGTTGGCTC	CCAGCCTACTCATTGGGATCAT
CD36	NM_007643	ATGCCAGTCGGAGACATGC	TGGTGCCTGTTTTAACCCAGT
COX-2	NM_011198	AGCCTTCTCCAACCTCTCCT	CAGGGATGAACTCTCTCCGT
FPR-1	NM_013521	GGAGATCAGCTGTAGATCTGT	GAGGTTCACTGCAGACTTGTT
HPGD	NM_008278	CATCGGATTCACACGCTCAG	CAATGGATTCAAGGATGG
IL-10	NM_010548	CAGAGAAGCATGGCCCAGA	TGCTCCACTGCCTTGCTCTTA
IL-12A	NM_008351	AGACCACAGATGACATGGTGA	GTCCCGTGTGATGTCTTCAT
IL-1RN	NM_031167	TCCTGTTTAGCTCACCCATGG	CCAGCAATGAGCTGGTTGTTT
IL-6	NM_031168	ACAAAGCCAGAGTCCTTCAGAGAG	GCTTATCTGTTAGGAGAGCATTGGA
IRAK-M	NM_028679	TGGACATTCGAAACCAAGCATA	GGGTGACGGAACAGGAGTAGAA
LCN2	NM_008491	CAGCTTTACGATGTACAGCACCA	TCCAGTAGCGACAGCCCTG
LTB4DH	NM_025968	CAACCGTACTGGACCATG	GCCACCGATTCACGATGAA
LTC4S	NM_008521	GGCAACATGAAGGACGAA	GAAAGCCCTTCGTGCAGAGA
PBGD	NM_013551	GGTACAAGGCTTTCAGCATCGC	ATGTCCGGTAACGGCGGC
PKR	NM_011163	AATCCCGAACAAGGAGAACAGG	AGCTAGAATAAGGCCCAAAGCA
PTGES	NM_022415	ACCACCTGCAGGAGATGTA	GGTCAGCAAGGTTAGGCTTC
SIGIRR	NM_023059	GTTAAGTGTCGGCTGAACATGC	GTCGCTATAGGACACGTAGGCA
SOCS-1	NM_009896	GGA GCA TGC GCG ACA GC	CCT ACT CGA GGG GCC AGC T
SOCS-3	NM_007707	GCC GGA GAT TTC GCT TCG	GAG CCA GCG TGG ATC TGC
SPP1	NM_009263	CTCGGAGGAAACCAGCCA	GGATCTGGGTGCAGGCTGTA
ST2	NM_010743	GCCACCAGATCATTCACAGTTG	TGAACAGGCAATACTTGCTGGT
TGFβI	NM_009369	ACCAATGAAGCGTTCCAAGC	TGCCTCCGCTAACCAGGAT
TLR2	NM_011905	CACCGGTCAGAAAACAACTTACC	CAGAACAGCGTTTGCTGAAGAG
TLR4	NM_021297	GAGCTTCAACCCCTTGAAGATCT	GCCATGCCATGCCTTGTCT
TNF-α	NM_013693	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC

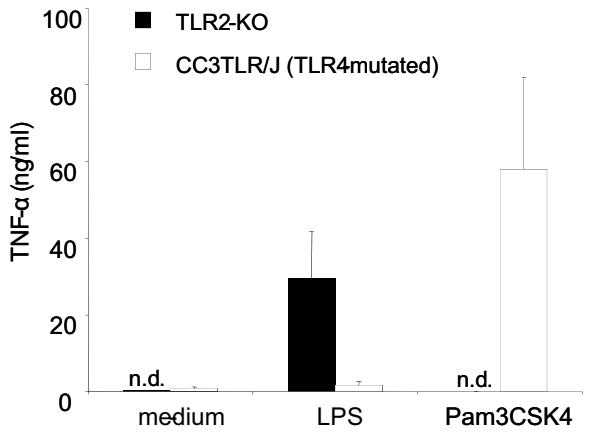

Figure 6.1 ELISA for TLR2/4 ligands

AMs isolated from TLR2-KO and TLR4 mutated (CC3TLR/J) mice were incubated with medium alone or treated with LPS (200 ng/ml) or Pam_3CSK_4 (2 µg/ml) for 6 h. Collected supernatants were assayed for TNF-α concentrations by ELISA as described in *Materials and Methods*. Data are represented as mean ± SD of at least three different experiments.

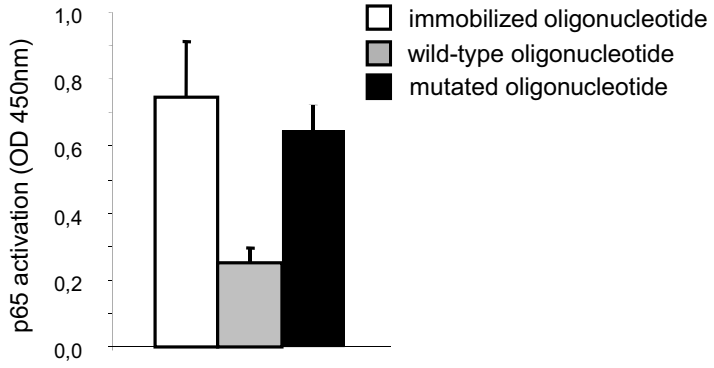

Figure 6.2 TransAM assay control

Activation of p65 subunit of NF-κB was monitored using nuclear extracts of Jurkat cells (5 µg) as described in *Materials and Methods*. Briefly, unlabeled wild-type and mutated oligonucleotides were used to perform competition experiments against the provided biotinylated immobilized oligonucleotide. The wild-type consensus oligonucleotide will prevent NF-κB binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on NF-κB binding. Results are presented as mean ± SD of at least three different experiments.

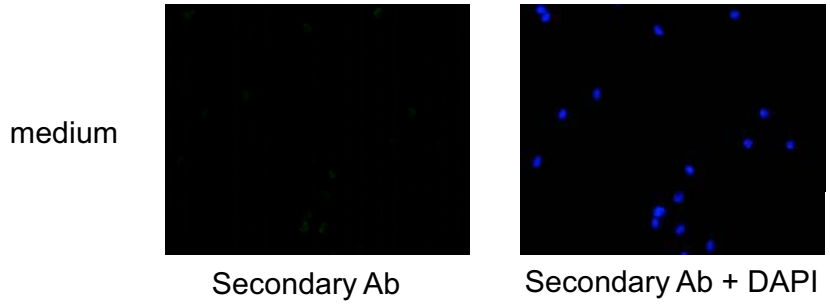

Figure 6.3 Secondary antibody control

AMs from WT mice were incubated with medium alone. After washing, cells were incubated only with secondary Alexa fluor 488 labelled goat anti-rabbit Ig antibody (green) as described in *Materials and Methods*. Nuclei were counterstained with DAPI (blue). The cells were imaged by conventional microscopy. Images are representative of at least three independent experiments.

Table 6.2 List of antibodies used for western blotting and immunofluorescence microscopy

Antibody	Host	Dilution	Company
Primary			
anti-phospho p38	rabbit	1:1000	Cell Signalling
anti-p38	rabbit	1:1000	Cell Signalling
anti-p65 NF-κB	rabbit	1:50	Santa Cruz
anti-cJun	rabbit	1:1000	Santa Cruz
anti-phospho ERK1/2	rabbit	1:1000	Cell Signalling
anti-ERK1/2	rabbit	1:1000	Cell Signalling
anti-phospho JNK1/2	rabbit	1:1000	Cell Signalling
anti-JNK1/2	rabbit	1:1000	Cell Signalling
anti-phospho MKK4	rabbit	1:1000	Cell Signalling
anti-phospho PKR	rabbit	1:1000	Abcam
anti-PKR	rabbit	1:1000	Santa Cruz
anti-β-actin	rabbit	1:2000	Biolegend
Secondary			
HRP-conjugated anti-rabbit IgG	goat	1:3000	Pierce
Alexa Fluor 488 anti-rabbit IgG	goat	1:3000	Invitrogen

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8 DECLARATION

“I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.”

Place and Date

Maciej Cabanski

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

Publications:

Maciej Cabanski, Mirko Steinmueller, Leigh Marsh, Ewa Surdziel, Werner Seeger, Juergen Lohmeyer. “PKR regulates TLR2/TLR4-dependent signaling in murine alveolar macrophages“. *Am J Respir Cell Mol Biol* (2008)

Maciej Cabanski, Jochen Wilhelm, Mirko Steinmueller, Werner Seeger, Juergen Lohmeyer. ”Genome-wide transcriptional profiling of mononuclear phagocytes recruited to mouse lungs in response to alveolar challenge with the TLR2 agonist Pam_3CSK_4”. *Am J Physiol Lung Cell Mol Physiol. In revision* (2008)

Poster presentations:

M. Cabanski, M. Steinmueller, L. Marsh, E. Surdziel, W. Seeger, J. Lohmeyer. “Double-Stranded RNA-Activated Protein Kinase (PKR) regulates TLR-dependent signalling in murine alveolar macrophages”. ATS 2007, 18-23 May, San Francisco, California

T. Shafiei Tabar, M. Steinmueller, M. Cabanski, W. Kuziel, W. Seeger, J. Lohmeyer. “Alveolär rekrutierte CCR2-positive Monozyten regulieren die LPS-induzierte pulmonale Inflammation in vivo“. Kongress der Deutschen Gesellschaft für Innere Medizin 2007, Wiesbaden, Germany

M. Cabanski, M. Steinmueller, L. Marsh, E. Surdziel, W. Seeger, J. Lohmeyer. “Double-Stranded RNA-Activated Protein Kinase (PKR) is involved in TLR4-mediated signaling in murine alveolar macrophages”. The 39th Annual

Meeting of Society for Leukocyte Biology (SLB), 9-11 November 2006, San Antonio, Texas

Scientific interests:

Signal transduction; innate and adaptive immunity; bacterial infections, inflammatory response of the lungs; allergic asthma; transcriptional profiling and regulation; mononuclear phagocytes, alveolar macrophages

Skills:

Basic techniques used in molecular biology: isolation of DNA, RNA, proteins; electrophoresis (DNA, RNA, PAGE, SDS-PAGE); PCR; qualitative and quantitative RT-PCR; transfection and siRNA, flow cytometry (FACS Canto) and cell sorting (FACS Vantage); mice models of TLR-mediated lung inflammation (LPS and Pam3CSK4 models); mouse model of allergic asthma (OVA model): maintaining primary cell cultures (alveolar macrophages) and cell lines (MHS alveolar macrophages); immunofluorescence; ELISA and ELISA-based assays (e.g. TransAM assay); Microsoft Office; knowledge of biological databases (e.g. NCBI, Ensembl, KEGG, Onto-Tools, GENECODIS)

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