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A New Model of Acute *Streptococcus Pneumoniae* Infection in Human Lung Tissue: Cellular and Molecular Mechanisms of the Pulmonary Inflammatory Response

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Abbreviations

AEC: Alveolar epithelial cell

AM: Alveolar macrophage

AP: Activator protein

APS: Ammonium persulfate

ARDS: Acute respiratory distress syndrome

ASIM: A model of acute *S. pneumoniae* infection

ATCC: American Type Culture Collection

ATP: Adenosine triphosphate

BALF: Bronchoalveolar lavage fluid

BEC: Bronchial epithelial cell

bp: Base pair

BSA: Bovine serum albumin

CAP: Community-acquired pneumonia

CBP: Choline binding protein

cDNA: complementary Deoxyriboneucleic acid

CFU: Colony forming unit

Cox: Cyclooxygenase

cp: Crossing point

DEPC: Diethylpyrocarbonate

DNA: Deoxyriboneucleic acid

ECL: Enhanced chemiluminescene

EDTA: Ethylenediamine tetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

ERK：Extracellular signal-regulated kinase

FCS: Fetal calf serum

Fig.: Figure

GM-CSF: Granulocyte-macrophage colony-stimulating factor

h: Hour

HCl: Hydrochloric acid

H_2O_2: Hydrogen peroxide

HOPE: Hepes-Glutamic acid buffer mediated **O**rganic solvent **P**rotection **E**ffect

HRP: Horseradish peroxidase

H_2SO4: Sulphuric acid

IFN: Interferon

Ig: Immunoglobulin

IKK: IκB kinase

IL: Interleukin

iNOS: inducible Nitric oxide synthase

IRAK: IL-1 receptor-associated kinase

IRF: Interferon regulatory factor

ISH: *In situ* hybridization

JNK: c-Jun N-terminal kinase

KCl: Potassium chloride

KH_2PO_4: Potassium dihydrogen phosphate

L: Liter

LDH: Lactate dehydrogenase

LPS: Lipopolysaccharide

LTA: Lipoteichoic acid

M: Molar

Mal: MyD88-adaptor-like

MAPK: Mitogen-activated protein kinase

$MgCl_2$: Magnesium Chloride

MHC: Major histocompatibility complex

min: Minute

MKK: MAPK kinase

MKKK: MAPK kinase kinase

mL: Milliliter

mRNA: messenger Riboneucleic acid

MyD88: Myeloid differentiation marker 88

NaCl: Sodium chlorid

$NaHCO_3$: Sodium bicarbonate

Na_2HPO_4: Sodium hydrogen phosphate

NaOH: Sodium hydroxide

ND: Non-determined

NEAA: Nonessential amino acid

NF-κB: Nuclear factor-κB

ng: Nanogram

PAF: Platelet activating factor

PAMP: Pathogen-associated molecular pattern

PBP: Penicillin binding protein

PBS: Phosphate-buffered saline

pg: Picogram

PGE_2: Prostaglandin E_2

PGN: Peptidoglycan

PMN: Polymorphonuclear leukocyte

PRR: Pattern recognition receptor

PspA: Pneumococcal surface protein A

PVA: Polyvinyl alcohol

rpm: Revolutions per minute

RT-PCR: Reverse transcription polymerase chain reaction

s: Second

SDS: Sodium dodecyl sulfate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SEM: Standard error of mean

SSC: Standard saline citrate

TA: Teichoic acid

Tab.: Table

TAK: Transforming growth factor β-activated kinase

TBS: Tris buffered saline

TEMED: Tetramethylethylenediamined

TICAM: TIR domain-containing adaptor molecular

TIR: Toll/IL-1 receptor

TIRAP: TIR domain-containing adaptor protein

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor-α

TRAF: Tumor necrosis factor receptor-associated factor

TRIF: TIR domain-containing adaptor-inducing IFN-β

Tris-Cl: Tris-chlorine

T-TBS: Tween-tris buffered saline

μg: Microgram

μM: Micromolar

V: Volt

VCAM: Vascular cell adhesion molecule

v/v: Volume per volume

w/v: Weight per volume

y: Year

1. Introduction

Despite the development of potent antimicrobial therapy, pneumonia is the sixth leading cause of death in the world and the main cause of infectious deaths (Wenger, 2001). As an encapsulated Gram-positive diplococcus, *Streptococcus pneumoniae* (the pneumococcus) is the most frequently isolated pathogen in community-acquired pneumonia (CAP) and one of the most common causes of death by infectious diseases such as septic shock, bacterial meningitis and acute respiratory distress syndrome (ARDS) (Schuchat et al., 1997; Fedson and Scott, 1999). Pneumococci cause 500,000 cases of pneumococcal pneumonia, 50,000 cases of bacteremia, 7,000,000 cases of otitis media and 3,000 cases of meningitis annually in United States (Austrian, 1999). Worldwide over 1 million children per year succumb to pneumococcal lung infection (Kadioglu and Andrew, 2004).The rise in antibiotic resistance of this pathogen and the limited efficacy of the widely used 23-valent polysaccharide vaccine urge further efforts to understand the host response mechanisms involved in pneumococcal pneumonia (Catterall 1999).

1.1 Bacterial factors in the pathogenesis of *S. pneumoniae* infection

The pathogenesis of *S. pneumoniae* is complex and the outcome of this infection depends on bacterial virulence factors and the effectiveness of the host response. The main factors of *S. pneumoniae* involved in the pathogenesis of pneumonia are the following:

1.1.1 The polysaccharide capsule

Since Avery found that the soluble substance surrounding the pneumococcus is composed of polysaccharide in 1925 (Avery and Heidelberger, 1925; Avery and Morgan, 1925), more than 90 serologically distinct polysaccharides have been found until now, each structurally and chemically different. Using genetically engineered pneumococci which differ only in capsular type, Kelly and colleagues proved that the virulence of the mutants compared to the parental strains is determined mainly, though not entirely, by the capsular type (Kelly et al., 1994). The capsule increases virulence by its antiphagocytic properties, but does not play a role in inducing host inflammation (Tuomanen et al., 1987). The level of virulence is determined more by the chemical composition of the capsule than its size (Knecht et al., 1970).

1.1.2 The cell wall

The pneumococcal cell wall is a potent inflammation inducer, probably via the activation of complement and the induction of cytokines such as tumor necrosis factor (TNF)-α and Interleukin (IL)-8 (Winkelstein and Tomasz, 1978; Heumann et al., 1994). The active component of cell wall is polysaccharide, a complex teichoic acid (TA) or lipoteichoic acid (LTA), composed of extended repeat carbohydates differing only in their attachment to the cell surface. TA links directly to the peptidoglycan (PGN) while LTA is hydrophobically anchored through its fatty acids to the plasma membrane (McCullers and Tuomanen, 2001). An unusual and important active component of cell wall among bacteria is phosphorylcholine which activates endothelial cells by attaching to platelet activating factor (PAF) receptor (Geelen et al., 1993). Cell wall components released during bacterial lysis induced by antibiotics are more potent inflammatory and chemotactic factors than are intact cell walls (Tomasz and Saukkonen, 1989).

1.1.3 Pneumolysin

As a cytoplasmic toxin, pneumolysin is released only when the cell wall undergoes lysis. This toxin can form large oligomeric pores and create transmembrane pores in cholesterol-containing membranes of eukaryotic cells (Rossjohn et al., 1998). Pneumolysin has been shown to cause respiratory ciliary slowing and epithelial damage, and impair the tight junctions of alveolar epithelial cells, facilitating bacterial proliferation and spread (Steinfort et al., 1989; Rayner et al., 1995). The laboratory pneumococcal strains deficient in pneumolysin reduce virulence compared to wild type strains (Benton, et al., 1995; Berry et al., 1995; Berry et al., 1999; Berry and Paton, 2000). Pneumolysin is also a main inducer of inflammation through both its cytotoxic activity and its characteristics to directly activate the classical pathway of complement (Paton et al., 1984; Cockeran et al., 2001). The molecular basis of pneumolysin induced complement activation may be related to the structural similarity of domain 4 of pneumolysin to IgG Fc fragment, rather than the presumed homology of the toxin to C-reactive protein (Rossjohn et al., 1998).

1.1.4 Choline binding proteins (CBPs)

CBPs are a family of surface proteins bound to the choline component of cell wall TA or LTA via a conserved ligand binding domain. Autolysin, the enzyme which is responsible for cell wall lysis during stationary phase or in response to antibiotics, consists of at least three kinds of hydrolases or lytic enzymes: LytA, LytB, LytC. Autolysin degrades cell wall and allows the release of intracellular such as pneumolysin into external environment. Reduced virulence was shown when the normal virulent pneumococci were transformed with inactivated LytA (Berry et al., 1989). Pneumococcal surface protein A (PspA) is a surface protein involved in inhibition of complement activation by a pathway independent of complement regulatory protein factor H whereas PspC is essential for pneumococcal carriage by acting as a bridge between pneumococcal phosphorylation and the activation of human cell glycoconjugates (Gillespie and Balakrishnan, 2000).

1.1.5 Other proteins

Other proteins that may contribute to the pathogenicity and virulence of pneumococci include neuraminidase, hyaluronidase, a neutrophil elastase inhibitor, IgA_1 protease, protein adhesins, and etc., but their precise roles have not been well determined (Catterall, 1999; McCullers and Tuomanen, 2001). In addition, penicillin binding proteins (PBPs) are the transcarboxypeptidases located in the cell wall which also bind penicillin. With alterations of PBPs to different extent, the level of resistance to penicillin can vary considerably (Appelbaum, 1996). This gradual nature of penicillin resistance has direct relevance to clinical practice. Interest in pneumococcal proteins lies not only in their pathogenicity but also in the fact that they are T cell-dependent antigens and have the potential to be used in producing new pneumococcal vaccines (Catterall, 1999).

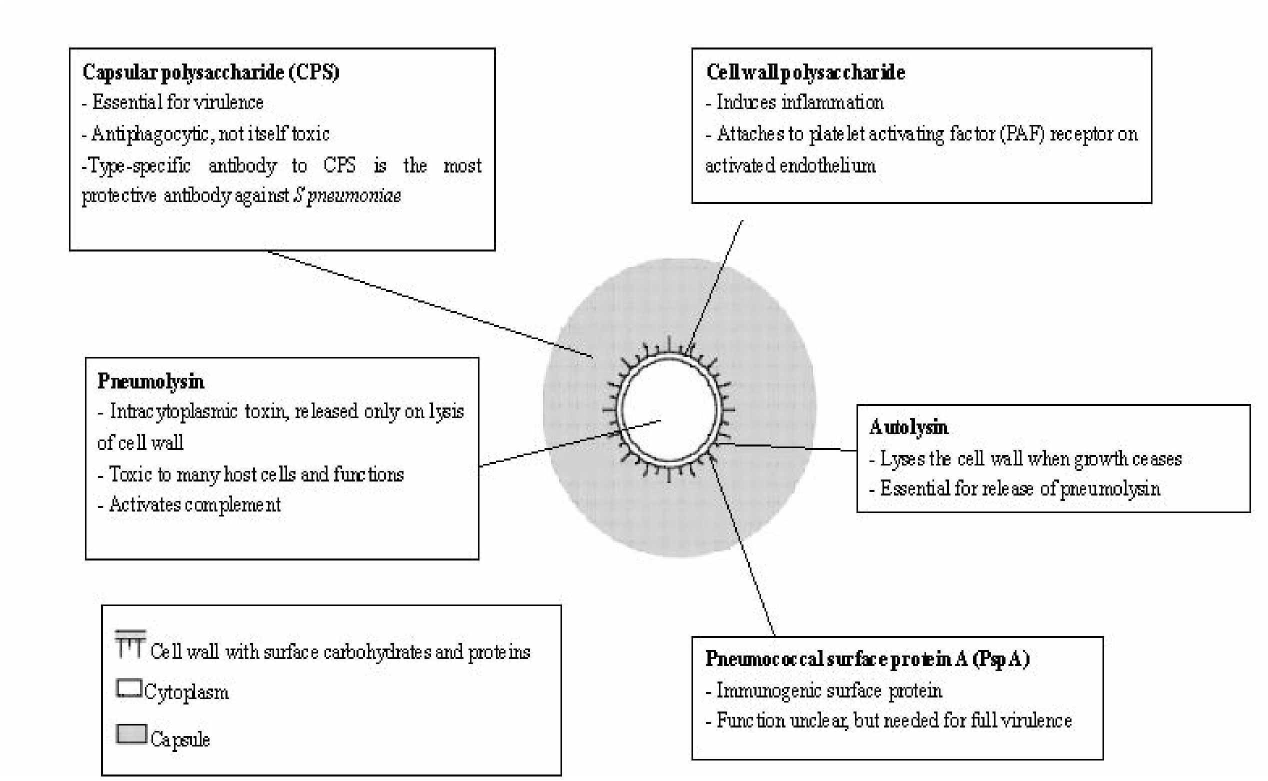


Fig. 1: Schematic figure of the known virulence factors of *Streptococcus pneumoniae* including their main functions and cellular location (Catterall 1999).

1.2 Host defense against *S. pneumoniae* infection

In recent years, it became clear that the interrelated and coordinated effort of multiple cell types, including respiratory epithelial cells lining the airways and alveoli, and both resident and recruited immune cells like macrophages, neutrophils, and lymphocytes, is implicated in the clearance of *S. pneumoniae* from the airways. Other than innate immunity, adaptive immune responses are also required for effective host defense, especially in cases of chronic bacterial infections (Sadikot et al., 2005). We illustrate the roles of different host cell types involved in *S. pneumoniae* infection and discuss individual host cell receptors and signaling cascades that have been shown to be important in host defense against this pathogen.

1.2.1 Respiratory epithelial cells

The respiratory epithelium is an important interface to environmental microorganisms. In addition to provide a physical barrier against microbial invasion and contribute to mucociliary clearance, respiratory epithelial cells are actively involved in inflammation and host defense of the lung in multiple ways: activation of a host of pro- and anti-inflammatory mediators; and secretion of a variety of antimicrobial substances including acute phase proteins, bacteriolytic lysozyme and antimicrobial peptides (Kagnoff et al, 1997; Knowles et al, 2002; Hiemstra and Bals , 2004).

In particular, type II alveolar epithelial cells (AECs) as a defender of the alveolus are strategically located in alveoli where they contribute to the innate immune response against invading pathogens. Human Type II AECs express functional Toll-like receptor (TLR) 2 and TLR4 which can be modulated by lipopolysaccharide (LPS) and TNF-α. This suggests that AECs recognize microorganisms by pathogen-associated molecular patterns (PAMPs) and play an important role in local host defense (Droemann et al., 2003; Armstrong et al, 2004). Interestingly, TLR4 expression was unresponsive to LPS in the bronchial cell line BEAS-2B (Guillot et al., 2004). This may be explained by the fact that bronchial epithelia usually are exposed to larger amounts of airborne pathogens and contaminators than alveolar epithelia. The recognition of the key mechanisms of inflammatory signaling in epithelial cells may provide novel targets for modulation of pulmonary inflammation.

1.2.2 Macrophages

Pulmonary resident alveolar macrophages (AMs) are the first line of host defense against microorganisms by phagocytosing bacteria, generating antimicrobial peptides, secreting inflammatory cytokines and presenting antigens to T cells that links innate to adaptive immunity (Underhill and Ozinsky, 2002). However, the exact role of macrophages in inflammatory response and host defense has not been well elucidated. Using a low-dose pneumococcal infection model that is characterized by the clearance of bacteria without polymorphonuclear leukocytes (PMNs) recruitment, the absence of bacteremia, and the survival of mice without development of pneumonia, Dockrell and colleagues showed that alveolar macrophages have a key role in bacterial clearance from lung and apoptosis of macrophages contributes to host defense against pneumococci (Dockrell et al., 2003).

Similar results about the role of macrophages in bacterial clearance were observed in a low-dose *Klebsiella pneumoniae* infection (Broug-Holub et al., 1997). Knapp et al found that AM^- mice displayed a higher mortality in line with a significantly increased pulmonary proinflammatory cytokine production and elevated and prolonged PMNs accumulation in the lung as compared with AM^+ control mice. These data suggest that AMs have a protective anti-inflammatory role by eliminating apoptotic PMNs. However, surprisingly, AM depletion did not alter bacterial clearance in AM^- mice (Knapp et al, 2003). The difference of bacterial clearance in two mouse models in the studies by Kanpp and Dockrell may be due to different models (fulminant vs resolving infection), differences in instillation methods (intranasal vs intratracheal) and mice with different genetic background (BALB/c vs C57BL/6 mice).

1.2.3 Polymorphonuclear leukocytes (PMNs)

PMNs play a critical role in host defense against microbial infection. The recruitment of PMNs as the key phagocytic cells involved in bacterial clearance into affected lung parenchyma is a major component of host response and appears to enhance other immune cells in the acute setting. Activated PMNs themselves are able to secret IL-8 in an autocrine/paracrine manner to keep up local inflammatory response. Droemann et al reported that pulmonary neutrophils from patients with CAP showed a decreased rate of apoptosis and increased activation compared to peripheral neutrophils (Droemann et al., 2000). Enhanced neutrophil survival and activation in the pulmonary compartment can increase the antimicrobial phagocytic function in these patients. Subsequently, PMNs undergoing apoptosis are removed from the site of inflammation by macrophage engulfment, which is strongly associated with the resolution of pulmonary inflammation (Cox et al, 1995; Zysk et al., 2000).

1.2.4 T lymphocytes

Although the mechanisms of antigen-specific T lymphocyte response to bacterial pathogens are well illustrated in adaptive immune response, less is known about its role in innate immunity. There is increasing evidence that T lymphocytes have also an important contribution to the early host response to pneumococci. Using major histocompatibility complex (MHC) class II-deficient mice which show CD4 T-cell-negative characteristics, Kadioglu et al have proved that knockout mice were more susceptible to pneumococcal infection at significantly earlier stage than wild-type strains. This suggests that CD4 T lymphocytes have a crucial protective role in the host response against *S. pneumoniae* (Kadioglu et al., 2004).

1.2.5 Toll-like receptors (TLRs)

The first line of defense against invading bacteria is provided by the innate immune system, which recognizes PAMPs, conserved microbial patterns shared by large groups of pathogens, but not found in higher eukaryotes (Zhang et al., 2000; Medzhitov, 2001). One of the central features of this system of microbial recognition is TLR-related signaling pathways that are critical in early host defense against the microbial invasion (Barton and Medzhitov, 2003; Iwasaki and Medzhitov, 2004). The Toll receptor was originally characterized in *Drosophila*, where it induces rapid induction of the antifugal peptide drosomycin in response to fungal infection (Lemaitre et al., 1996). At least 10 members of the TLR family (the homologues of the *Drosophila* Toll receptor) have been identified in humans until now. Different TLRs play crucial roles in the immune response by recognizing their distinct PAMPs. TLR1 recognizes triacyl lipopeptides from bacteria and mycobacteria (Takeuchi et al., 2002); TLR2 recognizes PGN, LTA and lipoproteins of Gram-positive bacteria whereas TLR4 recognizes LPS from Gram-negative bacteria. (Poltorak et al., 1998; Aliprantis et al., 1999; Schwandner et al, 1999); TLR3 has been identified as the receptor for virus double-stranded RNA (Kulka et al., 2004); TLR5 recognizes bacterial flagellin (Hayashi et al., 2001); TLR6 is shown to binds diacyl lipopeptides from mycoplasma, LTA from Gram-positive bacteria, zymosan from fungi (Schwadner et al, 1999; Ozinsky et al., 2000; Takeuchi et al., 2001); Imidazoquinolines and single-stranded RNA activate TLR7 and TLR8 (Jurk et al., 2002; Hemmi et al., 2002; Heil; 2004); CpG-containing DNA from bacteria and viruses is the ligand of TLR9 (Hemmi et al., 2000; Lund et al., 2003). However, the ligand of TLR10 has not yet been identified (Akira and Takeda, 2004). Recently, a *Toxoplasma gondii* profiling-like protein has been defined as the first ligand for TLR11 in mice whereas there may be no functional TLR11 protein in humans (Zhang et al., 2004; Yarovinsky et al., 2005).The chromosomal locations and the ligands of the known TLR family members in humans are shown in Tab.1.

TLRs are mainly expressed on myeloid cells, such as macrophages, neutrophils, and dendritic cells. However, it has become clear that other cell types such as epithelial cells, endothelial cells express TLRs upon stimulation. This indicates that these cells are implicated in innate immunity and play a role in host defense against microorganisms to different extents. Among TLR family, TLR2 and TLR4 are best documented. These two receptors are implicated in *S. pneumoniae*-associated host immune responses which are elucidated as below.

1.2.6 TLR2 and S. pneumoniae

TLR2 was reported to form a heterophilic dimer with TLR1 or TLR6. Functional TLR2 is expressed in monocytes/macrophages, alveolar epithelial cells and lymphoid tissue (Droemann et al., 2003; Armstrong et al., 2004; Blasi et al., 2005). Some authors have pointed out that TLR2 is the key pattern recognition receptor (PRR) in the immune response to Gram-positive bacteria and mycobacteria (Takeuchi et al., 1999; Schwandner et al., 1999; Takeuchi et al., 2000a). Moreover, TLR2 appears to induce cellular activation by atypical LPS from *Leptospira interrogans* and *Porphyromonas gingivalis* whose structures are different from LPS from enterobacteria such as *Escherichia coli* (Tabeta et al., 2000; Werts et al., 2001).

Some studies have attributed an important role to TLR2 in activating inflammatory response in immune cells upon stimulation with components of Gram-positive bacteria including *S. pneumoniae*. Schwandner et al. reported that nuclear factor-κB (NF-κB) in human embryonic kidney 293 cells expressing TLR2, but not in cells expressing TLR1 or TLR4, was activated by whole Gram-positive bacteria, PGN, and LTA (Schwandner et al., 1999). Similarly, the association of TLR2 gene expression in human HL60 cells and mouse

Tab. 1: Human TLR family ligands (Modification from Qureshi and Medzhitov, 2003; Akira and Takeda, 2004. ND: Non-determined)

	Chromosomal location	Ligands	Origin
TLR1	4p14	Triacyl lipopeptides	Bacteria, mycobacteria
		Soluble factors	*Neisseria meningitides*
TLR2	4q32	Lipoprotein/lipopeptides	A variety of pathogens
		Peptidoglycan	Gram-positive bacteria
		Lipoteichoic acid	Gram-positive bacteria
		Lipoarabinomannan	Mycobacteria
		A phenol-soluble modulin	*Staphylococcus epidermidis*
		Glycoinositolphospholipids	*Trypanosoma Cruzi*
		Glycolipids	*Treponema maltophilum*
		Porins	Neisseria
		Zymosan	Fungi
		Atypical lipopolysaccharide	*Leptospira interrogans*
		Atypical lipopolysaccharide	*Porphyromonas gingivalis*
		Atypical lipopolysaccharide	*Helicobacter pyroli*
		Heat shock protein 70	Host
TLR3	4q35	Double-stranded RNA	Viruses
TLR4	9q32-33	Lipopolysaccharide	Gram-negative bacteria
		Taxol	Plant
		Fusion proteins	Respiratory syncytical virus
		Envelope proteins	Mouse mammary tumor virus
		Envelope proteins	Moloney murine leukemia virus
		Heat shock protein 60	*Chlamydia pneumoniae*
		Heat shock protein 60	Host
		Heat shock protein 70	Host
		Extra domain A of fibronectin	Host
		Oligosaccharides of hyaluronic acid	Host
		Polysaccharide fragments of heparan sulfate	Host
		Fibrinogen	Host
		Collectin surfactant protein-A	Host
TLR5	1q41-42	Flagellin	Bacteria
TLR6	4p14	Diacyl lipopeptides	Mycoplasma
TLR7	Xp22.3	Imidazoquinoline	Synthetic compounds
		Loxoribine	Synthetic compounds
		Bropirimine	Synthetic compounds
		Single-stranded RNA	Viruses
TLR8	Xp22	Imidazoquinoline	Synthetic compounds
		Single-stranded RNA	Viruses
TLR9	3p21.3	CpG-containing DNA	Bacteria
TLR10	ND	ND	May interact with TLR2

RAW264.7 cells with NF-κB activation in response to PGN was observed (Liu et al., 2001). In addition, the activation of Chinese hamster ovary fibroblast cells expressing human TLR2 but not TLR4 was induced by heat-killed *S. pneumoniae* (Yoshimura et al., 1999). A recent report described that isolated AMs from $TLR2^{-/-}$mice failed to release TNF-α and keratinocyte chemoattractant (murine analogues of IL-8) upon stimulation with heat-killed *S. pneumoniae* compared to wild-type AMs. Therefore, TLR2 seems indispensable for alveolar macrophage responsiveness towards pneumococci and plays an important role in the induction of lung inflammatory response. These observations are in line with studies of Koedel and Schroder who also found a prominent role of TLR2 for pneumococci-related cell activation (Koedel et al., 2003; Schroder et al., 2003).

In addition, activation of TLR appears to be directly involved in induction of antimicrobial activity *in vitro*. The evidence demonstrates that TLR2 is directly involved in bacterial killing by monocytes/macrophages (Thoma-Uszynski et al., 2001). But some conflicting results about bacterial clearance still have been reported. Data from a murine *S. pneumoniae* meningitis model demonstrated that $TLR2^{-/-}$ mice had higher bacterial loads in brain and a reduced survival period than wild-type mice, suggesting TLR2 have a role in pneumococcal clearance at least in brain (Echchannaoui et al., 2002). However, $TLR2^{-/-}$ mice intranasally inoculated with *S. pneumoniae* at doses varying from non-lethal to lethal displayed an unaltered antibacterial defense, indicating TLR2 seems not to contribute to bacterial clearance and other PRRs likely are involved in the innate immune response to pneumococcal infection in this model (Knapp et al., 2004). Given the other factors involved in the innate immune response against *S. pneumoniae*, such as natural antibodies and complement, these mediators are likely candidates to compensate for the lack of TLR2 (Mold et al., 2002).

The activation of TLR2 which links innate immunity and adaptive immunity leads not only to the induction of inflammatory responses but also to the development of antigen-specific adaptive immune response. Activation of TLR2 by its synthetic ligand Pam3Cys was reported to induce the expression of Th2-associated effector molecules (Redecke et al., 2004). Khan et al suggested that TLR2 has a role in shaping a type 1 IgG humoral immune response to pneumococci, although the exact underlying mechanisms need to be further investigated (Khan et al., 2005).

1.2.7 TLR4 and S. pneumoniae

TLR4 is required for the recognition of LPS from Gram-negative bacteria and the mutations of TLR4 gene generate two mouse strains (C3H/HeJ and C57BL10/ScCr) which are hyporesponsive to LPS (Poltorak et al., 1998; Qureshi et al., 1999). $TLR4^{-/-}$ mice are also insensitive to LPS, confirming that TLR4 is an essential receptor for the recognition of LPS (Hoshino et al., 1999). The response to LPS is initiated upon its interaction with TLR4 in conjunction with the accessory proteins MD-2 and soluble or membrane-bound CD14.

Some investigations have shown that TLR4 still has a limited role in the innate immune response to *S. pneumoniae*. An *in vitro* study showed that TLR4-deficient macrophages lacked the response to Gram-positive bacterial cell wall components (Takeuchi et al., 1999), although this result was suspected by some authors because the LTA preparations used in earlier studies were easily contaminated with endotoxin (Gao et al., 2001; Morath et al., 2002). Indeed, an important role for TLR4 recognizing pneumolysin in the innate immune response to *S. pneumoniae* in the nasopharynx was reported by Malley and colleagues. They found that pneumolysin induced the inflammatory response of macrophages via TLR4. Furthermore, mutant mice lacking functional TLR4 were significantly more susceptible to lethal pneumococcal infection and displayed decreased survival after the challenge with wild-type pneumococci (Malley et al., 2003). Another investigation demonstrated that TLR4 mutant mice showed a reduced survival only after infection with low-level pneumococcal doses, which was associated with a higher bacterial burden in the lungs 48 h postinfection. But TLR4 mutant mice showed an unaltered inflammatory response in a model of pneumococcal pneumonia (Branger et al., 2004). Taken together, these findings suggest that the innate immune response to pneumococci is partly mediated by TLR4 (Kadioglu and Andrew, 2004).

1.2.8 TLR signaling pathways

The molecular mechanisms by which TLRs induce gene expression are being elucidated by using gene knockout mice. Increasing evidence indicates that there are myeloid differentiation marker 88 (MyD88)-dependent and MyD88-independent signaling pathways involved in TLR signal cascades.

MyD88, consisting of a N-terminal death domain and a C-terminal (Toll/IL-1 receptor) TIR domain, is a common adaptor to TLR family given the fact that most TLR ligands cannot induce inflammatory cytokine production in MyD88-deficient mice (Kawai et al., 1999; Hacker et al., 2000; Schnare et al., 2000; Takeuchi et al., 2000b; Hayashi et al., 2001; Hemmi et al., 2002). The MyD88-dependent pathway is analogous to IL-1 receptor signaling. Upon stimulation, MyD88 recruites IL-1 receptor-associated kinase (IRAK)-4 to the TLRs and initiates IRAK-4-mediated phosphorylation of IRAK-1. Then activated IRAK-1 associates with tumor necrosis factor receptor-associated factor (TRAF) 6 which leads to the activation of a mitogen-activated protein kinase kinase kinase (MKKK) named transforming growth factor β-activated kinase (TAK)-1. TAK-1 activates two distinct signaling pathways. One pathway activates the IκB kinase (IKK) complex consisting of IKKα, IKKβ, and IKKγ. The IKK complex phosphorylates and degradates IκB, inducing nuclear translocation of NF-κB which subsequently leads to the expression of inflammatory cytokines (Takeda and Akira, 2005). The other results in the activation of activator protein (AP)-1 transcription factors through the signaling of mitogen-activated protein kinase kinases (MKKs) and mitogen-activated protein kinases (MAPKs) including p38, p44/42 (extracellular signal-regulated kinase, ERK), and c-Jun N-terminal kinase (JNK). In addition, TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal) functions downstream of TLR2 and TLR4, but is not involved in other TLR signalings (Yamamoto et al., 2002a; Horng et al., 2002).

The investigation from MyD88-deficient mice showed that the MyD88-independent pathway is required for TLR3 and TLR4 to induce interferon (IFN)-β. Recent studies demonstrated that TIR domain-containing adaptor-inducing IFN-β (TRIF), also known as

TIR domain-containing adaptor molecule (TICAM)-1, appears to be responsible for inducing IFN-α/β genes by the activation of interferon regulatory factor (IRF) 3 (Kawai et al., 2001; Yamamoto et al., 2002b; Oshiumi et al., 2003), whereas two noncanonical IKKs: TBK1 and IKKε/IKKi have been revealed to function downstream of TRIF and upstream of IRF3 and NF-κB (Sharma et al., 2003; Sankar et al., 2005). The divergences among individual TLR signaling pathways cannot be well explained by the known signaling components. Additional signaling mechanisms have yet to be unraveled (Barton and Medzhitov, 2003).

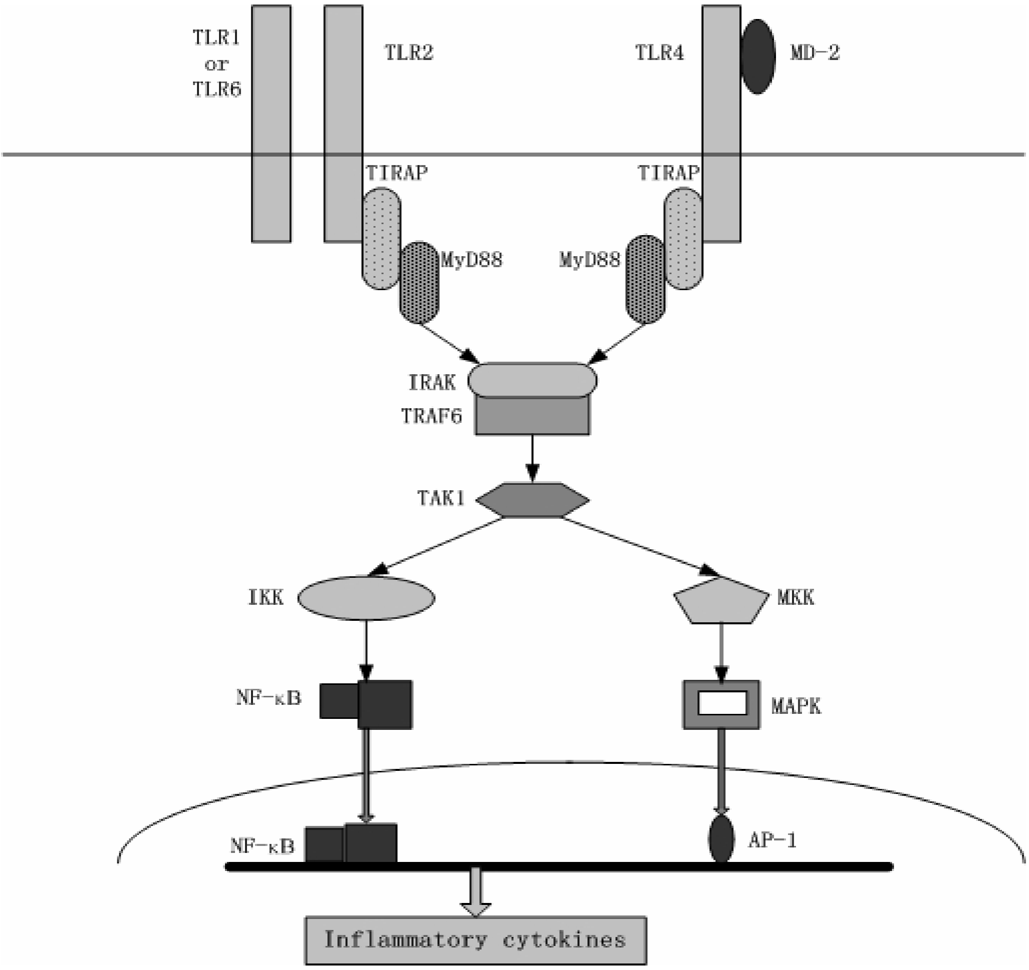


Fig. 2: The shared signaling pathways via TLR2 and TLR4 are shown. The main TLR2 and TLR4 signalings are transduced via IL-1 receptor signaling complex, which includes two essential adaptor proteins, MyD88 and TRAF6, then culminates in the activation of NF-κB transcription factors, as well as MAPKs. This signal transduction pathway further coordinates the induction of multiple genes encoding inflammatory mediators.

1.2.9 P38 MAPK and inflammation

The MAPKs are proline-directed serine and threonine protein kinases that are usually activated by phosphorylation of threonine and tyrosine residues by MKKs (Davis 1993; Cobb and Goldsmith, 1995; Ono and Han, 2000). The differences among three types of MAPKs are their intervening amino acids: glycine in p38, glutamic acid in p44/42, and proline in JNK (Saklatvala, 2004). As central signal pathways in many host cells, MAPKs are involved in inflammatory cytokine expression, cytoskeletal reorganization, and stress reaction in addition to the mitogenic response to growth factors (Robison and Cobb, 1997). Previous studies have demonstrated the essential role of MAPK activation in proinflammatory cytokine responses to a number of microbial stimuli and their cell components such as group B streptococcus, mycoplasma membrane lipoproteins, staphylococcal peptidoglycan and LPS from Gram-negative bacteria (Dziarski et al., 1996; Sweet and Hune, 1996; Garcia et al., 1998; Rawadi et al., 1998; Mancuso et al., 2002).

To date, four p38 MAPKs have been published. P38α was first isolated as 38-kDa protein which mediates the immune response to LPS stimulation (Han et al., 1993; Han et al., 1994). Three other members, p38β, p38γ, and p38δ, were subsequently found in mammals. Sequence analysis showed that each p38 isoform has more than 60 % homology, but only 40 %-50 % identity to the other MAPK members (Ono and Han, 2000). The p38α, p38β and p38δ are ubiquitously expressed whereas p38γ is predominantly expressed in skeletal muscle and involved in muscle differentiation. An important function of p38α (and probably p38β) is to regulate the expression of inflammatory response genes, but the exact role of p38γ and p38δ in regulation of inflammation is unclear (Saklatvala, 2004). There is much evidence to support an essential role of p38α (or simply p38) in inflammation. P38 signal pathway regulates the production of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β (Perregaux et al., 1995) and chemokines like IL-8, mediates the expression of intracellular enzymes such as cyclooxygenase (Cox)-2 and inducible nitric oxide synthase (iNOS) (Guan et al., 1998; Rupp et al., 2004; N'Guessan et al., 2006), and modulates the induction of adherent proteins including vascular cell adhesion molecule (VCAM)-1 and other inflammatory-related molecules (Pietersma et al., 1997). As p38 MAPK is implicated in the production of key inflammatory mediators, p38 and the major components of p38 pathway can become obvious therapeutic targets for inflammatory diseases. The widely used p38 inhibitor, SB203580, only blocks p38α and p38β and competes for the adenosine triphosphate (ATP)-binding pocket (Saklatvala, 2004). *In vivo* animal studies demonstrated that inhibition of p38 by SB203580 can decrease mortality in a murine model of endotoxin-induced shock and has an anti-inflammatory effect in mouse and rat models of rheumatoid arthritis (Badger et al., 1996).

It should be noted that the activation of p38 MAPK and its regulation of inflammatory response depend on cell type and stimulus. Emphasis in further study should be placed on how p38 functions in a specific cell type upon a specific stimulus. Little is known of the role of p38 pathways in *S. pneumoniae*-induced cell and tissue responses. Recently, Schmeck et al reported that pneumococci induced IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) gene expression in human bronchial epithelial cells (BEAS-2B) via p38 MAPK signaling pathway and activated NF-κB-dependent gene transcription in a p38 MAPK-dependent manner in HEK293 cells. Further experiments showed that blockade of p38 MAPK did not affect inducible nuclear translocation and recruitment of NF-κB/RelA to the IL-8 promotor but did reduce the level of phosphorylated p65/RelA (serine 536) at IL-8 promotor and inhibited pneumococci-mediated recruitment of RNA polymerase II to IL-8 promotor. These data suggested that p38 MAPK plays an important role in pneumococci-induced inflammatory cytokine transcription by modulating p65 NF-κB-mediated transactivation (Schmeck et al, 2004a). The components of signaling pathways and cross-talk between different signal molecules in the host immune response to *S. pneumoniae* are only partly understood. The critical signal molecules in the inflammatory response should be further investigated in order to modulate pneumococcal inflammation via small molecular approaches.

1.3 Aims of the current study

This study was undertaken to evaluate cellular and molecular mechanisms of host defense against *S. pneumoniae* in the human lung tissue. The specific aims of the present study were as follows:

- The establishment of cell culture models and a novel lung tissue model of acute *S. pneumoniae* infection for investigating interactions between pathogens and pulmonary host cells on the cell and tissue levels.

- Which pulmonary cell types are implicated as important host cells for acute *S. pneumoniae* infection?

- The role of alveolar macrophages in the inflammatory response was evaluated in human lung tissue depleted of macrophages by Clodronate/liposomes.

- The roles of TLR2, 4 and MAPK signalings in the inflammatory response of immune cells and human lung tissue stimulated with *S. pneumoniae* were investigated.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and Kits

All chemicals and reagents were used in analytic degree of purity.

Acetic acid..Merck, Darmstadt, Germany

Acetone……………………………………………..Merck, Darmstadt, Germany

Acrylamide (30 %)/Bisacrylamid (0.8 %)……………Bio-Rad, Munich, Germany

Agarose...Invitrogen, Karlsruhe, Germany

Ammonium persulfate (APS).......................................Sigma, Steinheim, Germany

Amphotericin-B…..PAA, Pasching, Austria

Bovine serum albumin (BSA)………………………....Roth, Karlsruhe, Germany

Bromophenol blue……………………………………..Bio-Rad, Muenichen, Germany

Calcium chloride.. ....Sigma, Steinheim, Germany

Diethylpyrocarbonate (DEPC) …................................Sigma, Steinheim, Germany

Dithiothreitol ....Sigma, Steinheim, Germany

EDTA...Merck, Darmstadt, Germany

Ethanol...Merck, Darmstadt, Germany

Ethidium bromide……………………………………. Sigma, Steinheim, Germany

Faramount mounting medium…………………………Dako, Hamburg, Germany

Formalin………………………………………………Sigma, Steinheim, Germany

Formamide………………….......................................Carl Roth, Karlsruhe, Germany

Fetal calf serum (FCS)……………………………....Biochrom, Berlin, Germany

Gentamicin..Sigma, Steinheim, Germany

Giemsa... Merck, Darmstadt, Germany

Glycerol ....Sigma, Steinheim, Germany

Glycine...Sigma, Steinheim, Germany

Hematoxylin..Chroma, Munich, Germany

HOPE-solution………………………………………....DCS, Hamburg, Germany

Hydrochloric acid (HCl)..Merck, Darmstadt, Germany

Hydrogen peroxide (H_2O_2).......... ....R&D, Minneapolis, USA

Isopropanol..Merck, Darmstadt, Germany

L-Glutamin ....PAA, Pasching, Austria

Lymphocyte separation medium...................................PAA, Pasching, Austria

Magnesium Chloride ($MgCl_2$).......................................Merck, Darmstadt, Germany

May-Grünwald……………………………………....Merck, Darmstadt, Germany

Methanol...Merck, Darmstadt, Germany

Milk powder (non-fat)…………………………….....Frema, Lueneburg, Germany

New-fuchsin………………………………………....Sigma, Steinheim, Germany

Nonessential amino acid (NEAA) ....PAA, Pasching, Austria

Paraformaldehyd..Merck, Darmstadt, Germany

Polyvinyl Alcohol (PVA).......................... ....Merck, Darmstadt, Germany

Potassium chloride (KCl) ....Merck, Darmstadt, Germany

Potassium dihydrogen phosphate (KH_2PO_4)…………...Merck, Darmstadt, Germany

RPMI 1640 ....Biochrom, Berlin, Germany

Sodium bicarbonate ($NaHCO_3$).......................................Merck, Darmstadt, Germany

Sodium chlorid (NaCl)…..Merck, Darmstadt, Germany

Sodium citrate…...Merck, Darmstadt, Germany

Sodium dodecyl sulphate (SDS)....................................Boi-Rad, Munich, Germany

Sodium hydrogen phosphate (Na_2HPO_4)……….........Merck, Darmstadt, Germany

Sodium hydroxide (NaOH) ....Merck, Darmstadt, Germany

Sucrose………………………………………….........Merck, Darmstadt, Germany

Sulphuric acid (H_2SO_4)……………………………....Merck, Darmstadt, Germany

Tetramethylbenzidine…………………………….......R&D, Minneapolis, USA

Tetramethylethylenediamined (TEMED)………..........Sigma, Steinheim, Germany

Tris-aminomethan……………………………….........Bio-Rad, Munich, Germany

Tris-chlorine (Tris-Cl)...Sigma, Steinheim, Germany

Trypan blue..Sigma, Steinheim, Germany

Trypsin/EDTA……………………………………………PAA, Pasching, Austria

Tween-20 ....…..Merck, Darmstadt, Germany

Yeast tRNA…………………………………………....Roche, Mannheim, Germany

Clinical Chemistry LDH kit...Abbott, Wiesbaden, Germany

ECL Chemiluminescent Substrate System......................Amersham, Freiburg, Germany

ELISA Kit…..R&D, Minneapolis, USA

First-Strand PCR kit …………………………………....Roche, Mannheim, Germany

LightCycler Detection System …………………………....Roche, Mannheim, Germany

NucleoSpin RNA II kit…………………………………Macherey-Nagel, Dueren, Germany

TMB Liquid Substrate System.......................................Sigma, Steinheim, Germany

2.1.2 Solutions and buffers

Running gel buffer: 1.5M Tris-Cl (PH 8.8)

Stacking gel buffer: 0.5M Tris-Cl (PH 6.8)

10 % APS: 0.1g APS in 1mL dH_2O

10 % SDS: 0.1g SDS in 1mL dH_2O

5 x SDS-PAGE running buffer: 0.125 M Tris-HCl, 0.96 M glycine, 0.5 % SDS

Protein lysis buffer: 4 % w/v SDS, 10 mM dithiothreitol, 20 % v/v glycerol, 0.125 M Tris-Cl (pH 7.8), 0.4 % bromophenol blue

Blotting buffer: 25 mM Tris-aminomethan, 193 mM glycine, 20 % methanol

10 x TBS: 80 g NaCl, 24.2 g Tris-aminomethan in 1 L dH_2O (pH 7.4)

1 x T-TBS: 100 ml 10 x TBS, 1 mL Tween-20 in 1L dH_2O

Blocking buffer for immunoblot: 5 g non-fat dried milk in 100 mL T-TBS

1 x PBS for ELISA: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4

Wash buffer: 0.05% Tween 20 in 1 x PBS, pH 7.2-7.4

Block buffer for ELISA: 1% BSA in 1 x PBS, 0.2 µm filtered

Diluent reagent: 0.1% BSA, 0.05% Tween 20 in 1 x TBS (20 mM Trizma-aminomethan, 150 mM NaCl), pH 7.2-7.4, 0.2 µm filtered (for IL-8); 1% BSA in 1 x PBS, 0.2 µm filtered

(for IL-6 and TNF-α)

Substrate solution: 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine)

Stop solution: 1M H_2SO_4

0.2 % DEPC: 0.2 mL DEPC in 100 mL dH_2O

20 x Standard saline citrate (SSC): 0.3 M Sodium citrate; 3 M NaCl

2.1.3 Bacterial strain and cell line

An encapsulated *S. pneumoniae* strain serotype 3 is obtained from American Type Culture Collection (ATCC 6303, Rockville, MD, USA).

Alveolar epithelial cell line A549 is obtained from European Collection of Cell Cultures.

2.1.4 Antibodies, inhibitors and their characteristics

Anti-phospho-p38 MAPK (rabbit monoclonal antibody) is from Cell Signaling Technology, Beverly, USA and used at 1:1000 dilution.

Anti-phospho-p44/42 MAPK (rabbit monoclonal antibody) is from Cell Signaling Technology, Beverly, USA and used at 1:1000 dilution.

Anti-β-actin (rabbit monoclonal antibody) is from Cell Signaling Technology, Beverly, USA and used at 1:2000 dilution.

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody is from Cell Signaling Technology, Beverly, USA and used at 1:4000 dilution.

Anti-TLR2 monoclonal antibody (functional grade) is from eBioscience, San Diego, USA and used at 5 μg/mL.

Anti-TLR4 monoclonal antibody (functional grade) is from eBioscience, San Diego, USA and used at 5 μg/mL.

Alkaline phosphatase-conjugated anti-digoxigenin antibody is from Roche, Mannheim, Germany.

SB203580 (p38 MAPK inhibitor) is from Calbiochem, CA, USA and used at 20 μM.

UO126 (p44/42 MAPK inhibitor) is from Calbiochem, CA, USA and used at 10 μM.

2.1.5 Markers for nucleic acids and proteins

DNA marker:

1 Kb ladder...Gibco, Karlsruhe, Germany

Protein marker:

MultiMark Standard……………………………Biolabs, Ipswich, USA

2.1.6 Instruments and equipments

Aeroset chemistry analyzer……………………..Abbott, Wiesbaden, Germany

Cell house 200…………………………………Heto, Allerod, Danmark

Cell counter AC-8………………………………Assistant, Frankfurt, Germany

Centrifuge Rotina 35……………………………Hettich, Tuttlingen, Germany

Cytocentrifuge Cytospin Ⅱ……………………..Shandon, Frankfurt, Germany

Eppendorf pipette………………………………Eppendorf, Hamburg, Germany

LightCycler...Roche, Mannheim, Germany

Light microscope………………………………..Carl Zeiss, Frankfurt, Germany

Mini-Protean Ⅱ Electrophoresis Cell…………...Bio-Rad, Munich, Germany

Photometer-340………………………………...SLT, Salzburg, Austria

Plate shaker……………………………………..Heidolph, Schwabach, Germany

TissueLyser…………………………….............Qiagen, Hilden, Germany

Weighter AE 200...Mettler-Toledo, Giessen, Germany

Cell culture flask………………………………....Greiner, Frickenhausen, Germany

Cell scraper……………………………………...Greiner, Frickenhausen, Germany

Combitip Plus……………………………………Eppendorf, Hamburg, Germany

ELISA plate……………………………………...Nalge-Nunc, Hereford, UK

Microlance………………………………………Becton Dickson, Heidelberg, Germany

Nitrocellulose membrane.....................................Sartorius, Goettingen, Germany

Nylon membranes..Roche, Mannheim, Germany

Sheep blood agar plate…………………………...Biomerieux, Nuertingen, Germany

Superfrost^{+} microscope slide…………..................Menzel-Gläser, Braunschweig, Germany

Syringe filter……………………………………...Nalge, Rochester, USA

Syringe..Becton Dickson, Heidelberg, Germany

24-well tissue culture plate………………………Biochrom, Berlin, Germany

48-well cell culture plate………………………....Greiner, Frickenhausen, Germany

2.2 Subjects and Methods

2.2.1 Patients

The study population consisted of 26 patients. Bronchopulmonary infection was excluded by clinical examination, systemic inflammatory markers and chest x-ray. The demographic data of the study population are summarized in Tab. 2. The protocol was approved by the Ethical Committee of University of Luebeck, Germany.

Tab. 2: Demographic data of the study population

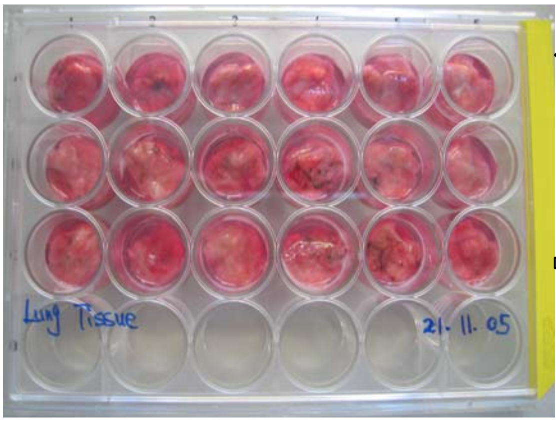
Patients	n = 26
Age (y)	64.0 ± 1.9
Gender	Male: 12 (46 %)
	Female: 14 (54 %)
FEV_1/IVC (%)	71.1±3.0
FEV_1 % predicted	77.5±3.8
Underlying diseases	Lung cancer: 20
	Metastases from extrapulmonary tumor: 5
	Parasite (Cystic echinococcosis): 1

2.2.2 Culture of *S. pneumoniae*

The encapsulated *S. pneumoniae* strain serotype 3 was obtained from American Type Culture Collection (ATCC 6303; Rockville, MD, USA). Pneumococci were grown on sheep blood agar plates at 37 °C and 5% CO_2. Bacteria at midlogarithmic phase were used for stimulation.

2.2.3 Establishment of a new model of acute *S. pneumoniae* infection (ASIM)

Vital lung specimens were obtained from pulmonary resections of the patients mentioned above without clinical signs of acute respiratory infection. The normal lung specimens at least 5 cm away from pulmonary suspected nodules were used in the experiments. Lung tissue (1 cm^3 size; 0.4-0.5g) was cultured in 800 µL of endotoxin-free RPMI1640 medium (Biochrom, Berlin, Germany) in 24-well flat-bottom, tissue culture plates (Biochrom). 50 µL of pneumococci were added into culture medium to a final concentration of 10^7 CFU/mL. Preliminary experiments using increasing pneumococcal concentrations demonstrated a dose-dependent increase in inflammatory response (IL-8 release from ASIM 24 h postinfection: 179 ng/mL [10^6 CFU/mL] *vs* 292 ng/mL [10^7 CFU/mL] *vs* 353 ng/mL [10^8 CFU/mL]); representative data from three independent experiments). After 4 h and 24 h stimulation, supernatants were harvested and stored at -70 °C, and lung specimens were fixed at 4 °C in the newly developed **H**epes-Glutamic acid buffer mediated **O**rganic solvent **P**rotection **E**ffect (HOPE)-solution (Goldmann et al., 2002). Schematic illustration of this *in vitro* lung tissue model infected with pneumococci is shown as Fig. 3.



Human lung tissue infected with *S. pneumoniae*

Analysis of lung tissue fixed with HOPE-solution:
- *S. pneumoniae* DNA detection by ISH;
- Detection of surface markers by RT-PCR;
- Immunoblot analysis of MAPK signaling pathways;
- Inflammatory mediators in the supernatants by ELISA.

Fig. 3: A novel model of acute *S. pneumoniae* infection in human lung tissue

The viability of lung cells in the ASIM was evaluated by the release of lactate dehydrogenase (LDH), an indicator of nonspecific necrotic cell death, into supernatants using AEROSET System (Abbott, Wiesbaden, Germany). The LDH level in RPMI 1640 was used as blank control whereas maximum LDH release from a piece of lung tissue ($1cm^3$) was performed by lung homogenization. The level of specific LDH was calculated by using the following formula: percentage of specific LDH release = ([experimental release - blank control] / [maximum release - blank control]). The relative LDH levels (expressed as the percentage of LDH release) demonstrated that the lung cells showed time-dependent necrosis in tissue culture whereas *S. pneumoniae* (10^7CFU/mL) stimulation aggravated cell necrosis and LDH release in the time frame tested (Fig. 4). These results suggest that vital lung specimens could be infected with *S. pneumoniae* for 48 h in our ASIM.

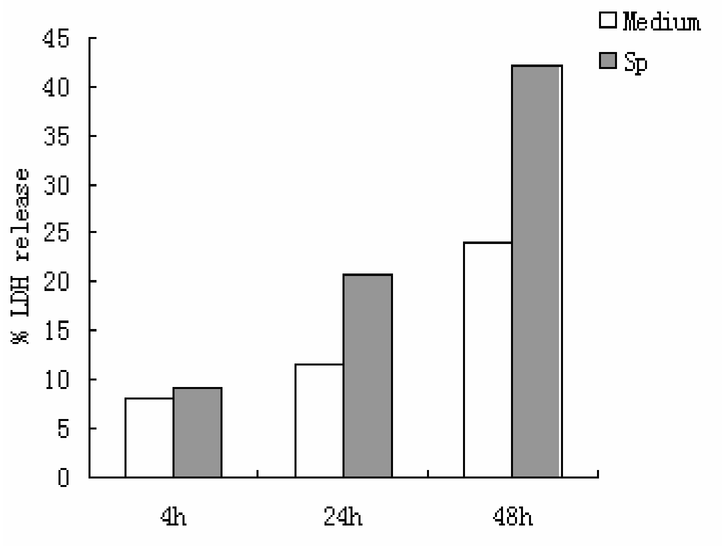


Fig. 4: Time-dependent necrosis of lung cells in the ASIM. LDH release (expressed as the percentage of LDH release) demonstrated that *S. pneumoniae* (10^7CFU/mL) infection aggravated cell necrosis and LDH release compared to non-infected lung tissue within 48 h. Representative data from three independent experiments are shown. Sp: *S. pneumoniae.*

2.2.4 Cell culture and stimulation

2.2.4.1 An epithelial cell line: A549 cell

A549 cells were grown in 175 cm^2 polystyrene flasks (Greiner, Frickenhausen, Germany) with RPMI 1640 supplemented with 10 % heat-inactivated fetal calf serum (FCS), 2 mM

L-glutamine, 1 x nonessential amino acid (NEAA), 10 mg/L gentamicin and 2 mg/L amphotericin-B. Cells were maintained under an atmosphere of 5 % CO_2 at 37 °C by routine passage every 3 days. Before stimulation, A549 cells were passaged by exposure to 0.05 % trypsin plus 0.02 % EDTA into 48-well flat-bottom, cell culture plates (Greiner). Cells were seeded at 1 x 10^5 cells per well. This produced an 80-90 % confluent monolayer after overnight incubation at 37 °C in a 5 % CO_2 humidified atmosphere.

2.2.4.2 Macrophages

Macrophages were got from bronchoalveolar lavage fluid (BALF) of 8 persons with no clinical signs of acute respiratory infections. Cells were differentiated counting a minimum of 600 cells on a cytocentrifuge smear (Cytospin II, Shandon, Frankfurt) stained with May-Grünwald/Giemsa solution, showing over 92 % of macrophages and less than 3 % of neutrophils. Gram stain was performed, and culture for bacteria and yeast was routinely done which did not show significant growth of pathogenic microorganisms. Macrophages were harvested after 2 h incubation at 37 °C and 5 % CO_2 for exclusion of nonadherent cells.

2.2.4.3 Blood leukocytes

Blood monocytes and PMNs were isolated from blood buffy coats of healthy donators as described before (Macey et al., 1995). 30 mL of blood, diluted 1:6 pyrogen-free PBS, was layered over 15 mL of lymphocyte separation medium (PAA, Pasching, Austria) and centrifuged at 1600 rpm for 20 min at room temperature. Mononuclear cells at the upper interface were collected and incubated at 37 °C and 5 % CO_2 for 2 h for exclusion of lymphocytes. The pellets containing PMNs were collected into a cylinder filled up with polyvinyl alcohol (PVA) to a total volume of 50 mL, and mixed well with a pipette. The supernatant was harvested from the cylinder after 20 min and centrifuged at 1600 rpm for 5 min at room temperature. The pellets were collected and suspended gently with 5ml of dH_2O for 45 s, then mixed with 5mL of pre-warmed 2 x PBS. PMNs were obtained after centrifuging at 1600 rpm for 5 min at room temperature. Determined by May-Grünwald/Giemsa stain, the purity of monocytes and PMNs obtained above is more than 90% and 92%, respectively.

2.2.4.4 Cell stimulation

A549 cells (5 x 10^5 cells/mL), PMNs (2.5 x 10^6 cells/mL) and monocytes/macrophages (1 x 10^6 cells/mL) were infected with *S. pneumoniae* (10 and/or 100 CFU/cell) and growth medium was replaced by an antibiotic-free medium (RPMI 1640 plus 10 % FCS). Inhibition experiments were carried out by 1 h pretreatment with the p38 MAPK inhibitor SB 203580 (20 µM; Calbiochem, CA, USA) and p44/42 MAPK inhibitor UO126 (10µM, Calbiochem) before stimulation.

2.2.5 *In situ* hybridization (ISH)

After overnight fixation at 4 °C in the HOPE-solution, sections of 4µm were cut, mounted on Superfrost$^+$ slides and deparaffinized with isopropanol and 70 % acetone for 1 h at 4 °C. Then lung specimens were dehydrated with acetone for 30 min for 6 times at 4 °C, followed by two incubations in isopropanol (10 min at 60 °C, 2 min at 60 °C) and air dryed. Rehydration was achieved by incubation in 70 % (v/v) acetone for 10 min at 4 °C, diethylpyrocarbonate (DEPC)-treated water for 10 min at 4 °C, and slides were air dried. An ISH probe targeting *S. pneumoniae* was kindly provided by Dr. Goldmann (Research Center Borstel, Germany), and hybridization was performed overnight at 46°C in moist chambers. Hybridization solution was composed of 2 ng/mL fresh denatured probe, 250 g/mL yeast tRNA, 0.1% SDS, and 50 % formamide in PBS. Slides were washed by the following steps: 2-SSC twice for 10 min at ambient temperature, then 0.2-SSC twice for 30 min at 50 °C. The specimens were then incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche, Mannheim, Germany) using new fuchsin as a chromogen. Lung specimens were then counterstained with hematoxylin. Enumeration of the infection rates of lung cells (infected cells/ total cells x 100 %) was done by two independent investigators.

2.2.6 Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from homogenised lung tissues using the NucleoSpin RNA II kit (Macherey-Nagel, Dueren, Germany) and reverse transcribed into cDNA using

First-Strand cDNA Synthesis Kit (Roche) for RT-PCR. PCR amplification was performed using the LightCycler® Detection System (Roche Molecular Biochemicals). Semi-quantification of TLR2 mRNA (forward: CCA TTC CCC AGC GCT TT; reverse: CCG CTG AGC CTC GTC CAT) and TLR4 mRNA (forward: AAG AAA TTA GGC TTC ATA AGC T; reverse: ACC CTT TCA ATA GTC ACA CTC A) expression was performed against the endogenous control 18S rRNA gene (forward: TCA AGA ACG AAA GTC GGA GG; reverse: GGA CAT CTA AGG GCA TCA CA). PCR was run using the following protocol: 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 10 s, yielding the product sizes of 200 bp (TLR2), 200 bp (TLR4), and 300 bp (18S rRNA) respectively. In a dissociation protocol single peaks were confirmed in each of the amplified sequences to exclude non-specific amplification. For the relative quantification crossing point (cp) values of targets were expressed as $2^{-\Delta\Delta cp}$ (fold) as described previously (Winer et al. 1999). The PCR products were analyzed on 1.5 % agarose gels, stained with ethidium bromide, and subsequently visualized. To assure the identity of the PCR-amplified fragments, the size of each amplified mRNA fragment was compared with the standard 1Kb ladder.

2.2.7 Western blot assay

Lung tissues were stimulated with *S. pneumoniae* (10^7 CFU/mL) and cells including A549 cells, alveolar macrophages, and peripheral blood leukocytes were infected with *S. pneumoniae* at a bacterium-to-cell ratio of 10:1 and/or 100:1. The lung tissues were homogenized in a tissue lyser and cells were harvested by scrapers after stimulation. Lung homogenates and cell pellets were lysed in lysis buffer (125mM Tris, pH 6.8, 4 % SDS, 20 % glycerol, 100mM dithiothreitol, and 0.05 % bromophenol blue) and heated for 5 min at 95 °C. Electrophoresis was performed at 200 V for 1 h with 12 % SDS-PAGE at room temperature. Proteins were transferred to nitrocellulose membrane at 75 V for 1.5 h by wet blot at 4 °C in Mini-Protean Ⅱ (Bio-Rad, Munich, Germany). The membrane was then blocked with 5 % non-fat dried milk in T-TBS for 1 h, washed three times with T-TBS and incubated with the primary antibody (p-p38, p-p44/42; Cell Signaling Technology, Beverly, USA) at 4 °C overnight. The blots were washed three times with T-TBS and incubated for 1 h with HRP-conjugated goat-anti-rabbit IgG antibody (Cell Signaling Technology) at room temperature. Immunoreactive bands were developed using an ECL chemiluminescent substrate (Amersham, Freiburg, Germany). Autoradiography was performed with exposure times of 30 s-15 min, whichever were adequate for visualization. In all experiments, β-actin (Cell Signaling) was detected simultaneously to confirm equal protein load.

2.2.8 Macrophage depletion experiments

Clodronate/liposomes, obtained from Dr. N. van Rooijen (Vrije University, Amsterdam, Netherlands), have been reported to be successfully used in animal models for the depletion of macrophages. Preliminary experiments showed that 24 h Clodronate/liposomes coincubation resulted in over 85 % reduction of alveolar macrophages *in vitro* whereas they exhibited no significant effect on an epithelial cell line (A549 cells), proving their specificity of phagocytic cells. In addition, there is no significant difference in cytokine release from pneumococci infected tissues between medium and PBS/liposomes pretreatment (Tab. 3). Therefore, tissue pretreated with PBS/liposomes was selected as a negative control in further macrophage depletion experiments. Lung tissue (1 cm^3) was incubated with 1 mL of Clodronate/liposomes-RPMI 1640 mixture (1:1; containing 2.5 mg Clodronate), PBS/liposomes-RPMI 1640 mixture (1:1) for 24 h and 48 h respectively. To enhance the interaction between alveolar macrophages and Clodronate/liposomes, part of reagents was injected into lung tissue by a microlance. After washing with RPMI 1640, lung specimens were put into the wells of 24-well culture plates (Biochrom) and incubated with pneumococcal suspensions (10^7 CFU/mL) for further 24 h. The supernatants were collected and stored at -70° C until detected. Lung specimens were harvested and fixed at 4 °C in HOPE-solution until pathological examination.

Tab. 3 Inflammatory cytokine release from infected lung tissues pretreated with medium, PBS/liposomes and Clodronate/liposomes for 24 h (A) and 48 h (B) respectively (n=3). Pre/Sti: Pretreatment/Stimulation; PBS-lipo: PBS/liposomes; Clo-lipo: Clodronate/liposomes; Sp: *S. pneumoniae*. **p* <0.05 *vs* medium/Sp treated tissue.

A. 24 h pretreatment

Pre/Sti	IL-8 (ng/mL)	TNF-α (pg/mL)	IL-6 (ng/mL)
Medium/*Sp*	416±43	526±152	1470±766
PBS-lipo/*Sp*	443±259	722±115	1282±817
Clo-lipo/*Sp*	541±63	167±110*	517±48

B. 48 h pretreatment

Pre/Sti	IL-8 (ng/mL)	TNF-α (pg/mL)	IL-6 (ng/mL)
Medium/*Sp*	325±81	597±278	1919±1099
PBS-lipo/*Sp*	507±174	615±184	2362±1413
Clo-lipo/*Sp*	190±24	<16*	285±96

2.2.9 Inhibition experiments in the ASIM

Lung specimens (1 cm^3 size) were pretreated for 1 h with the functional TLR antibodies (anti-TLR2: 5 µg/mL, anti-TLR4: 5 µg/mL; eBioscience, San Diego, USA) and MAPK inhibitors (SB203580: 20 µM, UO126: 10 µM; Calbiochem), then stimulated with *S. pneumoniae* (10^7 CFU/mL). The working concentrations of functional TLR2, 4 antibodies and MAPK inhibitors adopted in this experiment have been proved to efficiently block TLRs or MAPK signalings respectively in our previous work and other studies (Lien et al., 1999; Rupp et al., 2004; Droemann et al., unpublished). The supernatants were harvested 4 h and 24 h after pneumococcal stimulation, and stored at -70 ° C until ELISA.

2.2.10 Enzyme-linked immunosorbent assay (ELISA)

For the quantitative determination of IL-8, IL-6 and TNF-a, we performed ELISA assays as described by the manufacturer (R&D Systems, Minneapolis, USA). The detection limits were 1.2 pg/mL for IL-8, 9.4 pg/mL for IL-6, 15.6 pg/mL for TNF-a. Assay procedure was briefly as follows: Dilute the capture antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate with 100 µL of the diluted capture antibody per well. Seal the plate and incubate overnight at room temperature. Aspirate each well and wash with wash buffer (300µL) for three times. After the last wash, remove any remaining wash buffer by inverting the plate and blotting it against clean paper towels. Block plates by adding 300 µL of block buffer to each well. Incubate at room temperature for a minimum of 1 h, followed by the aspiration/washing step. Add 100 µL of sample or standards in diluent reagent per well, and incubate 2 h at room temperature, followed by the aspiration/washing step. Add 100 µL of the detection antibody, diluted in diluent reagent, to each well and incubate 2 h at room temperature, followed by the aspiration/washing step. Add 100 µL of the working dilution of Streptavidin-HRP to each well and incubate for 20 min in dark at room temperature, followed by the aspiration/washing step. Add 100 µL of substrate solution to each well and incubate for 20 min in dark at room temperature. Add 50 µl of stop solution (1 M H_2SO_4) to each well, followed by tapping gently the plate to ensure thorough mixing. Determine the optical density of each well immediately, using a microplate reader set to 450 nm (Photometer-340, SLT, Salzburg, Austria).

2.2.11 Statistical analysis

Data are presented as the mean ± SEM. For independent samples, One-way ANOVA with Posthoc tests by LSD was used for statistical analysis of the differences between groups. A p value <0.05 was considered statistically significant. Calculations were carried out with SPSS for Windows software program11.5.

3. Results

3.1 Detection of *S. pneumoniae* in the ASIM

ISH analysis showed that S. *pneumoniae* DNA was detected in 80-90% of AMs and 15-30% of AECs showing the morphologic characteristics of type II cells 24 h after stimulation. However, bronchial epithelial cells (BECs) were only sporadically infected (<1%) (Fig. 5).

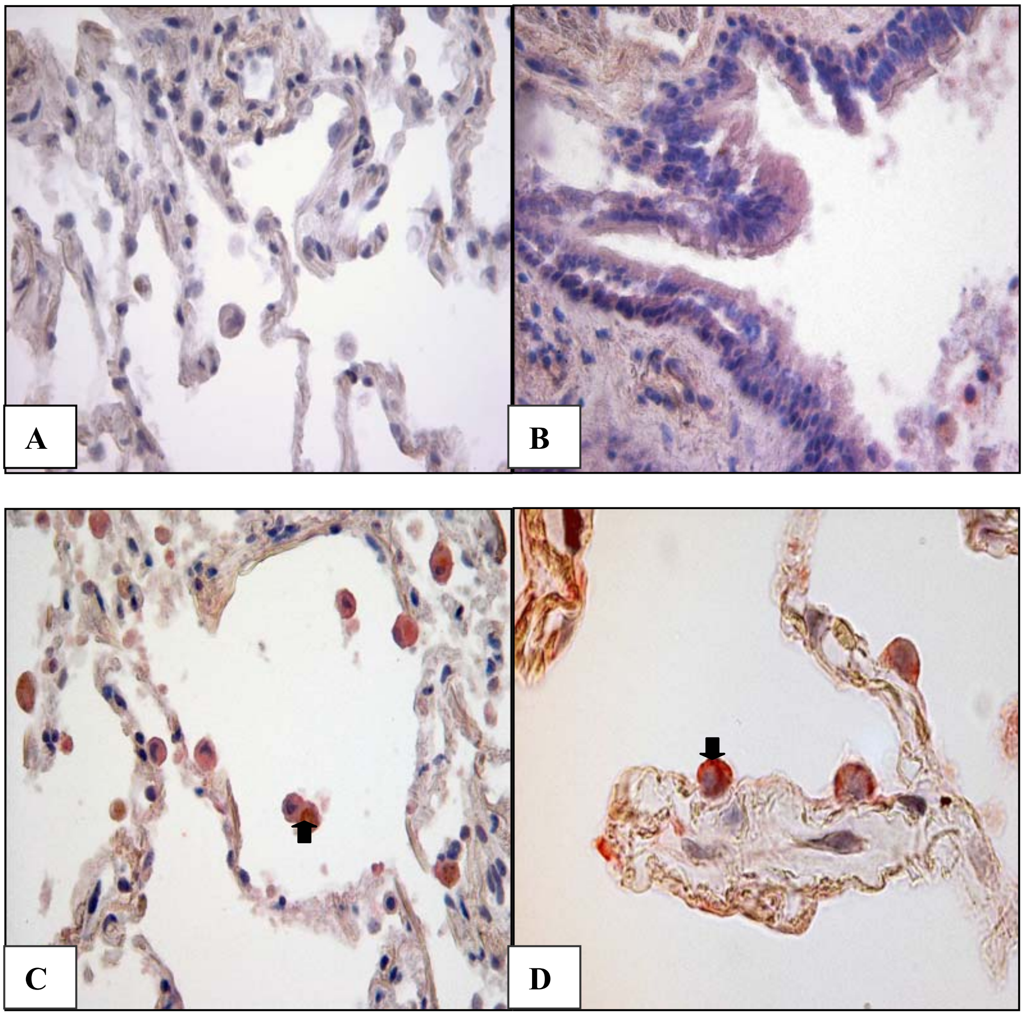


Fig. 5: ISH analysis of *S. pneumoniae* DNA in ASIM 24 h after stimulation. The human lung tissue without stimulation was used as a negative control (A: 400 x). Pneumococcal infection was sporadically found in BECs (B: 400 x). The presence of *S. pneumoniae* DNA was observed to be mainly located in AMs (C; 400 x, arrow) and AECs type II (D; 1000 x, arrow) in infected lung tissue 24 h after stimulation (n=26)

3.2 Macrophage depletion resulted in decreased inflammation in the ASIM

As shown in Methods 2.2.8, Clodronate/liposomes have the ability to deplete alveolar macrophages *in vitro*. In addition, neutrophils and epithelial cells cultured in the presence of Clodronate/liposomes were not affected (Van Rooijen and Sanders, 1994; our unpublished results). The macrophage-depleted lung tissue model was created using Clodronate/liposomes method. In the present study, Clodronate/liposomes inhibited the release of inflammatory mediators (TNF-α, IL-8 and IL-6) from infected lung tissue in a time-dependent manner. 48 h Clodronate/liposomes pretreatment fully ablated TNF-a production in infected lung tissue (Fig. 6). These date indicate that Clodronate/liposomes treatment results in decreased inflammation by suppressing macrophages in the ASIM, given the fact that pulmonary macrophages, not epithelial cell and neutrophil granulocytes, are the main cellular source of TNF-α (Knapp et al., 2004).

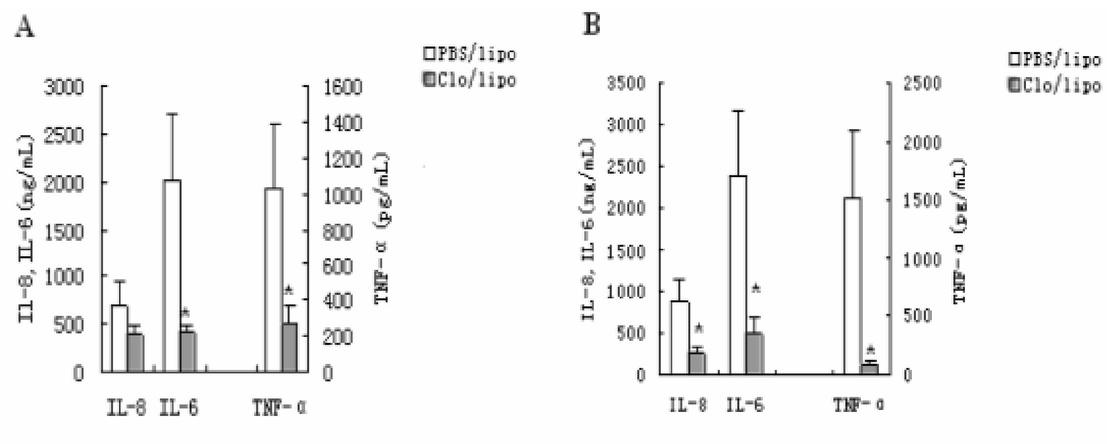


Fig. 6: Lung specimens were pretreated with PBS/liposomes or Clodronate/liposomes for 24 h (A, n=6) and 48h (B, n=5) respectively before 24 h *S. pneumoniae* infection. Clodronate/liposomes significantly inhibited the production of TNF-α, IL-8 and IL-6 in the ASIM in a time-dependent manner, suggesting macrophages hold an important role in the pulmonary inflammatory response. Especially, 48 h Clodronate/liposomes pretreatment fully ablated TNF-α production in infected lung tissue, indicating macrophage specificity of Clodronate/liposomes. PBS/lipo: PBS/liposomes; Clo/lipo: Clodronate/liposomes; Sp: *S. pneumoniae*. **p* <0.05 *vs* PBS/lipo-pretreated, Sp infected tissue.

3.3 RT-PCR analysis of TLR2 and TLR4 mRNA in the ASIM

Compared to non-infected lung specimens, *S. pneumoniae* stimulation resulted in a 2.9-fold increase in TLR2 mRNA expression 24 h postinfection. Similarly, there was a 2.7-fold increase in the expression of TLR4 mRNA in response to pneumococcal stimulation (Fig.7). These data indicate that TLR2 and TLR4 are both involved in the activation of lung cells in the ASIM upon pneumococcal infection.

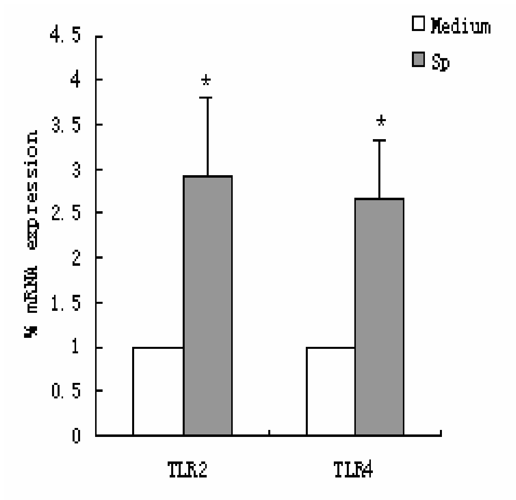


Fig. 7: Significantly enhanced TLR2 and TLR4 mRNA expression was detected by RT-PCR in lung tissue 24 h after pneumococcal infection (n=11). Sp: *S. pneumoniae*. **p* <0.05 *vs* medium-treated tissue.

3.4 Immunoblot assay of phosphorylation of p38 and p44/42 MAPKs in the ASIM and host cells

Activation of p38 MAPK is considered to participate in the regulation of pro-inflammatory cytokine expression. The significantly enhanced phosphorylation of p38 MAPK in lung tissue 4–24 h postinfection with pneumococci was observed in contrast to unchanged phospho-p44/42 expression. However, for all cell culture experiments, phosphorylation of the p38 and p44/42 MAPK was rapidly induced within 15-30 min after pneumococcal stimulation (Fig.8), suggesting that both p38 and p44/42 MAPK signalings are involved in the host cell activation upon pneumococcal infection.

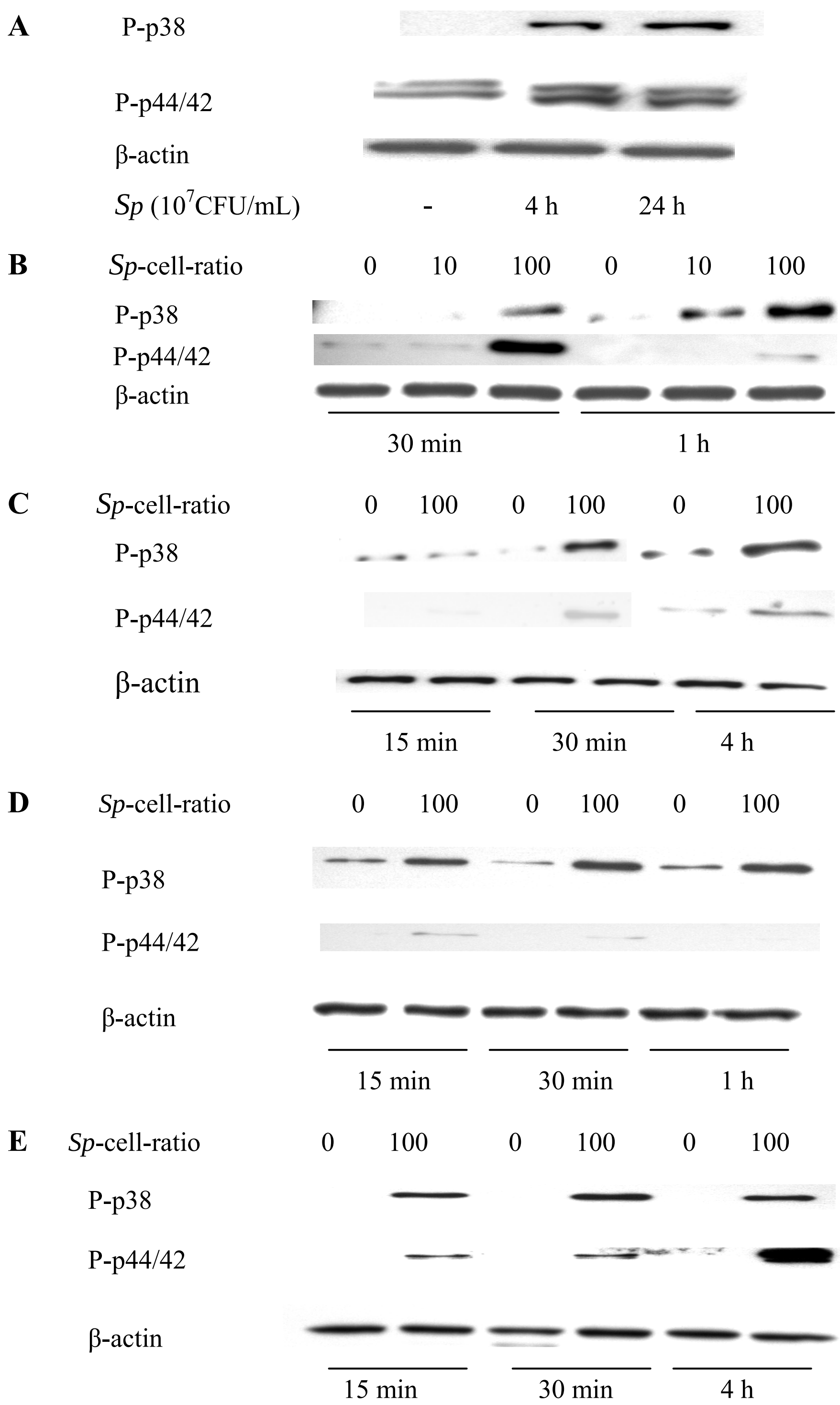


Fig.8: Immunoblot analysis of p38 and p44/42 MAPKs in the lung tissue and cells. The enhanced phosphorylation of p38 MAPK 4-24 h postinfection with pneumococci was observed in the ASIM (A). The expression of phospho-p38 and phospho-p44/42 was rapidly induced by *S. pneumoniae* 15-30 min postinfection in A549 cells (B), alveolar macrophages (C), blood monocytes (D) and PMNs (E), indicating that both p38 and p44/42 MAPKs are involved in host cell activation upon pneumococcal stimulation. Representative gels from each three independent experiments are shown as above. *Sp*: *S. pneumoniae*.

3.5 *S. pneumoniae* induced a time-dependent inflammatory response and the modulation of inflammation in the ASIM and host cells

S. pneumoniae (10^7 CFU/mL) induced a time-dependent inflammatory response within 24 h stimulation in the ASIM (Fig. 9). In order to further investigate the roles of TLR2, TLR4 and the p38, p44/42 MAPK pathways in the pulmonary inflammation, functional TLR2, 4 monoclonal antibodies, SB203580 (the p38 inhibitor) and UO126 (the p44/42 inhibitor), were adopted to block inflammatory cytokine production in the ASIM. ELISA data showed that infection of lung specimens with *S. pneumoniae* markedly enhanced the release of inflammatory cytokines after 24 h stimulation. TLR2 blockade reduced IL-8, TNF-α, and IL-6 production by 12.5 %, 29.8 %, and 23.0 % respectively without reaching statistical significance while blocking of TLR4 appeared to have no effect on pulmonary inflammatory response (Fig. 10A). Inhibition of p38 MAPK signaling by SB203580 significantly reduced inflammatory cytokine (IL-8, TNF-α, and IL-6) release from human lung tissues whereas blockade of p44/42 only inhibited the production of TNF-α, but not IL-8 and IL-6, in the ASIM (Fig. 10B).

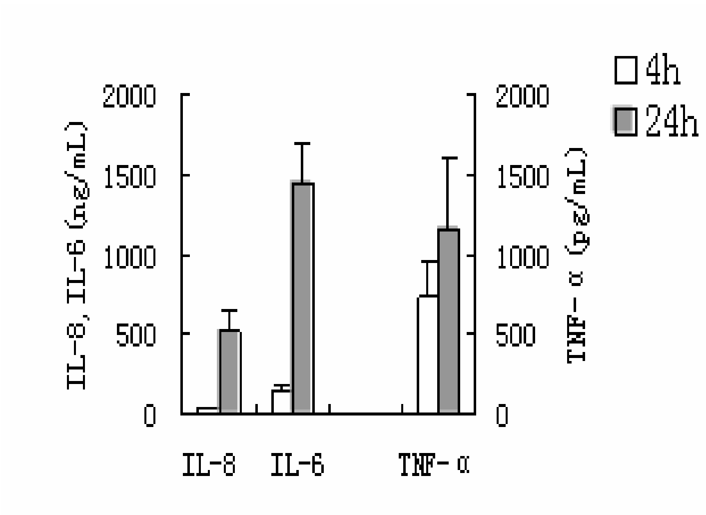


Fig. 9: *S. pneumoniae* (10^7 CFU/mL) induced a time-dependent inflammatory response within 24 h stimulation in the ASIM (n=8).

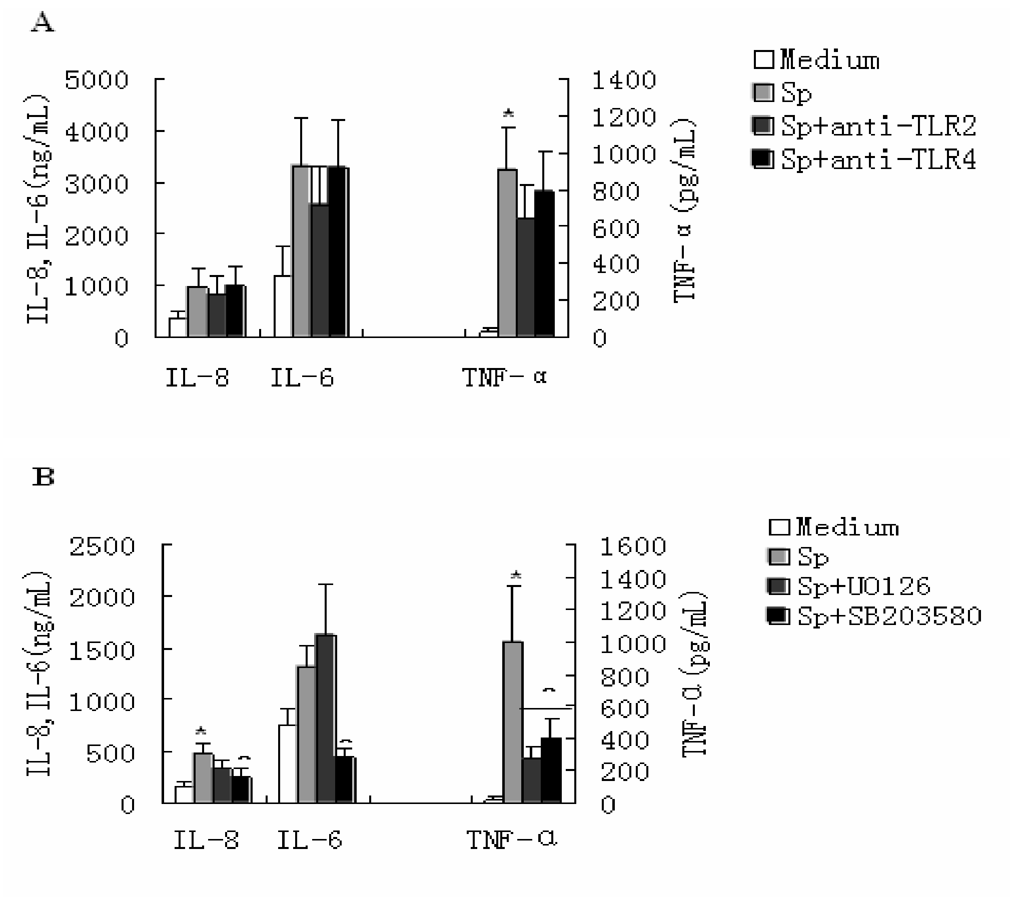


Fig. 10: The effect of anti-TLR2, 4 and MAPK inhibitors on inflammatory response in the ASIM. *S. pneumoniae* infection enhanced the release of inflammatory mediators such as IL-8, TNF-α and IL-6 from lung tissue after 24 h stimulation. Blockade of TLR2 generated a partially inhibitory effect on the production of IL-8, TNF-α, and IL-6 without reaching statistical significance whereas TLR4 blokade had no influence on pulmonary inflammation (A, n=5). The p38 MAPK inhibitor SB203580 markedly reduced the production of IL-8, TNF-α, IL-6 whereas blockade of p44/42 only inhibited the production of TNF-α, but not IL-8 and IL-6, in the ASIM (B, n=11). Sp: *S. pneumoniae*. *$p<0.05$ *vs* medium-treated tissue, ^$p<0.05$ *vs* *Sp*-treated tissue.

In order to confirm our finding from the ASIM that p38 MAPK holds a predominant role in the regulation of pneumococci-related pulmonary inflammation, we further analyzed an alveolar epithelial cell line (A549 cells), alveolar macrophages and blood leukocytes as potential sources of inflammatory cytokines, given the fact that different lung cell types contributed differentially to the overall results obtained from lung tissue. The same downregulation in inflammation by p38 inhibitor SB203580 was observed in these cell culture models whereas the p44/42 inhibitor UO126 had no significant effect on the inflammatory response (Fig. 11). Taken together, these results demonstrate that SB203580 has a potential to downregulate pulmonary inflammation upon pneumococcal infection.

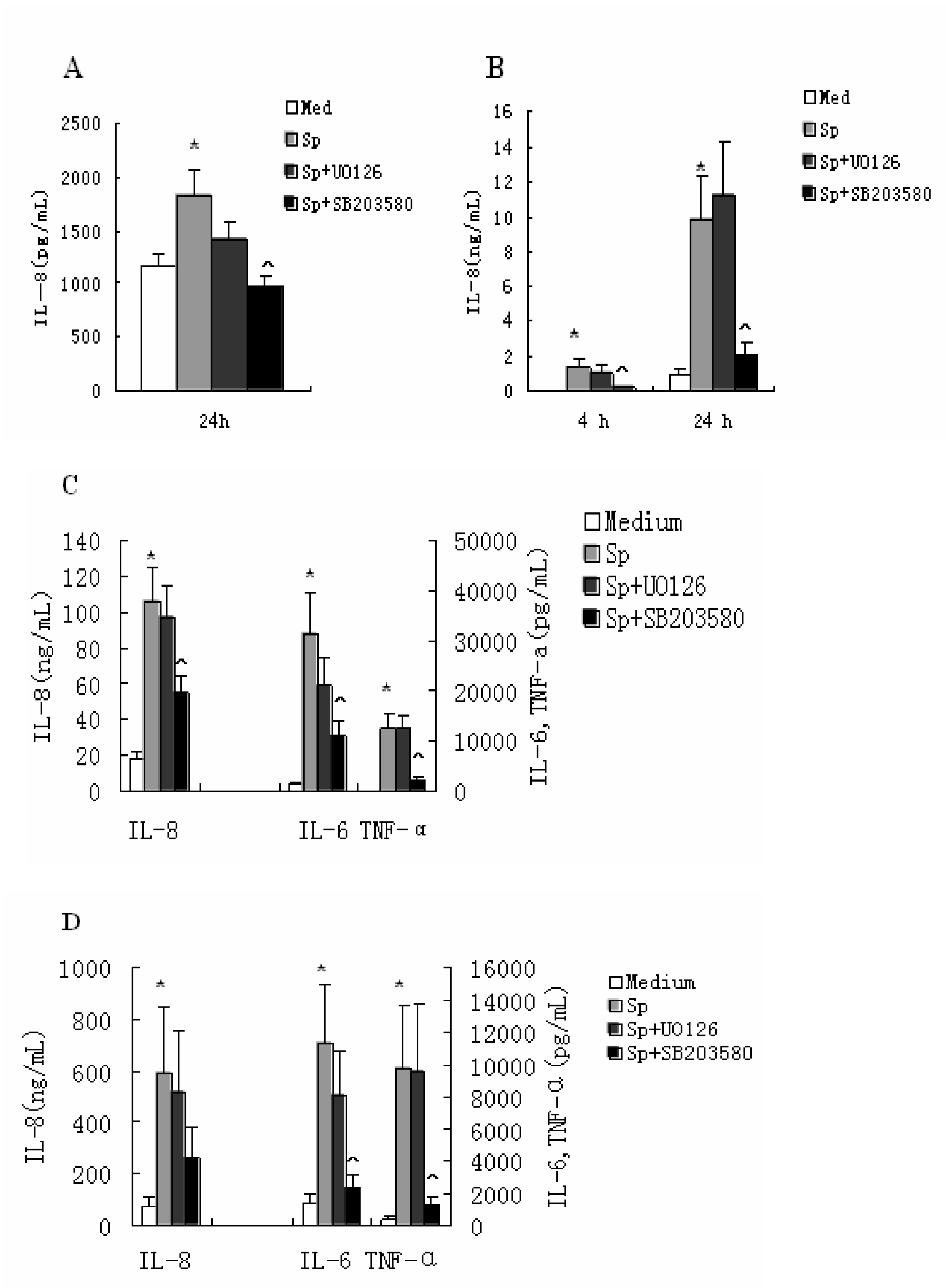


Fig. 11: The effect of p38 MAPK inhibitor on inflammatory response in different cell types. SB203580 significantly inhibits IL-8 release from A549 cells infected with *S. pneumoniae* (10 CFU/cell) (A, n=7) and PMNs stimulated with *S. pneumoniae* (100 CFU/cell) (B, n=6). The concentration of TNF-α in the supernatants of these two kinds of cells is undetectable. The proinflammatory cytokine expression was markedly suppressed by SB203580 in monocytes (C, n=7) and macrophages (D, n=8) infected with bacteria (100 CFU/cell) in contrast to UO126. *Sp*: *S. pneumoniae*. **p*<0.05 *vs* medium-treated cells, ^*p*<0.05 *vs* *Sp*-treated cells.

4. Discussion

S. pneumoniae is the most prevalent bacterial pathogen causing otitis media in children and community-acquired pneumonia in adults. Much of our knowledge about the pathogenesis of *S. pneumoniae* infection and pulmonary host defense is derived from animal studies on experimental pneumonia (Canvin et al., 1995; Rubins et al., 1995; Idanpaan-Heikkila e al., 1997; Kadioglu et al., 2000; Rijneveld et al., 2000; Wang et al., 2000; Dallaire et al., 2001; Gingles, et al., 2001; Kerr et al., 2002; Knapp et al., 2003; Dockwell et al., 2003; Knapp et al., 2004; Lysenko et al., 2005), whereas little is known about pathogen-cell interactions within the human pulmonary compartment. As lung tissue samples from patients with acute pneumococcal pneumonia are obviously not available we developed a novel model of acute *S. pneumoniae* infection to analyze the infection pattern and host cell response during acute infection, using vital lung specimens from patients undergoing lobectomy due to pulmonary nodules. This model was established according to a previously described model of short period stimulation of soft tissues (Olert et al., 2001; Goldmann et al., 2002). The tissue could be infected with pneumococci at least 48 h based on the results of LDH release. Recently, we had successfully used the same protocol to establish *in vitro* infection models with *Chlamydia pneumoniae* and nontypeable *Haemophilus influenzae* in human lung tissue (Rupp et al., 2004; Xu et al. 2005). Lung specimens after infection were treated with the recently developed HOPE-fixative, which provides an excellent preservation of proteins and antigenic structures for differential analysis by immunohistochemistry and Western blot (Goldmann et al., 2002). In addition, the most remarkable feature of HOPE is the extremely low degradation of nucleic acids leading to good results obtained by *in situ* hybridization (Olert et al., 2001; Uhlig et al., 2004).

The target cells within the lung in acute pneumococcal infection have not been well documented although they are crucial to the implicated pathology. Using various cell culture models, human pharyngeal epithelial cells, BECs, AECs type II and vascular endothelial cells have been proved as target cells for *S. pneumoniae* (Andersson et al., 1983; Cundell and Tuomanen; 1994; Cundell et al., 1995; Cundell et al., 1996; Adamou et al., 1998; N´Guessan et al., 2005). Hament et al demonstrated that pneumococci are able to adhere to human erythrocytes by which way the bacteria are transported to the reticulo-endothelial system in the spleen (Hament et al., 2003). In the present study, ISH analysis showed that *S. pneumoniae* was found to be mainly located in AMs (80-90%) and AECs II (15-30%) 24 h after infection. This indicates that AMs and AECs might be the most important host cells in primary pneumococcal infection. Surprisingly, only few BECs were infected in the ASIM. In contrast to a tumor-derived alveolar cell line (A549) and a simian virus 40-transformed bronchial epithelial cell line (BEAS-2B) (Lieber et al., 1976; Reddel et al., 1988), the infection pattern of primary lung cells more directly reflects the complex interactions between pneumococci and airway epithelia in our tissue model. An investigation from Schulz et al demonstrated that there are some differences in the activation and cytokine release of BECs and AECs type II stimulated with LPS (Schulz et al., 2002). We hypothesize that the differences of infection rates between AECs and BECs may be due to their distinct characteristics with respect to pneumococcal adherence and invasion. Indeed, unlike other respiratory pathogens such as *Haemophilus influenzae* which initiate infection in the nasopharynx and often descend into the bronchi causing bronchitis, pneumococci do not typically cause bronchial infections although they have been reported to be associated with a few cases of exacerbated chronic bronchitis (Cole 1987; Adamou et al., 1998). The primary site of colonization for pneumococci is the nasopharynx and bronchial cells may serve as transient sites for pneumococcal attachment when the pathogens pass from the nasopharynx to the lower respiratory tract (Adamou et al., 1998). Even a few investigators thought that pneumococci cannot adhere to the ciliated epithelia of the tracheo-bronchial tree (McCullers and Tuomanen, 2001).

There are increasing data showing that airway epithelia prevent colonization and invasion of *S. pneumoniae* by releasing innate antimicrobial molecules such as proinflammatory cytokines, antimicrobial peptides, neutrophil elastase, and reactive oxygen intermediates. Once an organism evades host mucociliary clearance, it must adapt to the milieu, compete for iron, and avoid professional phagocytic cells and complement (Sadikot et al., 2005).

Talbot et al demonstrated the capacity of *S. pneumoniae* to invade A549 cells. The bacteria in AECs could be protected from professional phagocytes and shielded themselves from antibiotics (Talbot et al, 1996). The uptake by epithelial cells may be helpful for *in vivo* survival of pneumococci at least in the short period. The clinical importance of this phenomenon deserves further investigations.

S. pneumoniae has been shown to induce the apoptosis of human monocytes/macrophages and neutrophil granulocytes (Zysk et al., 2000; Ali et al., 2003; Dockrell et al., 2003). Recent studies demonstrated that pneumococci caused apoptosis in lung epithelial cells in a caspase 6-dependent manner and endothelial cells in a p38 MAPK and JNK-mediated caspase-dependent way (Schmeck et al., 2004b, N´Guessan et al., 2005). These results are in line with a marked upregulation of caspase 3, a key effector protease, in A549 cells, monocytes and PMNs after 20 h of pneumococcal stimulation found in our experiments (data not shown). In addition, LDH data in the present study demonstrated that *S. pneumoniae* (encapsulated strain ATCC 6303) stimulation resulted in time-dependent necrosis of lung cells within at least 48 h in the tissue model. Similarly, Schmeck et al reported that an encapsulated pneumococcal strain caused a massive LDH release in AECs and BECs whereas unencapsulated pneumococci only induced apoptosis (Schmeck et al., 2004b). A study by Zysk et al. showed that viable wild-type pneumococci induced PMN necrosis whereas heat-inactivated bacteria caused apoptosis (Zysk et al., 2000). These results indicate that the switch from apoptosis to necrosis depends on the intensity of the stimulus. Among pneumococcal products, the cytotoxin pneumolysin and H_2O_2 have been well illustrated to contribute to apoptosis and/or necrosis in lung epithelial cells, monocytes and brain neuronal cells (Zysk et al., 2000; Braun et al., 2002; Schmeck et al., 2004b). Pneumococcus-related apoptosis and necrosis of lung cells seem to hold important roles in pneumococcal pneumonia, which needs further elucidation.

Taken together, a new ASIM was established to investigate pathogen-cell-interaction in the early stage of acute respiratory infections. Advantages of this model are listed as follows: (1) As human beings, but not mice, are natural hosts for *S. pneumoniae*, our *in vitro* ASIM provides a novel tool to investigate interactions between *S. pneumoniae* and lung host cells in the phase of acute infection, and allows us to modify environmental factors within the pulmonary compartment. (2) HOPE-fixative has been adopted in the preservation and treatment of lung specimens. It facilitates the analysis of genomic and proteomic structures of bacteria and human host cells, and provides a novel tool in the detection of respiratory pathogens in human lung tissue. (3) The protocol for establishing the ASIM is applicable to investigate respiratory pathogens other than *S. pneumoniae.* Indeed, *in vitro*-infection models with *Chlamydia pneumoniae* and nontypeable *Haemophilus influenzae* in human lung tissue were developed to investigate infection pattern (acute versus chronic infections in human lungs), surface marker expression of target cells, and inflammation modulation in our research group. (Rupp et al., 2004; Xu et al. 2005).

At the same time, some limitations in our ASIM deserve consideration. Firstly, as the *ex vivo* infected lung tissues do not remain vital for a prolonged period, we are not able to analyze the interaction between host cells and respiratory microorganisms in a subacute phase of infection using this model. In addition, the host immune response to pneumococcal pneumonia has usually been characterized as an intense inflammatory reaction, initially implicating resident AMs followed by a massive neutrophil influx into the pulmonary compartment (Kadioglu and Andrew, 2004). Unfortunately, the contribution of neutrophils to the immune response in primary *S. pneumoniae* infection cannot be investigated properly in this ASIM.

Macrophages play an essential role in pulmonary host defense. They generate antimicrobial molecules, secrete cytokines, present antigens and phagocytize foreign materials, hence contributing to specific and nonspecific immune response to combat invading microorganisms (Underhill and Ozinsky, 2002). However, the exact role of AMs in host defense against pneumococci is unclear (Dockrell et al., 2003). The infection pattern in the ASIM showed a high infection rate of AMs, indicating that AMs are the most important early immune cells and target cells upon *S. pneumoniae* infection. Clodronate is a bisphosphonate used for treatment of osteolytic bone diseases. However, phagocytic cells are killed by Clodronate through apoptosis, once the drug is delivered into phagocytic cells using liposomes as vehicles. Until now, Clodronate/liposomes have been proved to efficiently deplete AMs if they are adequately administered in a group of animal models of lung infections. (Van Rooijen and Sanders, 1994; Broug-Holub et al., 1997; Kooguchi et al., 1998; Cheung et al., 2000; Leemans et al., 2001; Dockrell et al., 2003; Knapp et al., 2003). Much of our knowledge about the role of alveolar macrophages in the pathogenesis of pneumonia is originated from animal studies of experimental pneumonia. Pulmonary macrophages, but not epithelial cells and granulocytes, have been shown to be the main cellular source of TNF-α in a mouse model (Knapp et al., 2004). The data from our cell stimulation experiments also demonstrated that TNF-α release from PMNs and A549 cells stimulated with *S. pneumoniae* was undetectable compared to higher expression of TNF-α in monocytes/macrophages. In the present study, Clodronate/liposomes were proved to have the ability to deplete human alveolar macrophages *in vitro*. Furthermore, the release of inflammatory cytokines including TNF-α from lung tissue upon pneumococcal stimulation was significantly suppressed in the ASIM pretreated with Clodronate/liposomes for over 24 h. Especially, 48 h Clodronate/liposomes treatment fully abolished TNF-α production in the ASIM. Overproduction of TNF-α has been shown to contribute to the pathogenesis of acute inflammatory states. The selective inactivation of AMs by Clodronate/liposomes resulted in a significantly decreased release of TNF-α from the lung tissue model, proving that AMs are the main source of proinflammatory cytokine production in the human lungs and hold an indispensable role in the host innate immune response against respiratory pathogens. Interestingly, an investigation from Knapp et al showed that AMs have a protective anti-inflammatory role by eliminating apoptotic PMNs in a mouse model of pneumococcal infection. The disparate findings between Knapp et al and our study might be due to the different experimental systems (mouse model *in vivo* and human lung tissue *in vitro*); the involvement of PMNs; and the complex dual function of AMs which initiate inflammatory response and recruit PMNs to alveolar space on the onset of pulmonary inflammation, and contribute to the resolution of inflammation in the final stage of inflammation.

The acute inflammatory response is a cornerstone in the innate immune response to infection. The identification of TLR family and illustration of their signal pathways provide insight as to how the airways respond to bacterial pathogens (Qureshi and Medzhitov, 2003; Koehler et al., 2004). However, knowledge of the role of TLR2 and TLR4 in host defense against respiratory tract pathogens is limited. Although some evidence from animal models showed that TLR2 is the predominant receptor involved in host immune response to *S. pneumoniae* and its cell wall components such as LTA and PGN (Yoshimura et al., 1999; Schwandner et al., 1999; Opitz et al., 2001; Schroder et al., 2003), to our knowledge, there are no definitive data confirming these observations in human lungs. TLR2 and TLR4 are constitutively expressed and are functional on alveolar macrophages and airway epithelia, indicating that they hold important roles in lung innate immune response against microorganisms. In the present study, we determined the role of TLR2, 4 and their downstream p38 and p44/42 MAPK signaling pathways in the innate immune response to *S. pneumoniae* in the novel ASIM. We found an upregulated expression of TLR2 and TLR4 mRNA in the lung tissue, followed by upregulated release of inflammatory mediators such as IL-8, IL-6 and TNF-α after pneumococcal infection. In addition, enhanced TLR2 expression on the surface of monocytes and neutrophils was observed compared to unaltered TLR4 expression after *S. pneumoniae* infection (Data not shown). In the study by Yoshimura et al, the activation of Chinese hamster ovary fibroblast cells expressing human TLR2 but not TLR4 was observed to be induced by heat-killed *S. pneumoniae* (Yoshimura et al., 1999). A recent report demonstrated that the isolated AMs from TLR2$^{-/-}$mice failed to release TNF-α and keratinocyte chemoattractant upon stimulation with heat-killed *S. pneumoniae* compared to wild-type AMs, suggesting that TLR2 is indispensable for alveolar macrophage responsiveness toward pneumococci and plays an important role in the induction of lung inflammatory response (Koedel et al., 2003). To delineate the roles of TLR2 and TLR4 in pathogen induced inflammation in human pulmonary compartment, we further performed blocking experiments using their monoclonal antibodies. TLR2 blockade showed a moderately decreased inflammation without reaching statistical difference compared to pathogen-infected lung tissue whereas TLR4 functional antibody had no effect on inflammatory response in our ASIM. This phenomenon suggests that the inflammatory cytokine response in this model is only partly mediated via TLR2 and other PRRs beyond TLR2 signaling through MyD88 are involved in host defense against Gram-positive bacteria. Indeed, MyD88$^{-/-}$, but not TLR2$^{-/-}$, mice were markedly defective in their induction of multiple splenic proinflammatory cytokine- and chemokine-specific mRNAs after intraperitoneal challenge with heat-killed *S. pneumoniae* (Khan et al., 2005). Similar results were reported by Takeuchi et al who found that the production of TNF-a and IL-6 by peritoneal macrophages in response to heat-killed *S. aureus* was completely absent in MyD88$^{-/-}$ mice and only reduced in TLR2$^{-/-}$ macrophages, indicating a more definitive proinflammatory signaling defect conferred by MyD88 deficiency (Takeuchi, et al., 2000a).

P38 MAPK is a key element in inflammatory response by regulating the production of proinflammatory cytokines, and modulating inflammation-related intracellular enzymes and adhesion molecules (Perregaux et al., 1995; Pietersma et al., 1997; Guan et al., 1998; Rupp et al., 2004; N'Guessan et al., 2006). But little is known of the role of p38 pathways in *S. pneumoniae*-induced human cell and tissue responses. A study by Monier et al. illustrated that p38 MAPK is a key signaling pathway in the upregulation of iNOS and TNF expreesion in murine macrophages stimulated with antibiotic-killed pneumococci and pneumococcal cell wall preparations (Monier et al., 2002). Schmeck et al reported that p38 MAPK was rapidly activated and phosphorylated in the lung of mice and human bronchial epithelial cell line BEAS-2B when exposed to *S. pneumoniae.* Pneumococci activated NF-κB-dependent gene transcription in HEK293 cells and induced IL-8 and GM-CSF expression in BEAS-2B cells via p38 MAPK signaling pathway (Schmeck et al., 2004a). A recent investigation showed that pneumococci induced Cox-2 expression and subsequent prostaglandin E_2 (PGE_2) synthesis via p38 MAPK and NF-κB in lung epithelial cells. Furthermore, the recruitment of NF-κB subunit p65 to the Cox-2 promoter depended on p38 activation (N'Guessan et al., 2006). Our data showed that phosphorylation and upregulation of p38 MAPK in the ASIM was detectable after 4 h infection and increased up to 24 h in contrast to unstimulated normal lung tissue. We also found that phospho-p38 and phospho-p44/42 MAPKs were rapidly increased in *in vitro* cell models such as blood leucocytes, resident macrophages and A549 cells within 15-30 min upon stimulation and persisted to 1 h or 4 h respectively, suggesting that both p38 and p44/42 signaling pathways are implicated in pneumococci-related lung cell activation. The blockade of p38 MAPK using SB203580 markedly inhibited the release of proinflammatory cytokines such as IL-8, TNF-α, and IL-6 from lung tissue. The same downregulation of inflammation by the p38 inhibitor was proved in *in vitro* cell models. These results are in line with the finding from Schmeck et al that p38 MAPK plays an important role in pneumococci-induced inflammatory cytokines transcription by modulating p65 NF-κB-mediated transactivation (Schmeck et al., 2004a). *In vivo* experiments deserve to be performed to verify the efficacy of p38 inhibitors which may aid in the identification of novel therapeutic intervention strategies to attenuate the pathology induced by pneumococcal infection. In addition, UO126, the p44/42 MAPK inhibitor, suppressed TNF-α production in the tissue model whereas it had no influence on the release of IL-8 and IL-6. But data from *ex vivo* cell models showed UO126 exerted no inhibitory effect on pneumococci-induced release of TNF-α, IL-8 and IL-6 from monocytes /macrophages. The reasons for the differential effects of UO126 on TNF-α production between the tissue and cell model are not clear. GM-CSF produced by tissue cells is increased in inflammation and participates in the inflammatory response by direct activation of leukocytes and resident macrophages at the local site of infection (Chung and Barnes, 1999). As p44/42 MAPK is rapidly phosphorylated and activated in response to stimulation with GM-CSF (Welham et al., 1992), we extrapolate that some cytokines such as GM-CSF released from airway epithelium upon *S. pneumoniae* are able to stimulate and strengthen p44/42 MAPK signaling in AMs within pulmonary compartment while there is no ample stimulation of these cytokines in the environment of *in vitro* AM culture.

TLRs and their downstream MAPK signalings have considerable implications with regard to innate immune responses and disease pathogenesis as central mediators. Identification of all relevant TLR ligands, combined with a comprehensive understanding of the pathways involved in pneumococcal induction of inflammation and their cross-talk could deepen our understanding of host-pathogen-interaction and lead to more specific agents to be used in pneumococcal pneumonia. Inflammation is an important hallmark of pneumonia. Although appropriate inflammatory response is beneficial to bacterial clearance, inflammation itself can damage host cells that in turn stimulate inflammation if not properly mounted (Nathan, 2002). Uncontrolled host defenses and inflammatory responses have been shown to contribute to pulmonary disorders such as ARDS and systemic sepsis. For efficient control of airway inflammation, a fine balance between the need for clearance of the inflammatory stimulus and the risk of lung injury is important (Droemann et al., 2000). Modulating inflammation using drugs targeting multiple molecules or key inflammatory pathways could be effective, but this concept will need to be weighted against the risk of impairing the innate immune response (Koehler et al., 2004). Therefore, the combination of adjuvant immunotherapy that downregulates or upreguletes inflammation as appropriate and conventional treatment with powerful antibiotics may provide an innovative way to treat severe bacterial pneumonia (Cazzola et al., 2005). In fact, the success from the use of dexamethasone to inhibit inflammatory response in the treatment of infants and children with bacterial meningitis has set a good example that intentionally downmodulating the host response during the early phase of antibiotic therapy has a beneficial effect on the outcome of infectious diseases (Lebel et al., 1988). Further studies on the mechanisms and pathways of lung inflammation will increase the understanding of respiratory physiology and pathology and potentially develop novel diagnostic markers and therapeutic strategies.

Summary

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia. The lung plays an important role in initiating innate immune responses and clearing microbes. However, little is known concerning *S. pneumoniae*-host cell interactions within the human pulmonary compartment. Toll-like receptor (TLR) 2 is a key pattern recognition receptor for pneumococci-related cell activation. Mitogen-activated protein kinases (MAPKs) are involved in TLR-mediated signal transduction and contribute to nuclear factor-κB transactivation and inflammatory cytokine expression. In the present study, we established a novel model of acute *S. pneumoniae* infection (ASIM) in vital lung specimens from pulmonary lobectomy. *In situ* hybridization analysis showed that S. *pneumoniae* DNA was detected in 80-90% alveolar macrophages and 15-30% of alveolar epithelial cells type II 24h after stimulation (n=26). The lung tissue depleted with Clodronate/liposomes exhibited a decreased TNF-α release upon pneumococcal infection. In addition, enhanced phosphorylation of p38 MAPK and increased TLR2 and 4 mRNA expression were observed in infected lung tissues using Western blot and RT-PCR. Inhibition of p38 MAPK significantly reduced inflammatory cytokine release from lung tissue in contrast to TLR2 and TLR4 blockade. To confirm findings from the ASIM, we further analyzed lung epithelial cells, alveolar macrophages and blood leukocytes as potential sources of inflammatory cytokines and demonstrated that p38 MAPK is a key element in the inflammatory response to intact pneumococci.

Altogether, alveolar macrophages are the important host cells in the human ASIM and are the main source of proinflammatory cytokine release. TLR2 appears to be implicated in cell activation although blockade of TLR2 only elicits a slight decrease of inflammatory response. P38 MAPK holds a major role in the pneumococci-induced pulmonary inflammation and could become a potential molecular target to modulate lung inflammation.

References

Adamou JE, Wizemann TM, Barren P, Langermann S: Adherence of *Streptococcus pneumoniae* to human bronchial epithelial cells (BEAS-2B). Infect Immun 66, 820-822 (1998)

Akira S, Takeda K: Toll-like receptor signalling. Nat Rev Immunol 4, 499-511 (2004)

Ali F, Lee ME, Iannelli F, Pozzi G, Mitchell TJ, Read RC, Dockrell DH: *Streptococcus pneumoniae*-associated human macrophage apoptosis after bacterial internalization via complement and Fc gamma receptors correlates with intracellular bacterial load. J Infect Dis 188, 1119-1131 (2003)

Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, Klimpel GR, Godowski P, Zychlinsky A: Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor 2. Science 285, 736-739 (1999)

Andersson B, Dahmen J, Frejd T, Leffler H, Magnusson G, Noori G, Eden CS: Identification of an active disaccharide unit of a glycoconjugate receptor for pneumococci attaching to human pharyngeal epithelial cells. J Exp Med 158, 559-570 (1983)

Appelbaum PC: Epidemiology and *in vitro* susceptibility of drug-resistant *Streptococcus pneumoniae*. Pediatr Infect Dis J 15, 932-934 (1996)

Armstrong L, Medford AR, Uppington KM, Roberston J, Witherden IR, Tetley TD, Millar AB: Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells. Am J Respir Cell Mol Biol 31, 241-245 (2004)

Austrian R: The pneumococcus at the millennium: not down, not out. J Infect Dis 179 (Suppl 2), S338-341 (1999)

Avery OT, Heidelberger M: Immunological relationships of cell constituents of pneumococcus. J Exp Med 42, 367-376 (1925)

Avery OT, Morgan HJ: Immunological reactions of the isolated carbohydrate and protein of pneumococcus. J Exp Med 42, 347-353 (1925)

Badger AM, Bradbeer JN, Votta B, Lee JC, Adams JI, Griswold DE: Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. J Pharmacol Exp Ther 279, 1453-61 (1996)

Barton GM, Medzhitov R: Toll-like receptor signaling pathways. Science 300, 1524-1525 (2003)

Benton KA, Everson MP, Briles DE: A pneumolysinnegative mutant of *Streptococcus pneumoniae* causes chronic bacteraemia rather than acute sepsis in mice. Infect Immun 63, 448-455 (1995)

Berry AM, Lock RA, Hansman D, Paton JC: Contribution of autolysin to virulence of *Streptococcus pneumoniae*. Infect Immun 57, 2324-2330 (1989)

Berry AM, Alexander JE, Mitchell TJ, Andrew PW, Hansman D, Paton JC: Effect of defined point mutations in the pneumolysin gene on the virulence of *Streptococcus pneumoniae*. Infect Immun 63, 1969-1974 (1995)

Berry AM, Ogunniyi AD, Miller DC, Paton JC: Comparative virulence of Streptococcus pneumoniae strains with insertion-duplication, point, and deletion mutations in the pneumolysin gene. Infect Immun 67, 981-985 (1999)

Berry AM, Paton JC: Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. Infect Immun 68, 133-140 (2000)

Blasi F, Tarsia P, Aliberti S: Strategic targets of essential host-pathogen interactions.

Respiration 72, 9-25 (2005)

Branger J, Knapp S, Weijer S, Leemans JC, Pater JM, Speelman P, Florquin S, van der Poll T: Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. Infect Immun 72, 788-794 (2004)

Braun JS, Sublett JE, Freyer D, Mitchell TJ, Cleveland JL, Tuomanen EI, Weber JR: Pneumococcal pneumolysin and H_2O_2 mediate brain cell apoptosis during meningitis. J Clin Investig 109, 19-27 (2002)

Broug-Holub E, Toews GB, van Iwaarden JF, Strieter RM, Kunkel SL, Paine R 3rd, Standiford TJ: Alveolar macrophages are required for protective pulmonary defenses in murine Klebsiella pneumonia: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. Infect Immun 65, 1139-1146 (1997)

Canvin JR, Marvin AP, Sivakumaran M, Paton JC, Boulnois GJ, Andrew PW, Mitchell TJ：The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type 2 pneumococcus. J Infect Dis 172, 119-123 (1995)

Catterall JR: *Streptococcus pneumoniae*. Thorax 54, 929-937 (1999)

Cazzola M, Matera MG, Pezzuto G: Inflammation-a new therapeutic target in pneumonia. Respiration 72, 117-126 (2005)

Cheung DO, Halsey K, Speert DP: Role of pulmonary alveolar macrophages in defense of the lung against *Pseudomonas aeruginosa*. Infect Immun 68, 4585-4592 (2000)

Chung KF, Barnes PJ: Cytokines in asthma. Thorax 54, 825-857 (1999)

Cobb MH, Goldsmith EJ: How MAP kinases are regulated. J Biol Chem 270, 14843-14846 (1995)

Cockeran R, Theron AJ, Steel HC, Matlola NM, Mitchell TJ, Feldman C, Anderson R: Proinflammatory interactions of pneumolysin with human neutrophils. J Infect Dis 183, 604-611 (2001)

Cole PJ: Significance of *Haemophilus influenzae* and other microorganisms for the pathogenesis and therapy of chronic respiratory infection. Infection 15 (Suppl 3), S99–102 (1987)

Cox G, Crossley J, Xing Z: Macrophages engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation in vivo. Am J Respir Cell Mol Biol 12, 232-237 (1995)

Cundell DR, Tuomanen EI: Receptor specificity of adherence of *Streptococcus pneumoniae* to human type-II pneumocytes and vascular endothelial cells *in vitro*. Microb Pathog 17, 361-374 (1994)

Cundell DR, Pearce BJ, Sandros J, Naughton AM, Masure HR: Peptide permeases from *Streptococcus pneumoniae* affect adherence to eucaryotic cells. Infect Immun 63, 2493-2498 (1995)

Cundell DR, Gerard C, Idanpaan-Heikkila I, Tuomanen EI, Gerard NP: PAF receptor anchors *Streptococcus pneumoniae* to activated human endothelial cells. Adv Exp Med Biol 416, 89-94 (1996)

Dallaire F, Ouellet N, Bergeron Y, Turmel V, Gauthier MC, Simard M, Bergeron MG: Microbiological and inflammatory factors associated with the development of pneumococcal pneumonia. J Infect Dis 184, 292-300 (2001)

Davis RJ: The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 268, 14553-14556 (1993)

Dockrell DH, Marriot HM, Prince LR, Ridger VC, Ince PG, Hellewell PG, Whyte MK; Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection. J Immunol 171, 5380-5388 (2003)

Droemann D, Aries SP, Hansen F, Moellers M, Braun J, Katus HA, Dalhoff K: Decreased apoptosis and increased activation of alveolar neutrophils in bacterial pneumonia. Chest 117, 1679-1684 (2000)

Droemann D, Goldmann T, Branscheid D, Clark R, Dalhoff K, Zabel P, Vollmer E: Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung. Histochem Cell Biol 119, 103-108 (2003)

Dziarski R, Jim YP, Gupta D. Differential activation of extracellular signal-regulated kinase (ERK) 1, ERK 2, p38, and c-Jun NH2-terminal kinase mitogen-activated protein kinases by bacterial peptidoglycan. J Infect Dis 174, 777-785 (1996)

Echchannaoui H, Frei K, Schnell C, Leib SI, Zimmerli W, Landmann R: Toll-like receptor 2-deficient mice are highly susceptible to *Streptococcus pneumoniae* meningitis because of reduced bacterial clearing and enhanced inflammation. J Infect Dis 186, 798-806 (2002)

Fedson DS, Scott GA: The burden of pneumococcal disease among adults in developed and developing countries: what is and is not known. Vaccine 17 (Suppl 1), S11-18 (1999)

Gao JJ, Xue Q, Zuvanich EG, Haghi KR, Morrison DC: Commercial preparations of lipoteichoic acid contain endotoxin that contributes to activation of mouse macrophages *in vitro*. Infect Immun 69, 751-757 (2001)

Garcia J, Lemercier B, Roman-Roman S, Rawadi G: A Mycoplasma fermentans-derived synthetic lipopeptide induces AP-1 and NF-κB activity and cytokine secretion in macrophages via the activation of mitogen-activated protein kinase pathways. J Biol Chem 273, 34391-34398 (1998)

Geelen S, Bhattacharyya C, Tuomanen E: The cell wall mediates pneumococcal attachment to and cytopathology in human endothelial cells. Infect Immun 61, 1538-1543 (1993)

Gillespie SH, Balakrishnan I: Pathogenesis of pneumococcal infection. J Med microbial 49, 1057-1067 (2000)

Gingles NA, Alexander JE, Kadioglu A, Andrew PW, Kerr A, Mitchell TJ, Hopes E, Denny P, Brown S, Jones HB, Little S, Booth GC, McPheat WL: Role of genetic resistance in invasive pneumococcal infection: identification and study of susceptibility and resistance in inbred mouse strains. Infect Immun 69, 426-434 (2001)

Goldmann T, Wiedorn KH, Kuhl H, Olert J, Branscheid D, Pechkovsky D, Zissel G, Galle J, Muller-Quernheim J, Vollmer E: Assessment of transcriptional gene activity *in situ* by application of HOPE-fixed, paraffin-embedded tissues. Pathol Res Pract 198, 91-95 (2002)

Guan Z, Buckman SY, Pentland AP, Templeton DJ, Morrison AR: Induction of cyclooxygenase-2 by the activated MEKK1→SEK1/MKK4→p38 mitogen-activated protein kinase pathway. J Biol Chem 273, 12901-12908 (1998)

Guillot L, Medjane S, Le-Barillec K, Balloy V, Danel C, Chignard M, Si-Tahar M: Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. J Biol Chem 279, 2712-2718 (2004)

Hacker H, Vabulas RM, Takeuchi O, Hoshino K, Akira S, Wagner H: Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF) 6. J Exp Med 192, 595-600 (2000)

Hament JM, van Dijk H, Fleer A, Aerts PC, Schoenmakers M, de Snoo MW, Dekker BH, Kimpen JL, Wolfs TF: Pneumococcal immune adherence to human erythrocytes. Eur J Clin Invest 33, 169-175 (2003)

Han J, Lee JD, Tobias PS, Ulevitch RJ: Endotoxin induces rapid protein tyrosine phosphorylation in 70Z/3 cells expressing CD14. J Biol Chem 268, 25009-25014 (1993)

Han J, Lee JD, Bibbs L, Ulevitch RJ: A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265, 808-811 (1994)

Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A: The innate immune response to bacterial flagellin is mediated by Toll-like receptor-5. Nature 410, 1099-1103 (2001)

Heil F, Hemmi H, Hochrein H, Amepenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S: Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303, 1526-1529 (2004)

Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S: A Toll-like receptor recognizes bacterial DNA. Nature 408, 740-745 (2000)

Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K, Akira S: Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol 3, 196-200 (2002)

Heumann D, Barras C, Severin A, Glauser MP, Tomasz A: Gram-positive cell walls stimulate synthesis of tumor necrosis factor and interleukin-6 by human monocytes. Infect Immun 62, 2715-2721 (1994)

Hiemstra PS, Bals R: Series introduction: innate host defense of the respiratory epithelium. J Leuko Biol 75, 3-4 (2004)

Horng T, Barton GM, Flavell RA, Medzhitov R: The adaptor molecule TIRAP provides signaling specificity for Toll-like receptors. Nature 420, 329-333 (2002)

Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takada K, Akira S: Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol 162, 3749-3752 (1999)

Idanpaan-Heikkila I, Simon PM, Zopf D, Vullo T, Cahill P, Sokol K, Tuomanen E: Oligosaccharides interfere with the establishment and progression of experimental pneumococcal pneumonia. J Infect Dis 176, 704-712 (1997)

Iwasaki A, Medzhitov R: Toll-like receptor control of the adaptive immune responses. Nat Immunol 5, 987-995 (2004)

Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, Lipford G, Bauer S: Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol 3, 499 (2002)

Kadioglu A, Gingles NA, Grattan K, Kerr A, Mitchell TJ, Andrew PW: Host cellular immune response to pneumococcal lung infection in mice. Infect Immun 68, 492-501 (2000)

Kadioglu A, Andrew PW: The innate immune response to pneumococcal lung infection: the untold story. Trends Immunol 25, 143-149 (2004)

Kadioglu A, Coward W, Colston MJ, Hewitt CR, Andrew PW: CD4-T-lymphocyte interactions with pneumolysin and pneumococci suggest a crucial protective role in the host response to pneumococcal infection. Infect Immun 72, 2689-2697 (2004)

Kagnoff MF, Eckmann L: Epithelial cells as sensors for microbial infection. J Clin Invest 100, 6-10 (1997)

Kawai T, Adachi O, Ogawa T, Takeda K, Akira S: Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 11, 115-122 (1999)

Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, Hoshino K, Akira S: Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J Immunol 167, 5887-5894 (2001)

Kelly T, Dillard PJ, Yother J: Effect of genetic switching of capsular type on virulence of *Streptococcus pneumoniae*. Infect Immun 62, 1813-1819 (1994)

Kerr AR, Irvine JJ, Search JJ, Gingles NA, Kadioglu A, Andrew PW, McPheat WL, Booth CG, Mitchell TJ: Role of inflammatory mediators in resistance and susceptibility to pneumococcal infection. Infect Immun 70, 1547-1557 (2002)

Khan AQ, Chen Q, Wu ZQ, Paton JC, Snapper CM: Both innate immunity and type 1 humoral immunity to *Streptococcus pneumoniae* are mediated by MyD88 but differ in their relative levels of dependence on toll-like receptor 2. Infect Immun 73, 298-307 (2005)

Knapp S, Leemans JC, Florquin S, Branger J, Maris NA, Pater J, van Rooijen N, van der Poll T: Alveolar macrophages have a protective antiinflammatory role during murine pneumococcal pneumonia. Am J Respir Crit Care Med 167, 171-179 (2003)

Knapp S, Wieland CW, van't Veer C, Takeuchi O, Akira S, Florquin S, van der Poll T: Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense. J Immunol 172, 3132-3138 (2004)

Knecht JC, Schiffman GR, Austrian R: Some biological properties of pneumococcus type 37 and the chemistry of its capsular polysaccharide. J Exp Med 132, 475–487 (1970)

Knowles MR, Boucher RC: Mucus clearance as a primary innate defense mechanism for mammalian airways. J Clin Invest 109, 571-577 (2002)

Koedel U, Angele B, Rupprecht T, Wagner H, Roggenkamp A, Pfister HW, Kirschning CJ: Toll-like receptor 2 participates in mediation of immune response in experimental pneumococcal meningitis. J Immunol 170, 438-444 (2003)

Koehler DR, Downey GP, Sweezey NB, Tanswell AK, Hu J: Lung inflammation as a therapeutic target in cystic fibrosis. Am J Respir Cell Mol Biol 31, 377-381 (2004)

Kooguchi K, Hashimoto S, Kobayashi A, Kitamura Y, Kudoh I, Wiener-Kronish J, Sawa T: Role of alveolar macrophages in initiation and regulation of inflammation in *Pseudomonas aeruginosa* pneumonia. Infect Immun 66, 3164-3169 (1998)

Kulka M, Alexopoulou L, Flavell RA, Metcalfe DD: Activation of mast cells by double-stranded RNA: evidence for activation through Toll-like receptor 3. J Allergy Clin Immunol 114, 174-182 (2004)

Lebel MH, Freij BJ, Syrogiannopoulos GA, Chrane DF, Hoyt MJ, Stewart SM, Kennard BD, Olsen KD, McCracken GH Jr: Dexamethasone therapy for bacterial meningitis. Results of two double- blind, placebo-controlled trials. N Engl J Med 319, 964-971 (1988)

Leemans JC, Juffermans NP, Florquin S, van Rooijen N, Vervoordeldonk MJ, Verbon A, van Deventer SJ, van der Poll T: Depletion of alveolar macrophages exerts protective effects in pulmonary tuberculosis in mice. J Immunol 166, 4604-4611 (2001)

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA: The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifugal response in *Drosophila* adults. Cell 86, 973-983 (1996)

Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro G: A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer 17, 62-70 (1976)

Lien E, Sellati TJ, Yoshimura A, Flo TH, Rawadi G, Finberg RW, Carroll JD, Espevik T, Ingalls RR, Radolf JD, Golenbock DT**:** Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J Biol Chem 274, 33419-33425 (1999)

Liu Y, Wang Y, Yamakuchi M, Isowaki S, Nagata E, Kanmura Y, Kitajima I, Maruyama I: Upregulation of toll-like receptor 2 gene expression in macrophage response to peptidoglycan and high concentration of lipopolysaccharide is involved in NF-kappa B activation. Infect Immun 69, 2788-2796 (2001)

Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A: Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J Exp Med 198, 513-520 (2003)

Lysenko ES, Ratner AJ, Nelson AL, Weiser JN: The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. PLOS

Pathogens 2005, 1: 3-11

Macey MG, McCarthy DA, Vordermeier M, Newland AC, Brown KA: Effects of cell purification methods on CD11b and L-selectin expression as well as the adherence and activation of leucocytes. J Immunol Methods 181, 211-219 (1995)

Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, Kurt-Jones E, Paton JC, Wessels MR, Golenbock DT: Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proc Natl Acad Sci USA 100, 1966-1971 (2003)

Mancuso G, Midiri A, Beninati C, Piraino G, Valenti A, Nicocia G, Teti D, Cook J, Teti G: Mitogen-activated protein kinases and NF-κB are involved in TNF-α responses to *group B streptococci*. J Immunol 169, 1401-1409 (2002)

McCullers JA, Tuomanen EI: Molecular pathogenesis of pneumococcal pneumonia. Front Biosci 6, D877-889 (2001)

Medzhitov R: Toll-like receptors and innate immunity. Nat Rev Immunol 1, 135-145 (2001)

Mold C, Rodic-Polic B, Du Clos TW: Protection from *Streptococcus pneumoniae* infection by C-reactive protein and natural antibody requires complement but not Fc gamma receptors. J Immunol 168, 6375-6381 (2002)

Monier RM, Orman KL, Meals EA, English BK: Differential effects of p38- and extracellular signal-regulated kinase mitogen–activated protein kinase inhibitors on inducible nitric oxide synthase and tumor necrosis factor production in murine macrophages stimulated with *Streptococcus pneumoniae*. J Infect Dis 185, 921-926 (2002)

Morath S, Geyer A, Spreitzer I, Hermann C, Hartung T: Structural decomposition and heterogeneity of commercial lipoteichoic acid preparations. Infect Immun 70, 938-944 (2002)

Nathan C: Points of control in inflammation. Nature 420, 846-852 (2002)

N´Guessan PD, Schmeck B, Ayim A, Hocke AC, Brell B, Hammerschmidt S, Rosseau S, Suttorp N, Hippenstiel S: *Streptococcus pneumoniae* R6x induced p38 MAPK and JNK-mediated caspase-dependent apoptosis in human endothelial cells. Thromb Haemost 94, 295-303 (2005)

N´Guessan PD, Hippenstiel S, Etouem MO, Zahlten J**,** Beermann W, Lindner D, Opitz B, Witzenrath M, Rosseau S, Suttorp N, Schmeck B: *Streptococcus pneumoniae*-induced p38 MAPK- and NF-κB-dependent COX-2 expression in human lung epithelium. Am J Physiol Lung Cell Mol Physiol (2006, Epub ahead of print)

Olert J, Wiedorn KH, Goldmann T, Kuhl H, Mehraein Y, Scherthan H, Niketeghad F, Vollmer E, Muller AM, Muller-Navia J: HOPE fixation: a novel fixing method and paraffin-embedding technique for human soft tissues. Pathol Res Pract 197, 823-826 (2001)

Ono K, Han J: The p38 signal transduction pathway: activation and function. Cell Signal 12, 1-13 (2000).

Opitz B, Schroder NW, Spreitzer I, Michelsen KS, Kirschning CJ, Hallatschek W, Zahringer U, Hartung T, Gobel UB, Schumann RR: Toll-like receptor-2 mediates Treponema glycolipid and lipoteichoic acid-induced NF-kappaB translocation. J Biol Chem 276, 22041-22047 (2001)

Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T: TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-β induction. Nat Immunol 4, 161-167 (2003)

Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A: The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci USA 97, 13766-13771 (2000)

Paton JC, Rowan-Kelly B, Ferrante A: Activation of human complement by the pneumococcal toxin pneumolysin. Infect Immun 43, 1085-1087 (1984)

Perregaux DG, Dean D, Cronan M, Connelly P, Gabel CA: Inhibition of interleukin-1 beta production by SKF86002: evidence of two sites of *in vitro* activity and of a time and system dependence. Mol Pharmacol 48, 433-442 (1995)

Pietersma A, Tilly BC, Gaestel M, de Jong N, Lee JC, Koster JF, Sluiter W: p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. Biochem Biophys Res Commun 230, 44-48 (1997)

Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Frendenburg M, Ricciardi-Castagnoli P, Layton B, Beutler B: Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282, 2085-2088 (1998)

Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P, Malo D: Endotoxintolerant mice have mutations in Toll-like receptor 4 (Tlr4). J Exp Med 189, 615–625 (1999)

Qureshi ST, Medzhitov R: Toll-like receptors and their role in experimental models of microbial infection. Genes Immun 4, 87-94 (2003)

Rawadi G, Ramez V, Lemercier B, Roman-Roman S: Activation of mitogen-activated protein kinase pathways by *Mycoplasma fermentans* membrane lipoproteins in murine macrophages: involvement in cytokine synthesis. J Immunol 160, 1330-1339 (1998)

Rayner CF, Jackson AD, Rutman A, Dewar A, Mitchell TJ, Andrew PW, Cole PJ, Wilson R: Interaction of pneumolysin-sufficient and -deficient isogenic variants of *Streptococcus pneumoniae* with human respiratory mucosa. Infect Immun 63, 442-447 (1995)

Reddel RR, Ke Y, Gerwin BI, McMenamin MG, Lechner JF, Su RT, Brash DE, Park JB, Rhim JS, Harris CC: Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res 48, 1904-1909 (1988)

Redecke V, Hacker H, Datta SK, Fermin A, Pitha PM, Broide DH, Raz E: Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. J Immunol 172, 2739-2743 (2004)

Rijneveld AW, Levi M, Florquin S, Speelman P, Carmeliet P, van Der Poll T: Urokinase receptor is necessary for adequate host defense against pneumococcal pneumonia. J Immunol 168, 3507-3511 (2002)

Robinson MJ, Cobb MH: Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 9, 180-186 (1997)

Rossjohn J, Gilbert RJ, Crane D, Morgan PJ, Mitchell TJ, Rowe AJ, Andrew PW, Paton JC, Tweten RK, Parker MW: The molecular mechanism of pneumolysin, a virulence factor from *Streptococcus pneumoniae*. J Mol Biol 284, 449-461 (1998)

Rubins JB, Charboneau D, Paton JC, Mitchell TJ, Andrew PW, Janoff EN: Dual function of pneumolysin in the early pathogenesis of murine pneumococcal pneumonia. J Clin Invest 95, 142-150 (1995)

Rupp J, Droemann D, Goldmann T, Zabel P, Solbach W, Vollmer E, Branscheid D, Dalhoff K, Maass M: Alveolar epithelial cells type II are major target cells for C. pneumoniae in chronic but not in acute respiratory infection. FEMS Immunol Med Microbiol 41, 197-203 (2004)

Sadikot RT, Blackwell TS, Christman JW, Prince AS: Pathogen–host interactions in *Pseudomonas aeruginosa* Pneumonia. Am J Respir Crit Care Med 171, 1209-1223 (2005)

Saklatvala J: The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. Curr Opin Pharmacol 4, 372-377 (2004)

Sankar S, Chan H, Romanow WJ, Li J, Bates RJ: IKK-i signals through IRF3 and NFkappaB to mediate the production of inflammatory cytokines. Cell Signal (Epub ahead of print, 2005)

Schmeck B, Zahlten J, Moog K, Laak V, Huber S, Hocke AC, Opitz B, Hoffmann E, Krach M, Zerrahn J, Hammerschmidt S, Rosseau S, Suttorp N, Hippenstiel S: *Streptococcus pneumoniae*-induced p38 MAPK-dependent phosphorylation of RelA at the interleukin-8 promotor. J Biol Chem 279, 53241-53247 (2004a)

Schmeck B, Gross R, N'Guessan PD, Hocke AC, Hammerschmidt S, Mitchell TJ, Rosseau S, Suttorp N, Hippenstiel S: *Streptococcus pneumoniae*-induced caspase 6-dependent apoptosis in lung epithelium. Infect Immun 72, 4940-4947 (2004b)

Schnare M, Holt AC, Takeda K, Akira S, Medzhitov R: Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. Curr Biol 10, 1139-1142 (2000)

Schroder NW, Morath S, Alexander C, Hamann L, Hartung T, Zahringer U, Gobel UB, Weber JR, Schumann RR: Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. J Biol Chem 278, 15587-15594 (2003)

Schuchat A, Robinson K, Wenger JD, Harrison LH, Farley M, Reingold AL, Lefkowitz L, Perkins BA: Bacterial meningitis in the United States in 1995. Active Surveillance Team. N Engl J Med 337, 970-976 (1997)

Schulz C, Farkas L, Wolf K, Kraetzel K, Eissner G, Pfeifer M: Differences in LPS-induced activation of bronchial epithelial cells (BEAS-2B) and type-like pneumocytes (A-549). Scand J Immunol 56, 294-302 (2002)

Schwadner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ**:** Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem 274, 17406-17409 (1999)

Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J: Triggering the interferon antiviral response through an IKK-related pathway. Science 300, 1148-1151 (2003)

Steinfort C, Wilson R, Mitchell T, Feldman C, Rutman A, Todd H, Sykes D, Walker J, Saunders K, Andrew PW, Boulnois GJ, Cole PJ: Effect of *streptococcus pneumoniae* on human respiratory epithelium *in vitro*. Infect Immun 57, 2006-2013 (1989).

Sweet MJ, Hune DA: Endotoxin signal transduction in macrophages. J Leukoc Biol 60, 8-26 (1996)

Tabeta K, Yamazaki K, Akashi S, Miyake K, Kumada H, Umemoto T, Yoshie H: Toll-like receptors confer responsiveness to lipopolysaccharide from *Porphyromonas gingivalis* in human gingival fibroblasts. Infect Immun 68, 3731-3735 (2000)

Takeda K, Akira S: Toll-like receptors in innate immunity. Int Immunol 17, 1-14 (2005)

Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, and Akira S: Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11**,** 443-451 (1999)

Takeuchi O, Hoshino K, Akira S: Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. J Immunol 165, 5392-5396 (2000a)

Takeuchi O, Takeda K, Hoshino K, Adachi O, Ogawa T, Akira S: Cellular response to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. Int Immunol 12, 113-117 (2000b)

Takeuchi O, Kawai T, Mulhradt PF, Morr M, Radolf JD, Zychlinsky A, Takeda K, Akira S: Discrimination of bacterial lipopeptides by Toll-like receptor 6. Int Immunol 13, 933-940 (2001)

Takeuchi O, S Sato S , Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, Akira S: Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 169, 10-14 (2002)

Talbot UM, Paton AW, Paton JC: Uptake of *Streptococcus pneumoniae* by respiratory epithelial cells. Infect Immun 64, 3772-3777 (1996)

Thoma-Uszynski S, Stenger S, Takeuchi O, Ochoa MT, Engele M, Sieling PA, Barnes PF, Rollinghoff M, Bolcskei PL, Wagner M, Akira S, Norgard MV, Belisle JT, Godowski PJ, Bloom BR, Modlin RL: Induction of direct antimicrobial activity through mammalian Toll-like receptors. Science 291, 1544-1547 (2001)

Tomasz A, Saukkonen K: The nature of cell wall-derived inflammatory components of peumococci. Pediatr Infect Dis J 8, 902-903 (1989)

Tuomanen E, Rich R, Zak O: Induction of pulmonary inflammation by components of the pneumococcal cell surface. Am Rev Respir Dis 135, 869-874 (1987)

Uhlig U, Uhlig S, Branscheid D, Zabel P, Vollmer E, Goldmann T: HOPE technique enables Western blot analysis from paraffin-embedded tissues. Pathol Res Pract 200, 469-472 (2004)

Underhill DM, Ozinsky A: Phagocytosis of microbes: complexity in action. Annu Rev Immunol 20, 825-852 (2002)

Van Rooijen N, Sanders A: Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 174, 83-93 (1994)

Wang E, Simard M, Ouellet N, Bergeron Y, Beauchamp D, Bergeron MG: Modulation of cytokines and chemokines, limited pulmonary vascular bed permeability, and prevention of septicemia and death with ceftriaxone and interleukin-10 in pneumococcal pneumonia. J Infect Dis 182, 1255-1259 (2000)

Welham MJ, Duronio V, Sanghera JS, Pelech SL, Schrader JW: Multiple hemopoietic growth factors stimulate activation of mitogen-activated protein kinase family members. J Immunol 149, 1683-1693 (1992)

Wenger JD: Vaccines for the developing world: current status and future directions. Vaccine 19, 1588-1591 (2001)

Werts C, Tapping RI, Mathison JC, Chuang TH, Kravchenko V, Saint Girons I, Haake DA, Godowski PJ, Hayashi F, Ozinsky A, Underhill DM, Kirschning CJ, Wagner H, Aderem A, Tobias PS, Ulevitch RJ: Leptospiral lipopolysaccharide activatescells through a TLR2-dependent mechanism. Nat Immunol 2, 346-352 (2001)

Winer J, Jung CK, Shackel I, Williams PM: Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring of gene expression in cardiac myocytes *in vitro*. Anal Biochem 270, 41–49 (1999)

Winkelstein JA, Tomasz A: Activation of the alternative complement pathway by pneumococcal cell wall teichoic acid. J Immunol 120, 174–178 (1978)

Xu F, Droemann D, Rupp Y, Goldmann T, Krueger S, Maass M, Zabel P, Dalhoff K: *In vitro* infection with nontypeable *Haemophilus influenzae* in a model of human lung tissues and cells. Eur Respir J 26 (Suppl49), 405 (2005)

Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, Hoshino K, Takeuchi O, Kobayashi M, Fujita T, Takeda K, Akira S: Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature 420, 324-329 (2002a)

Yamamoto M, Sato S, Mori K, Takeuchi O, Hoshino K, Takeda K, Akira S: Cutting edge: a novel TIR domain-containing adaptor that preferentially activates the interferon-β promoter. J Immunol 169, 6668-6672 (2002b)

Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS, Hieny S, Sutterwala FS, Flavell RA, Ghosh S, Sher A: TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science 308, 1626-1629 (2005)

Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D: Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 163, 1-5 (1999)

Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, Ghosh, S: Toll-like receptor that prevents infection by uropathogenic bacteria. Science 303, 1522-1526 (2004)

Zhang P, Summer WR, Bagby GJ, Nelson S: Innate immunity and pulmonary host defense. Immunol Rev 173, 39-51 (2000)

Zysk G, Bejo L, Schneider-Wald BK, Nau R, Heinz H: Induction of necrosis and apoptosis of neutrophil granulocytes by *Streptococcus pneumoniae*. Clin Exp Immunol 122, 61-66 (2000)

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