Toll-like receptors and innate immunity in pneumonia

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Toll-like receptors and innate immunity in pneumonia

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Contents **page**

Chapter 1

General introduction and outline of the thesis

Introduction

Infectious diseases are a major cause of morbidity and mortality worldwide (1). Respiratory tract infections by (myco)bacteria and viruses are a frequent cause of such diseases. Improved hygiene and the introduction of antibiotics and vaccination programs have successfully reduced the morbidity and mortality of many infectious diseases during the twentieth century. However, the increasing incidence of (multi)drug resistant bacterial strains hampers adequate treatment. In addition, vaccines are less effective in the very young and the elderly, both being susceptible to pulmonary infections. To further improve treatment against infectious (pulmonary) disease it is mandatory to expand our understanding of host-pathogen interactions (2, 3). Higher vertebrates have two protective systems to combat invading microbes: the innate and the adaptive immune system. Innate immunity consists of soluble proteins, such as complement components which bind microbacterial products, and phagocytotic leukocytes, which both contribute to the rapid killing and eradication of invading pathogens. The adaptive immune system consists of lymphocytes which respond to signals from the innate immunity resulting in the production of highly specific antibodies against bacteria and viruses. These antibodies opsonize microbes and viruses and facilitate their destruction by leukocytes during (re)infection (review(4)). Although the innate and adaptive immune system provide immediate and rapid protection (innate), as well as specific and prolonged protection (adaptive) against many bacteria and viruses, some microbes have the ability to escape from these protective host defense mechanisms.

This thesis is focused on the role of the innate immune system in pulmonary infections with the bacterium *Streptococcus pneumoniae* and Influenza A virus. This chapter will summarize (a) current knowledge of the pulmonary innate immune system, (b) the characteristics of *Streptococcus pneumoniae* and Influenza A virus and their structures that are recognized by the innate immune system, and (c) the experimental pneumonia models in normal and genetically modified mice that we have used to study the interactions between the innate immune system and these pathogens.

1. Innate immunity in the lungs

1.1 Innate immune cells

The alveolar membrane of the lungs has a large surface of approximately 100-140 m^2 and is continuously exposed to (in)organic particles and microbes like bacteria and viruses. In the upper respiratory tract, physical mechanisms such as coughing and sneezing are used to remove potential pathogens. Particles smaller than 1 μm, like bacteria and viruses, which enter the lower respiratory tract will encounter cells from the innate immune system. In normal conditions, alveolar macrophages (AM) account for approximately 95% of the resident leukocytes, with 1-4% lymphocytes and only 1% neutrophils. Moreover, epithelial cells can also be regarded as part of the innate immune system in the lung as a source of surfactants (review(4)). During pulmonary infection, AM and epithelial cells produce several cytokines and chemokines after recognition of pathogens resulting in the recruitment of more immune cells like polymorphonuclear cells (PMNS) and lymphocytes from the lung capillary network into the alveolar space. Both AM and PMNs have phagocytotic capacities to engulf and eradicate bacteria from the pulmonary compartment. In addition, AM can phagocytose apoptotic PMNs and thereby contribute to the resolution of pneumonia. Depletion of AM or PMNs in a number of murine pneumonia models resulted in increased lethality and worsened outcome of the disease showing the significant role of these cells during infection (review (5)). Recognition and elimination of the pathogens by phagocytic leukocytes is brought about by several receptors, including Fc-receptors, complement receptors, scavenger receptors, lectins and Toll-like receptors of which the latter ones will be further discussed here.

1.2.1 Toll-like receptors

Toll-like receptors (TLRs) are pattern recognition receptors (PRR) which recognize specific molecules expressed by microbes like bacteria and viruses (so-called pathogen-associated molecular patterns -PAMPs-). This receptor family is conserved throughout evolution from fruit flies to human (review (6-8)). TLRs are member of the IL-1 receptor family and are expressed by a wide range of leukocytes including cells from the innate immune system like macrophages/monocytes, PMNs, dendritic cells, and also epithelial cells. So far 10 different TLRs have been identified in humans and 13 in mice (table 1). TLR1, 2, 4, 5, and 6 are expressed on the cell

surface whereas TLR3, 7, 8 and 9 are expressed almost exclusively intracellular. In general, binding of a PAMP to a TLR induces the recruitment of intracellular adaptor proteins to the TLR and the activation of several kinases, which ultimately leads to the translocation of nuclear transcription factors and the transcription of several genes encoding pro- and anti-inflammatory cytokines and chemokines. Each TLR recognizes distinct PAMPs (table 1), either independently or in combination with another TLR.

Table 1: TLRs and their PAMPs. Adapted and modified from (6, 8).

Receptor	**Ligand**	**Origin of Ligand**
TLR1 + TLR2	Triacyl lipopeptides	Bacteria and mycobacteria
	Soluble factors	*Neisseria meningitidis*
	Non-capped lipoarinomanna	Atypical mycobacteria
TLR2	Lipoprotein/lipopeptides	Various pathogens
	Lipoteichoic acid	Gram-positive bacteria
	Lipoaribonomannan	Mycobacteria
	Soluble tuberculose factor	*Mycobacterium tuberculosis*
	Phosphatidylinositolmannan	Mycobacteria
	Peptidoglycan	Gram-positive bacteria
	Phenol-soluble modulin	*Staphylococcis epidermis*
	Glycoinositolphospholipids	*Trypanosome cruzi*
	Glycolipids	*Trepenema maltophilum*
	Porins	*Neisseria*
	Zymosan	Fungi
	Phospholipomannan	*Candida albicans*
	Glucuronoxylomannan	*Cryptococcus neoformans*
	tGPI-mutin	*Trypanosoma*
	Hemagglutinin protein	Measles virus
TLR3	Double-stranded RNA (polyI:C)	Viruses
TLR4	Lipopolysaccharide	Gram-negative bacteria
	Fusion protein	Respiratory syncytial virus
	Envelope protein	Mouse mammary-tumor virus
		Respiratory synsitial virus
	Pneumolysin	*Streptococcus pneumoniae*
	Mannan	*Candida albicans*
	Glucuronoxylomannan	*Cryptococcus neoformans*
	Glycoinositolphospholipids	*Trypanosoma*
TLR5	Flagellin	Bacteria

TLR6 + TLR2	Diacyl lipopeptides Lipoteichoic acid zymosan	Mycoplasma Gram-positive bacteria Fungi
TLR7	Single-stranded RNA	Viruses
TLR8	Single-stranded RNA	Viruses
TLR9	Unmethylated CpG-containing DNA Hemozozoin	Bacteria and viruses *Plasmodium*
TLR10	Not determined	Not determined
TLR11	Not determined Profiling	Uropathogenic *Escherichia coli* *Toxoplasma gondii*
TLR12	Not determined	Not determined
TLR13	Not determined	Not determined

1.2.2. Toll-like receptor expression

TLRs are widely expressed in most tissues. Lung tissue harbors relatively high levels of mRNA of all TLRs (9). Peripheral blood leukocytes and phagocytes express the largest variety of TLR mRNA's (9) and human epithelial cells express mRNA of TLRs 1 to 6 (10). This shows that TLRs can be found in the three most important cell types involved in innate immunity in the lung, i.e. macrophages, neutrophils and epithelial cells. Among the TLRs, TLR2 and TLR4 are the most investigated receptors and considered to be the major receptors involved in protective immunity against bacterial infections. Both TLR2 and TLR4 are predominerly expressed by human monocytes/macrophages and PMNs. Expression of TLRs in other leukocytes, endothelial cells, epithelial cells and fibroblast are expressed to a lower extent. In vivo studies with immunohistochemistry and in situ hybridization have shown expression of both TLR2 and TLR4 on murine bronchial epithelium (11). In addition, human bronchial and alveolar epithelial cells and macrophages have been shown to express TLR2 (12). Immunohistochemical staining of lungs from naïve mice showed low expression of TLR2 and TLR4 mainly on bronchial epithelium and tissue macrophages. Expression of both TLRs increased after inhalation of LPS (11).

1.2.3. Toll-like receptor signaling

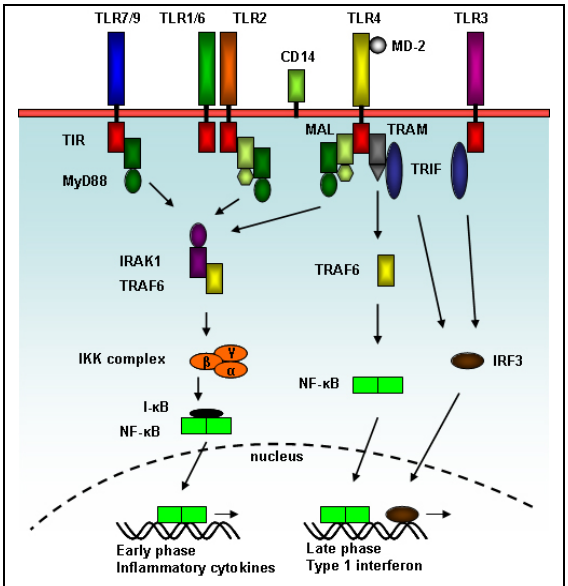

Figure 1: Toll-like receptor signaling: details are described in text (adapted from (7))

Activation of the TLRs activates a cascade of intracellular kinases, leading to diverse gene expression. The intracellular portion of each TLR contains a TIR domain (Toll-IL-1 receptor motif). So far, four functionally known different cytoplasmic adaptor proteins have been shown to bind to TLRs and initiate intracellular signaling. These adaptor proteins are MyD88 (myeloid differentiation factor 88), MAL (MyD88 adaptor-like protein) and TRIF (TIR domain-containing adaptor protein including interferon), TRAM (TRIF-related adaptor molecule). Recruitment of these adaptor proteins leads to activation of IRAK-4 (IL-1 receptor associated kinase 4) which facilitates phosphorylation of IRAK-1 and association with TRAF-6 (TNF receptor-associated factor 6). Then, phosphorylation of IKK-γ (I-kappa kinase-γ) and the phosphorylation and degradation of IκB results in translocation of NF-κB (nuclear factor- κB) to the nucleus and the transcription of several pro- and anti-inflammatory cytokines. Additionally there is a MyD88 independent signaling pathway which is dependent of TRIF and TRAM. Signaling via TRIF and TRAM requires IRF-3 (Interferon regulatory factor 3) and results in a ‘late’ NF-κB activation. The MyD88 independent pathway results in gene expression of type 1 interferons.

Down-regulation of TLR-induced cell activation is mediated by various molecules, including, SIGIRR (single immunoglobulin IL-1 receptor-related molecule), MyD88short, ST2, IRAK-M, A20, PI3 kinase (PI3K), Toll-interacting protein (TOLLIP) and SOCS-1 (suppressor of cytokine signaling), that act on different levels in the intracellular TLR signalling pathway (review (13)).

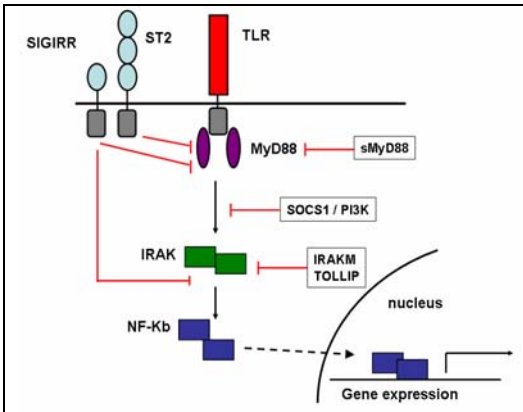

Figure 2: Negative regulation of Toll-like receptor signaling, details are described in text (modified from (13))

1.3 CD14

CD14 is a glycosyl phosphatidylinositol surface anchored molecule expressed by myeloid cells, in particular monocytes/macrophages and to a lesser extent PMNs (14, 15)(review (16)). CD14 is mainly known as a scavenger receptor which recognizes lipopolysaccharide (LPS),proinflammatory constituent of the gram-negative bacterial cell wall. LPS may bind to a serum protein, LBP (LPS-binding protein), which facilitates the binding to CD14 (17). Binding of LPS-LBP complex to CD14 leads to leukocyte activation and release of several cytokines/chemokines and upregulation of adhesion molecules. But since membrane bound (m)CD14 lacks an intracellular domain, it requires interaction with other receptors, like TLRs, for signal transduction (18). Besides as a membrane bound receptor, CD14 can exist as a soluble protein (sCD14). sCD14 can also bind LPS and the LPS/sCD14 complex can stimulate cells which lack mCD14 (19, 20). Two isoforms of this sCD14 have been identified: one that is formed by shedding from the cell surface and one that is released from cells before addition of the glycosyl phosphatidylinositol anchor (15, 21-25). Later on other ligands besides LPS were found to interact with (s)CD14 namely lipoteichoic acid (LTA) and phosphatidyl inositol (26).

1.3.1 CD14 expression

CD14 is mainly expressed on monocytes, tissue macrophages and to a lesser extent on PMNs, whereas sCD14 is mainly present is serum and cerebrospinal fluid (27, 28). Expression of CD14 and release of sCD14 are subjected to cytokine- and bacteria-induced regulation (review (16)). In naïve mice, CD14 mRNA and/or protein could not be detected in plasma, blood leukocytes, epithelial cells or liver cells; however, it could be detected at low levels in the lungs (29). After treatment with LPS both CD14 mRNA and protein levels increased in leukocytes, epithelial cells and plasma (29).sCD14 is mainly present in plasma and during bacterial meningitis, elevated concentrations of sCD14 were detected in the cerebrospinal fluid which originated from intrathecal leukocytes (28). sCD14 is elevated in septic patients; (24) a specific form of sCD14 has been correlated with the severity of sepsis (30).

1.4 Monocyte chemoattractant protein 1

Both cytokines and chemokines play an important role during pulmonary infection. Cytokines can be arbitrarily divided in pro-inflammatory and anti-inflammatory mediators. Pro-inflammatory cytokines such as TNF-α, IL-1β and IL-18, contribute to protective immunity against *Streptococcus. pneumoniae* pneumonia whereas the prototypic anti-inflammatory cytokine IL-10 impairs host defense against this infection (review (5)).

Chemokines can be divided into four families depending on their tetra cysteine motif, according to the configurations of the cysteine residues at their amino terminus; of these CXC and CC chemokines represent the largest groups (31, 32). Chemokines are produced by several cells types, including leukocytes, epithelial cells, endothelial cells and fibroblasts (31, 32). Monocyte chemoattractant protein 1 (MCP-1 or CCL2) is a CC chemokine with pleiotropic activities (33). MCP-1 primarily attracts monocytes and memory T cells, but during severe bacterial infection may also contribute to neutrophil recruitment (34, 35). During endotoxemia and pulmonary infection, MCP-1 levels have been shown to increase significantly (36-38); MCP-1 has been found to exert anti-inflammatory effects during murine endotoxemia (39).

2. Pneumonia

2.1.1 Pneumococcal pneumonia

Streptococcus (S.) pneumoniae, or pneumococcus, is a gram-positive bacterium which is present in the upper respiratory tract of 5-10 % in healthy adults and 20-40 % of healthy children (40). It is related to commensal members of the oral streptococci and usually do not cause symptomes. However, when translocated to the lower respiratory tract it may cause pathogenic infections, like pneumonia, sepsis and meningitis. Community-acquired pneumonia (CAP) affects 3 to 4 million people in the United States alone each year and up to 20% of the patients are admitted to the hospital with a 15 - 20% mortality rate for those who enter the intensive care unit (41). *S. pneumoniae* is the most isolated pathogen in CAP with a prevalence of 20-60 % (42). Especially the very young, elderly and immuno-compromised patients are susceptible to pneumococcal pneumonia. Moreover, the increasing incidence of (multi)drug resistant *S. pneumoniae* hampers adequate treatment. Based on differences in the composition of the polysaccharide capsule, approximately 90 serotypes have been identified. Unfortunately the 23-valent vaccine is ineffective in the very young due to the absence of memory-forming adaptive immune response. More frequently used are the conjugated vaccines. The 7 conjugated vaccine induces a memory-forming immune response and even though only 7 strains out of approximately 90 strains are covered, it is these strains that cause 80% to 90% of cases of severe pneumococcal disease.

2.1.2 Lipoteichoic acid and peptidoglycan

Lipoteichoic acid (LTA) and peptidoglycan (PGN) are both found in the cell wall of gram-positive bacteria, like the pneumococcus. LTA is anchored to the bacterial plasma membrane by hydrophobic interaction through its acyl chain (43). Both PGN and LTA are considered to be ligands for TLR2 and may be released when bacteria are killed by autolysis, host immune cells or antibiotic treatment (44). Release of large amounts of LTA is involved in postinfectious sequelae by inducing an exuberant

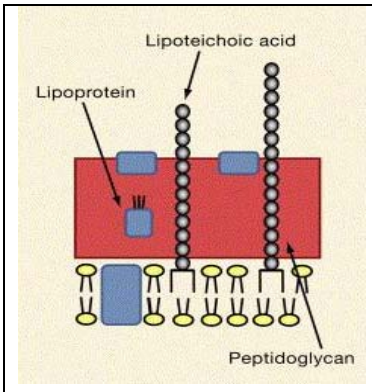

Figure 3: drawing of pneumo-coccal cell wall (adapted from (8))

host-derived inflammatory response by leukocytes (44). LTA also contributes to binding of pneumococci to host cells. LTA has a binding moiety containing choline and binds to specific choline-binding domains on epithelial cells. This binding can facilitate colonization and invasion of the pneumococcus which deteriorates the outcome of pneumonia (44). Pulmonary inoculation of *Staphylcoccus (S.) aureus* LTA induced cytokine production and PMN influx (45, 46). Moreover, simultaneous inoculation of both LTA and PGN synergistically induced PMN influx into the lungs and enhanced multiple organ failure in sepsis (46-48). Studies about the biological properties of LTA and TLR-dependency have only recently begun to elucidate LTA-TLR interaction since commercially available LTA preparations used in preceding studies were contaminated with endotoxin (49). So far, most studies focused on *S. aureus* LTA and information about the potency and TLR-dependency of pneumococcal LTA *in vivo* is lacking.

2.1.3 Pneumolysin

Pneumolysin is an intracellular toxin of pneumococci that is recently has been shown to be a TLR4 ligand (50). Pneumolysin is produced by all clinical isolates and is released when the bacteria is killed either by host immune cells, antibiotic treatment or by autolysis. Pneumolysin is an important virulence factor since mice infected with a pneumolysin-deficient strain of *S. pneumoniae* showed a reduced lethality and a diminished inflammatory response compared to mice infected with a normal, pneumolysin-producing strain (51-56). At sublytic dose, pneumolysin affects polymorphonuclear cell activity including respiratory burst, degranulation, chemotaxis and bactericidal activity (57). Furthermore, pneumolysin activates the classical pathway of complement and induces cytokine production by macrophages and monocytes (58-60). At lytic dose, pneumolysin forms ring-shaped pores in cholesterol containing cell membranes which results in apoptotic cell death (61, 62).

2.1.4 TLR and related molecules in pneumococcal infections

A number of TLRs (TLR1, TLR2, TLR4, TLR6 and TLR9) and related molecules (CD14 and MyD88) have been implied to contribute in the recognition of the pneumococcus or ligands originating from this bacterium.

TLR2: Both LTA and PGN are recognized by the innate immune system through TLR2 and CD14 (63-66). During murine meningitis, mice deficient of TLR2 (TLR2 knock-out (KO)) displayed increased disease severity and decreased survival compared to wild type mice (67, 68). Surprisingly, TLR2 only played a modest role during pneumococcal pneumonia (69). Although TLR2 KO mice displayed a reduced cytokine and chemokine production, bacterial clearance and morbidity did not differ compared to wild type mice (69). Similar as in pneumococcal pneumonia, intraperitonial injection of pneumococci in TLR2 KO mice resulted in similar survival and minor impaired immune response compared to wild type mice (70).

TLR4: Pneumolysin is recognized by the innate immune system and both pneumolysin-induced cytokine production and apoptosis are mediated through TLR4 (50, 71). In a murine nasopharyngeal carriage model, mice with a non-functional TLR4 protein (TLR4-mutant mice) were more heavily colonized and developed more invasive disease. In these TLR4-mutant mice, mortality was increased and more bacteria were present in the nasopharyngeal cavity compared to wild type mice (50). However, in a similar model TLR4 had no contribution to the clearance of pneumococci during nasopharyngal carriage (72), which can possibly be explained by the use of different strains of *S. pneumoniae*. In a murine pneumococcal pneumonia model it was also found that TLR4 had a benificial role. TLR4-mutant mice had a higher bacterial burden in the lungs and an increased mortality as compared to wild type mice (73). The effect however was only seen after infection with a low dose of pneumococci. In a sepsis model, TLR4 had no contribution to mortality or bacterial clearance after intravenous infection (74).

TLR1/TLR6: So far, few studies have been performed to determine the role of TLR1 or TLR6 in the recognition of the pneumococcus. TNF-α production by human peripheral blood mononuclear cells after stimulation with pneumococcal LTA was TLR1, as well as TLR2 and CD14 dependent (64). In a mouse macrophages cell line, TNF-α production induced by heat killed pneumococci was TLR6 dependent (75). A recent study has shown no contribution of TLR1 or TLR6 in pneumococal pneumonia (76).

TLR9: Human embryonic kidney (HEK)-293 cells were responsive to live pneumococci when transfected with merely TLR9 or TLR2 (77). Although mice deficient of TLR1/2/4/6 are only marginally affected in their susceptibility to pneumococcal pneumonia, mice deficient of the intracellular TLR9 are more susceptible to this infection (76); TLR9 KO mice displayed an increased bacterial load and mortality after intranasal instillation of *S. pneumoniae*.

MyD88: MyD88 plays a crucial role in signaling of TLRs except TLR3. Both intranasal and intraperitoneal injection of *S. pneumoniae* in MyD88 KO mice showed a significant contribution of MyD88 in controlling bacterial outgrowth and the immune response during infection (70, 78). In addition, MyD88 also contributed to severity of disease and bacterial clearance in pneumococcal meningitis (79).

CD14: Various *in vitro* studies studies revealed that CD14 contributes to the recognition of the pneumococcus (63-65, 80). Murine meningitis model induced by *S. pneumoniae* revealed a potential function of soluble CD14 (sCD14) in the brain during bacterial meningitis (28, 81). Whereas intracerebral infection caused only a minor and/or transient increase of sCD14 levels in serum, dramatically elevated concentrations of sCD14 were detected in cerebrospinal fluid. In addition, simultaneous intracerebral inoculation of recombinant sCD14 and *S. pneumoniae* resulted in a markedly increased local cytokine response. This shows that sCD14 can play an important role in the pathogenesis of this pneumococal infection (28). Moreover, CD14 KO mice had higher disease severity scores and mortality, and displayed a higher bacterial burden in the brains 24 hours after infection as compared to wild type mice (81).

2.2.1 Influenza A pneumonia

The influenza virus is a negative single-stranded RNA virus and a member of the Orthomyxoviridae family. Influenza viruses can be divided into subtypes A, B and C of which Influenza A is the most virulent. Infection with influenza virus may cause symptoms like fever, headache, sore throat, sneezing and general malaise. Symptoms may last for 4 to 10 days before the virus is cleared but in rare cases may lead to severe pneumonia. Especially the very young, elderly and the immuno-compromised patients are more susceptible to influenza infection and may display a more severe outcome of pneumonia. Millions of people in the United States (about 10% to 20% of U.S. residents) are infected with influenza each year. An average of about 36,000 people per year in the United States die from influenza, and 114,000 per year are admitted to a hospital as a result of influenza. According to estimates by the World Health Organization, between 250,000 and 500,000 humans die from influenza infection each year worldwide. Influenza pandemic outbreaks were notorious for their mortality rates. The most famous outbreak (and the most lethal) was the so-called Spanish Flu pandemic (type A influenza, H1N1 strain), which lasted from 1918 to 1919, and is believed to have killed more people in total than World War I. The pandemic took most of its toll over a period of weeks. Lesser flu epidemics included the 1957 Asian Flu (type A, H2N2 strain) and the 1968 Hong Kong Flu (type A, H3N2 strain). There have been no major pandemics subsequent to the 1968 infection. Increased immunity from antibodies and the development of influenza vaccines have limited the spread of the virus, and so far prevented any further pandemics.

Influenza virus is an enveloped virus containing 8 segmented genes, which encodes 10 viral proteins. The hemagglutinin and neuraminidase protein expression are expressed on the outer membrane and are required to infect target cells. Neuraminadase is used to attach to cells and hemagglutin is used to enter them. So far, 16 different hemagglutinin proteins and 9 neuraminidase proteins are discovered.

2.2.2 TLRs and related molecules in influenza infections

Our understanding of the functional role of TLRs in viral pathogenisis is still in its infancy. Several ligands from viruses have been characterised as PAMPs, like viral proteins and nucleic acids which stimulate through specific TLRs (review (6, 8, 82)). TLR3 and TLR7, as well as CD14 and MyD88 are involved in the induction of immune response by influenza A or singel-stranded RNA (ssRNA).

TLR3: Leukocytes from TLR3 KO mice are less responsive to poly I:C, a synthetic analogue of viral dsRNA (83). In addition, TLR3 KO mice were resistant to poly I:C-induced shock compared to wild type and displayed reduced IL-12 production in blood compared to wild type mice(83). Of note, influenza is a ssRNA and poly I:C mimics dsRNA. Dendritic cells (DC) from TLR3 KO mice equally produced IFN-α compared to DC from normal mice when stimulated with influenza virus (84, 85). Recently it was shown that influenza-infected TLR3 KO mice displayed significantly reduced inflammatory mediators as well as a lower number of CD8+ T lymphocytes in the bronchoalveolar space. More important, despite a higher viral production in the lungs, mice deficient in TLR3 had an unexpected survival advantage (86)

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TLR7: DC from TLR7 KO mice produced less cytokines compared to DC from normal mice when stimulated with ssRNA or influenza virus (84, 85, 87). Interestingly, TLR7 KO mice were less responsive to another ssRNA virus VSV, but influenza infection was not investigated (85).

CD14: Pauligk et al. showed that CD14 is required for influenza-induced cytokine production during infection (88). Macrophages from CD14 KO mice were less responsive to influenza compared to WT macrophages. In addition, treatment of human monocytes with CD14 antibody abolished inflammatory response (88). Interestingly, a resent study done by Lee et al. showed that CD14 can bind both ssRNA and dsRNA and mediates uptake of poly I:C (pIpC), a synthetic mimic of viral double stained (ds)RNA (89). CD14 has been suggested as a transporter of viral particles to (intracellular) TLRs (90).

MyD88: Leukocytes from MyD88 KO mice were also less responsive to Poly I:C stimulation (83) or influenza virus (85). Recently it was shown that influenza H2

heamagglutinin activated B-cells in a MyD88-dependent way although no specific TLR was found responsible for the activation and proliferation of these B-cells (91).

2.3 Postinfluenza pneumonia

Secondary bacterial pneumonia is a feared complication of respiratory tract infection by influenza A, responsible for at least 20,000 deaths annually in the United States alone (92). The most important pathogens causing postinfluenza pneumonia are *Staphylococcus aureus, Haemophilus influenzae* and in particular *Streptococcus (S.) pneumoniae* (93). Although *S. pneumoniae* is the most common pathogen isolated from previously healthy patients with community-acquired pneumonia (94), such primary pulmonary infections with the pneumococcus are usually less severe than secondary infections following influenza A (95). Thus far, knowledge about the precise mechanism by which influenza modulates the innate immune response to facilitate secondary bacterial infection in the lung is limited. One hypothesis is that the virus affects the epithelial barrier in the lungs thereby facilitating colonization of the pneumococcus during secondary infection (96). However, it is unclear why infection with *S. pneumoniae* results in an exegerated inflammatory response in the lungs of mice that have recovered from influenza.

3. Outline of this thesis

The general aim of this thesis was to investigate host-pathogen interactions during viral or bacterial pneumonia. Individual aspects of the innate immune system during pneumonia are studied in separate chapters. In this thesis we use *S. pneumoniae* and influenza A infections as models of respiratory tract infections.

The first part of this thesis is focused on innate immunity during pulmonary infection and inflammation with *S. pneumoniae* orcomponents of this pathogen. Earlier in vitro studies have shown that recognition of LTA from other gram-positive pathogens is TLR2 specific. **In Chapter 2** we investigated the recognition of *S. pneumoniae* LTA by TLRs and CD14. Pneumolysin is an intracellular toxin found in the pneumococcus and is shown to be a ligand for TLR4. **Chapter 3** discribes the contribution of TLRs in pneumolysin-induced inflammation and lung injury using purified pneumolysin. In **Chapter 4** we investigated the combined contribution of TLR2 and TLR4-signalling during pneumococcal pneumonia. CD14 is a coreceptor of TLRs and has been investigated in several gram-negative bacterial infections but less in gram-positive

infections. The contribution of CD14 in pneumococcal pneumonia was investigated in **Chapter 5.** During pulmonary infection, chemokine MCP-1 level is significantly increased. In **Chapter 6,** the role of MCP-1 during pneumococcal pneumonia is described. The second part of the thesis is focused on pulmonary infection with influenza A virus. As earlier investigated in bacterial pneumonia, the contribution of MCP-1 (**Chapter 7**) and CD14 (**Chapter 8**) was investigated in pneumonia caused by influenza A. In **Chapter 9** we investigated the expression of a broad range of genes during influenza infection in three different compartments: whole lung, bronchoalveolar lavage cells and epithelial cells. The third part of the thesis is focused of postinfluenza pneumonia. **Chapter 10** describes the role of TLR2 during (primary influenza infection and) postinfluenza pneumococcal pneumonia.

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Part I

Pneumococcal pneumonia

Chapter 2

Role of Toll-like receptors 2 and 4 in lipoteichoic acid-induced lung inflammation and coagulation

Submitted

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Abstract

The cell wall of gram-positive bacteria like *Streptococcus pneumoniae* consists of lipoteichoic acid (LTA) which is released when bacteria are killed by either the host immune system or antibiotic treatment. Release of excessive amounts of LTA has been implicated in the toxic sequelae of severe gram-positive infection by virtue of its proinflammatory properties. Several in vitro studies have shown that LTA is recognized by the pattern recognition receptors Toll-like receptor (TLR)-2 and CD14. However, data on receptor related LTA recognition in vivo are not available. To investigate the inflammatory properties of *S. pneumoniae* LTA in vivo and the role of TLR2, TLR4 and CD14 herein. Wild type (WT), TLR2 knock out (KO), TLR4 KO, TLR2x4 double KO and CD14 KO mice were intranasally inoculated with highly purified pneumococcal LTA. LTA induced a dose dependent neutrophil influx, cytokine and chemokine release and activation of the coagulation and fibrinolytic pathways in the bronchoalveolar compartment in a TLR2 dependent fashion. Surprisingly, TLR4 KO mice also displayed a somewhat diminished pulmonary inflammatory and coagulant response compared to WT mice, possibly as a result of absent TLR4 signaling through LTA-induced release of endogenous mediators. Pneumococcal LTA induces a profound inflammatory response and activation of the coagulation pathway in the lung in vivo by a TLR2 dependent route, which likely is amplified by endogenous TLR4 ligands.

Introduction

Streptococcus (S.) pneumoniae is the most commonly isolated pathogen in community acquired pneumonia, causing more than 500.000 cases each year in the United States (1, 2). Lipoteichoic acid (LTA) is a structure found in the cell wall of gram-positive bacteria, including the pneumococcus, anchored to the bacterial plasma membrane by hydrophobic interaction (3). LTA is a prominent mediator of the inflammatory response against gram-positive bacteria and in this respect is equivalent to lipopolysaccharide (LPS), a structure found in gram-negative bacteria. Moreover, LTA can be released from the cell wall when bacteria are killed by autolysis, host immune cells or antibiotic treatment (4-6). Release of large amounts of LTA has been implicated in systemic sequelae of infection, such as septic shock, by inducing an exuberant host-derived inflammatory response by leukocytes and as such contributes to mortality (6). For example, LTA levels in cerebral spinal fluid were significantly associated with neurological sequelae and mortality in *S. pneumoniae* meningitis (5).

The immune system recognizes pathogen associated molecular patterns through a repertoire of pattern recognition receptors, among which the family of Toll-like receptors (TLRs) prominently features (7, 8). Several studies have documented that LTA activates primary and transfected cells via TLR2 in collaboration with CD14 (9-15). Virtually all investigations on the biological properties of LTA have been done with LTA from *Staphylococcus (S.) aureus.* Only very recently, studies have begun to elucidate the biological properties of *S. pneumoniae* LTA. Whereas earlier studies reported a relatively low biological potency of pneumococcal LTA (13, 16, 17), some of us (C.D., S.v.A.) showed that the D-alanine substituents of LTA (present in *S. aureus* LTA and *S. pneumoniae* Fp23 LTA, but not in *S. pneumoniae* R6 LTA used in earlier investigations (13, 16, 17)) determined the cytokine-inducing potency of LTA (15).

The *in vivo* effect of *S. pneumoniae* LTA has never been investigated. In particular the effects of *S. pneumoniae* LTA within the intact pulmonary compartment is of relevance, considering that the pneumococcus is the most common pathogen in community-acquired pneumonia (1, 2). Therefore, in the present study we sought to determine the effect of highly purified LTA from *S. pneumoniae* Fp23 in the mouse

lung *in vivo* and the roles of CD14, TLR2 and TLR4 herein. We studied not only the pulmonary effects of pneumococcal LTA on lung inflammation, but also investigated bronchoalveolar coagulation, considering that an altered balance between coagulation and fibrinolysis has been implicated in the pathogenesis of pneumonia and lung injury (18, 19) and that staphylococcal LTA has been found to induce procoagulant activity in human mononuclear cells *in vitro* (20).

Methods

Animals: Specific pathogen free 8-10 week old C57BL/6 mice (WT) were purchased from Charles River (Maastricht, The Netherlands). TLR2 knockout (KO) mice and TLR4 KO mice were generated as described previously (21) (22) and backcrossed to a C57BL/6 genetic background 6 times. TLR2x4 KO mice were generated by crossing TLR2 KO and TLR4 KO mice. CD14 KO mice, backcrossed to a C57BL/6 genetic background, were obtained from Jackson Laboratory (Bar Harbor, Maine). All mice were bred in the animal facility of the Academic Medical Center in Amsterdam. In all experiments age and sex matched mice were used. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, the Netherlands).

Material: LTA from *S. pneumoniae* Fp23 (serotype 4) was prepared using butanol extraction and hydrophobic interaction chromatography as described earlier (15). Contamination of LPS in our LTA preparation was < 50 pg LPS/mg LTA as determined with the chromogenic Limulus Amoebocyte Lysate assay (LAL assay).

Experimental design: Mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, the Netherlands) after which 50 µl of sterile phosphate-buffered saline (PBS) or LTA dissolved in PBS was administered intranasally. The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). Bronchoalveolar lavage (BAL) was performed by instilling two 0.5-ml aliquots of sterile isotonic saline. Lavage fluid (0.9–1 ml/mouse) was retrieved, and total cell numbers were counted using Z2 Coulter particle count and size analyzer (Beckman-Coulter Inc., Miami, FL). Differential cell counts were determined in BAL fluid (BALF) using cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, IL).

Assays: Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and macrophage inflammatory protein (MIP)-2 were measured by ELISA (R&D Systems, Minneapolis, MN). Total protein level was measured using the BCA protein kit (Pierce, Rockford, IL). Thrombin-antithrombin complexes (TATc) (Dade Behring, Marburg, Germany), D-dimer (Asserachrom D-dimer, Roche, Woerden, the Netherlands) and plasminogen activator inhibitor type I (PAI-1) (23-25) were measured by ELISA. Myeloperoxidase (MPO) was measured by ELISA (Hycult Biotechnology BV, Uden, The Netherlands).

Statistical analysis: Data were analyzed by using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA. Mann Whitney U test or, where applicable, one-way ANOVA was used. Data are expressed as means ± SEM. A value of $P < 0.05$ was considered statistically significant.

Results

LTA induces a dose-dependent inflammatory response.

To determine the pulmonary inflammatory response to pneumococcal LTA *in vivo* we first inoculated WT mice with 0, 10 or 100 μg LTA via the intranasal route (Table I). BALF was harvested 6 hours later, since we previously established that this time point provides representative information on induction of inflammation and coagulation in models of lung inflammation (26-28). Intranasal inoculation with LTA induced an increase in total cell count using 100 μg LTA although this was not statistically significant. Surprisingly, whereas neutrophil counts increased dose dependently, macrophage counts decreased dose-dependently. The inflammatory response in the lungs, as reflected by neutrophil influx and release of cytokines (TNF-α, IL-1β, IL-6) and a chemokine (MIP-2) in BALF, increased dose dependently upon LTA administration, whereby the responses to LTA 100 μg were significantly stronger than the responses to LTA 10 μg. Moreover, LTA 100 μg but not LTA 10 μg elicited local activation of coagulation and fibrinolysis, as indicated by increases in BALF concentrations of TATc, D-dimer and PAI-1. Further experiments were done with LTA 50 μg. Considering that the LTA preparation used contained < 50 pg LPS per mg LTA (see Methods), LTA 50 μg contained < 2.5 pg LPS. In separate experiments we established that intranasal administration of LPS 2.5 pg did not induce neutrophil

influx or cytokine/chemokine release in WT mice (data not shown), confirming previous findings from our laboratory (26).

Table I: LTA induces a dose-dependent inflammatory response in the lungs of wild-type mice

LTA dose (µg/mouse)	0	10	100
Cell composition (x10^4 cells/ml)			
Total cell count	6.7 ± 1.1	5.8 ± 1.1	12.8 ± 3.2
Macrophage count	6.6 ± 1.1	4.1 ± 0.6	1.5 ± 0.5 * †
Neutrophil count	0.1 ± 0.1	1.7 ± 0.6 *	11.3 ± 2.9 * ‡
Cytokines and chemokines (pg/ml)			
TNF-α	57 ± 12	270 ± 33 *	2095 ± 538 * ‡
IL-1β	53 ± 9	115 ± 11 *	174 ± 25 *
IL-6	28 ± 5	93 ± 11 *	369 ± 90 * ‡
MIP-2	80 ± 17	126 ± 18	382 ± 75 * ‡
Coagulation and fibrinolysis			
TATc (ng/ml)	0.46 ± 0.05	0.66 ± 0.07	2.12 ± 0.26 * ‡
D-dimer (µg/ml)	0.12 ± 0.02	0.14 ± 0.03	0.38 ± 0.04 * ‡
PAI-1 (IU/ml)	1.08 ± 0.16	1.38 ± 0.14	4.26 ± 0.40 * ‡

WT mice were inoculated intranasally with 0, 10 or 100 µg LTA and killed 6 hours later. Data are mean ± SEM (N=5 per group). * P<0.01 vs. control, † P<0.05 versus 10 µg LTA, ‡ P<0.01 versus 10 µg LTA.

Role of TLR2, TLR4 and CD14 in LTA-induced lung inflammation

To investigate the role of TLR2, TLR4 and CD14 in the pulmonary inflammatory response to *S. pneumoniae* LTA we inoculated WT, TLR2 KO, TLR4 KO, TLR2x4 double KO and CD14 KO mice intranasally with LTA 50 µg and sacrificed them 6 hours later (Figures 1 and 2). TLR2 KO and TLR2x4 double KO mice displayed an equally strongly reduced inflammatory response after intrapulmonary delivery of LTA. Neither mouse strain demonstrated neutrophil influx (P<0.001 versus WT mice) in BALF, whereas surprisingly more macrophages were retrieved from BALF of these KO strains (both P<0.001 compared to WT mice).

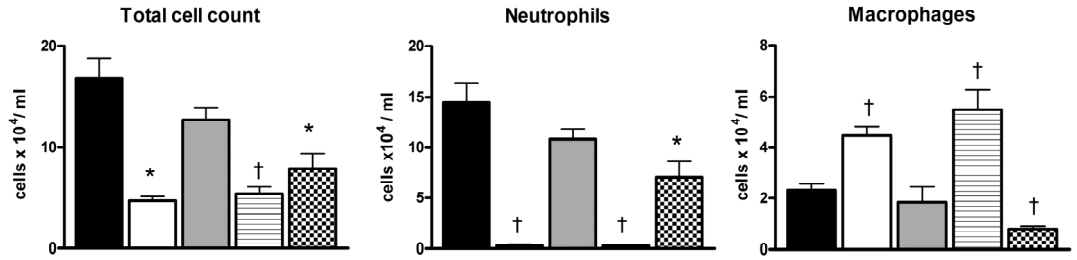

Figure 1: Cell composition in BALF of WT and TLR/CD14 KO mice. Total cell counts, macrophage counts and neutrophil counts in BALF of WT (black bars), TLR2 KO (white bars), TLR4 KO (grey bars), TLR2x4 double KO (horizontally-lined bars) and CD14 KO (blocked bars) mice 6 hours after inoculation of 50 μg LTA. Data are mean ± SEM (N=7-8 per group). * P<0.01, † P<0.001 vs. WT mice.

The release of TNF-α, IL-1β, IL-6 and MIP-2 into BALF from TLR2 and TLR2x4 double KO mice was strongly diminished (P< 0.01 to P<0.001 versus WT mice). CD14 KO mice displayed a reduced neutrophil influx (P<0.01) and lower IL-1β levels in BALF (P<0.05) compared to WT mice, whereas BALF TNF-α and MIP-2 concentrations tended to be lower (P=0.12 and P=0.07 respectively). Neutrophil influx upon LTA administration was not significantly altered in TLR4 KO mice. Remarkably, however, TLR4 KO mice did display reduced BALF levels of IL-1β, IL-6 and MIP-2 compared to WT mice (all P<0.05), although the differences with WT mice clearly were not as profound as for TLR 2 KO mice.

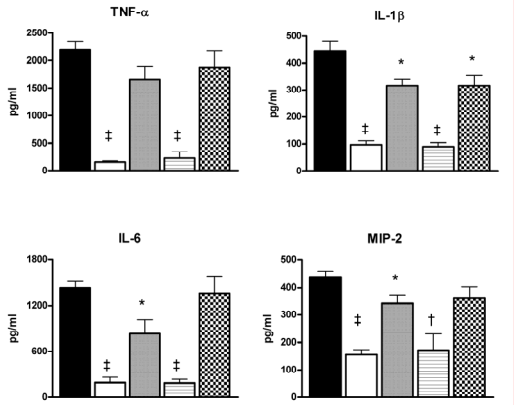

Figure 2: Role of TLR2, TLR4 and CD14 in LTA-induced cytokine release. Cytokine and chemokine concentrations in BALF of WT (black bars), TLR2 KO (white bars), TLR4 KO (grey bars), TLR2x4 double KO (horizontally-lined bars) and CD14 KO (blocked bars) mice 6 hours after inoculation of 50 μg LTA. Data are mean ± SEM (N=7-8 per group).
* P<0.05, † P<0.001, ‡ P<0.001 vs. WT mice.

In line, total protein levels in BALF, indicative of pulmonary vascular leakage, tended to be lower in both TLR2 KO and TLR2x4 double KO mice (P=0.05 and P=0.06 versus WT mice respectively) but unaltered in CD14 KO and TLR4 KO mice (WT: 564 ± 44, TLR2 KO: 454 ± 30, TLR4 KO: 631 ± 77, TLR2x4 double KO: 465 ± 28, CD14 KO: 583 ± 45 μg/ml, data are mean ± SEM, N=7-8 per group).

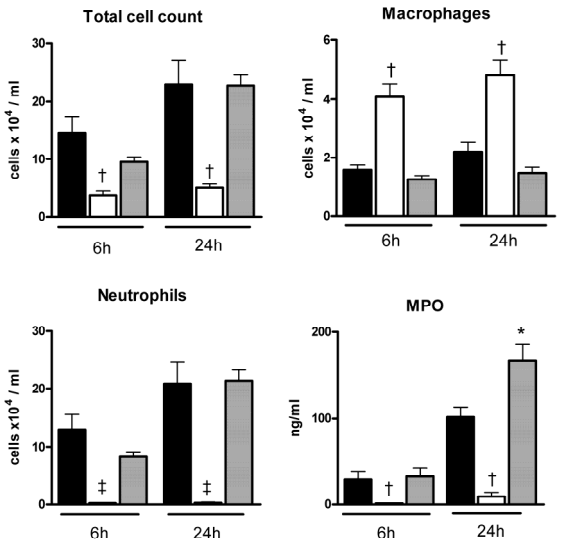

Figure 3: Cell composition and MPO in BALF of WT, TLR2 KO and TLR4 KO mice. Total cell counts, macrophage counts, neutrophil counts and MPO in BALF of WT (black bars), TLR2 KO (white bars) and TLR4 KO (grey bars) mice 6 and 24 hours after inoculation of 50 μg LTA. Data are mean ± SEM (N=7-8 per group). * P<0.01, † P<0.001 vs. WT mice.

To confirm and extend these data, we conducted additional studies in WT, TLR2 and TLR4 KO mice, obtaining BALF 6 and 24 hours after intranasal administration of LTA 50 μg. Again we established that the recruitment of neutrophils into BALF was strongly TLR2 dependent: TLR2 KO mice did not display neutrophil influx at either 6 or 24 hours (P<0.001 versus WT mice), whereas the number of neutrophils recovered from BALF of TLR4 KO mice did not differ from that in WT mice (Figure 3). Again, macrophage counts were higher in BALF from TLR2 and TLR2x4 double KO mice (both P<0.001 versus WT mice). In addition, to obtain insight into the capacity of LTA to elicit neutrophil degranulation and the roles of TLR2 and TLR4 herein, we measured MPO concentrations in cell-free BALF supernatants. Whereas MPO was not detectable in BALF from healthy mice (data not shown), LTA induced a time-dependent rise in BALF MPO concentrations, reaching maximal values at 24 hours (Figure 3). Local MPO release was delayed and strongly attenuated in TLR2 KO mice (P<0.001 versus WT mice). Interestingly, whereas at 6 hours BALF MPO levels in TLR4 KO and WT mice were indistinguishable, at 24 hours TLR4 KO mice demonstrated higher BALF MPO levels than WT mice (P<0.05). In these experiments cytokine and chemokine release elicited by LTA again proved to be largely TLR2 dependent (Figure 4). In accordance with the findings presented in Figure 2, relative to WT mice, TLR4 KO mice showed reduced cytokine/chemokine release in BALF, significantly so for MIP-2.

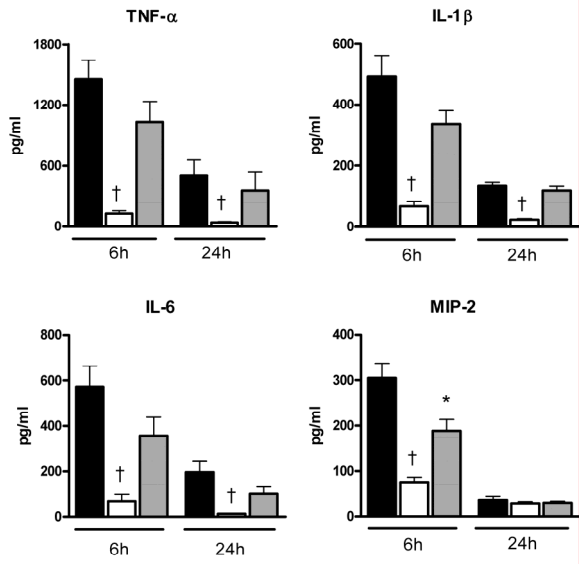

Figure 4: Role of TLR2 and TLR4 in the early and late inflammatory response to LTA. Cytokine and chemokine concentrations in BALF of WT (black bars), TLR2 KO (white bars) and TLR4 KO (grey bars) mice, 6 and 24 hours after inoculation of 50 µg LTA. Data are mean ± SEM (N=7-8 per group). * P<0.05, † P<0.001 vs. WT mice.

Role of TLR2 and TLR4 in LTA-induced pulmonary coagulation

Finally, to obtain insight into the role of TLR2 and TLR4 in LTA-induced activation of coagulation and fibrinolysis in the lung, we measured the concentrations of TATc, D-dimer and PAI-1 in BALF harvested 6 and 24 hours after the local LTA challenge (Figure 5). TLR2 KO mice demonstrated strongly reduced BALF levels of all three markers (P<0.01 to P<0.001 versus WT mice). TLR4 KO mice displayed a somewhat diminished hemostatic response in their bronchoalveolar space; in particular BALF TATc concentrations were lower than in WT mice (P<0.01 to P<0.001), whereas PAI-1 was modestly but significantly reduced at 24 hours (P<0.05).

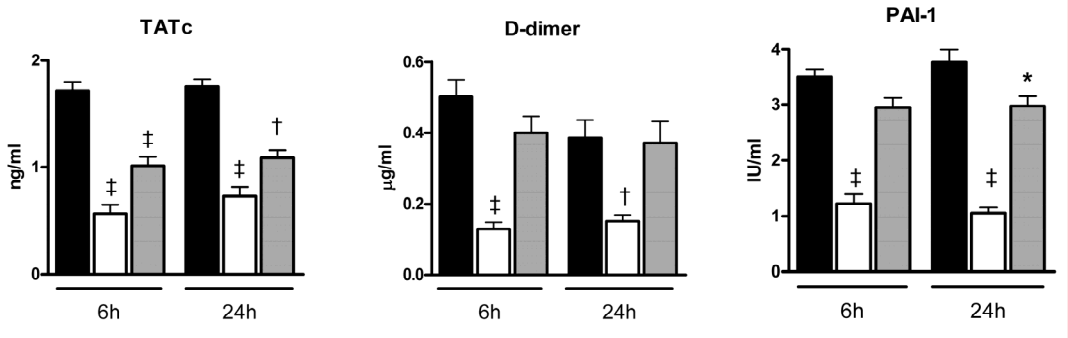

Figure 5: Role of TLR2 and TLR4 in the early and late activation of coagulation and fibrinolysis. BALF concentrations of TATc, D-dimer and PAI-1 in WT (black bars), TLR2 KO (white bars) and TLR4 KO (grey bars) mice, 6 and 24 hours after inoculation of 50 µg LTA. Data are mean ± SEM (N=7-8 per group). * P<0.05, † P<0.01, ‡ P<0.001 vs. WT mice.

Discussion

LTA is an important component of the pneumococcal cell wall and a potent inducer of cell activation *in vitro* via a TLR2 and partially CD14 dependent route (14, 15). Although LTA, released upon the killing of pneumococci by autolysis, host defense mechanisms, antibiotics or a combination of these, has been implicated in the toxic sequelae of pneumococcal infections (6), thus far studies on the biological effects of *S. pneumoniae* LTA *in vivo* had not been performed. We here show that pneumococcal LTA induces a dose dependent inflammatory response in the lung in a TLR2 dependent manner. Moreover, we show for the first time that pneumococcal LTA induces activation of the coagulation and fibrinolytic system in the bronchoalveolar compartment in a TLR2 dependent fashion. CD14 KO mice displayed only a mild reduction in the pulmonary inflammatory response compared to WT mice. Much to our surprise, TLR4 KO mice also had a modestly diminished inflammatory and procoagulant response to pneumococcal LTA.

Both *S. pneumoniae* and *S. aureus* LTA have inflammatory properties. Several studies have compared the biological potency of pneumococcal and *S. aureus* LTA *in vitro*, showing that pneumococcal LTA was less potent than *S. aureus* LTA; this difference originally was related to differences in their structures (13, 14). Indeed, stimulation of human peripheral blood mononuclear cells with *S. aureus* LTA induced more TNF-α production compared to pneumococcal LTA (13, 14). However, these earlier investigations used pneumococcal LTA derived from *S. pneumoniae* strains R6 or R36A. These strains lack D-alanine, which appears essential for the immunostimulatory potency of LTA: D-alanine containing LTA's from *S. aureus* and *S. pneumoniae* Fp23 proved equally potent in inducing cytokine release in human whole blood (15). We here demonstrate similar biological potency of *S. aureus* and *S. pneumoniae* Fp23 LTA in mice *in vivo*: *S. pneumoniae* Fp23 LTA induced a comparable dose dependent neutrophil influx and cytokine release in BALF as found for *S. aureus* LTA in earlier studies (27, 29, 30).

TLRs are a family of pattern recognition receptors that are capable of recognizing conserved molecular patterns expressed by pathogens (review (7, 8)). TLR2 has been implicated as the major pattern recognition receptor for gram-positive bacteria by

virtue of its capacity to recognize products of gram-positive organisms like LTA and peptidoglycan (11, 13, 14). To investigate whether pneumococcal LTA induces a TLR2-dependent inflammatory response *in vivo*, we inoculated LTA in WT and TLR2 KO mice. Neutrophil recruitment and cytokine and chemokine production were strongly reduced in TLR2 KO mice as compared to WT mice. Together with the fact that the early inflammatory response to intact pneumococci in the lower airways at least in part is dependent on TLR2 signaling (31), these data strongly support a role of LTA in the initiation of lung inflammation during respiratory tract infection by *S. pneumoniae*. Of note, this early interaction between TLR2 and LTA and possible other TLR2 ligands expressed by *S. pneumoniae* is not essential for induction of antibacterial defense mechanisms, as indicated by studies from our and another laboratory showing that TLR2 deficiency does not impact on the growth of pneumococci or the outcome in mouse models of *S. pneumoniae* pneumonia (31-33). Taken together, these data show that even though in pneumococcal pneumonia TLR2 can be compensated for by other receptors, recognition of pneumococcal LTA *in vivo* is clearly TLR2 dependent.

Interestingly, inoculation of pneumococcal LTA in WT mice resulted in a reduced recovery of alveolar macrophages from BALF. It is conceivable that local instillation of LTA into the lungs causes adhesion of alveolar macrophages to the respiratory epithelium thereby making them less easy to harvest by BAL. Clearly, this response was TLR2 dependent since it did not occur in TLR2 KO or TLR2x4 double KO mice. Further studies are warranted to study the mechanisms underlying this phenomenon.

Remarkably, compared to WT mice, TLR4 KO mice tended to display a diminished neutrophil recruitment and cytokine production 6 hours after inoculation of LTA. Moreover, especially MIP-2 was decreased in TLR4 KO mice compared to WT mice. Earlier studies showed contradictory results about the recognition of LTA by TLR2 (10, 13-15, 21, 34), possibly due to contamination of the LTA preparations with LPS (35). In our study a role for possible LPS contamination is highly unlikely for several reasons. First, inoculation of the LPS dose that, based on the LAL assay, could maximally contaminate the LTA preparation did not induce neutrophil influx or cytokine/chemokine production, confirming a previous report (26). Second, neutrophil recruitment and cytokine and chemokine production were similar in TLR2 and

TLR2x4 double KO mice, which argues against LPS-TLR4 signaling. Third, polymyxin B (an established inhibitor of LPS effects) did not influence cytokine release in human whole blood induced by the LTA preparation used here (15).
A possible explanation for the reduced inflammation in TLR4 KO mice could be the release of endogenous TLR ligands during LTA-induced inflammation (36-44). Several such endogenous mediators have been identified as TLR4 ligands, including fragmented hyaluronan, oxidation products, biglycans and heat shock proteins (review (44)); these could synergize in a TLR4 dependent way with LTA to cause an enhanced inflammatory response. However, it is not yet studied whether any of these factors are in fact induced by LTA stimulation. Other evidence for indirect effects of LTA in lungs *in vivo* comes from our finding of MPO release in BALF. Indeed, pneumococcal LTA (15), like *S. aureus* LTA (30), did not induce MPO release from isolated neutrophils *in vitro*. Likely, LTA-induced cytokines and chemokines are involved in these secondary effects relating to neutrophil degranulation.

CD14 is a glycosyl phosphatidylinositol surface anchored molecule and a pattern recognition receptor for several conserved bacterial motifs, including LPS, peptidoglycan and LTA (9, 45, 46). Membrane bound CD14 lacks an intracellular domain and requires interaction with other TLRs for signal transduction (47). CD14 is known to facilitate the recognition of and immune response to LTA *in vitro* (14); however, the contribution of CD14 to LTA signaling *in vivo* was previously unknown. We recently showed that CD14 plays an important role in the pathogenesis of pneumococcal pneumonia by a mechanism that does not rely on TLR signaling: CD14, either cell-bound or soluble, facilitated invasive respiratory tract infection by *S. pneumoniae* (48). We here demonstrated that the inflammatory response to pneumococcal LTA was only modestly attenuated in CD14 KO mice. Together these studies suggest that a possible CD14-LTA interaction does not contribute to TLR dependent lung inflammation during pneumococcal pneumonia to a significant extent.

Infection not only leads to an inflammatory response, but also to activation of the coagulation system, which has been considered to reflect an attempt of the host to limit the spread of bacteria and keep the inflammatory reaction local (49). Local activation of the coagulation system has been implicated in the pathogenesis of bacterial pneumonia (18, 19). Our laboratory previously showed that both patients and

mice with pneumococcal pneumonia display a compartmentalized activation of coagulation, reflected by elevated BALF levels of TATc, with a concurrent inhibition of fibrinolysis, reflected by elevated BALF PAI-1 concentrations, within their lungs (50, 51). We here demonstrate that intrapulmonary delivery of pneumococcal LTA reproduces these findings, implicating this cell wall constituent as a contributor to the altered hemostatic balance in the lung during respiratory tract infection by *S. pneumoniae*. Moreover, our data indicate that these local procoagulant responses to LTA are largely TLR2 dependent. The slightly reduced response in TLR4 KO mice may be explained by additional effects of endogenous mediators induced by LTA-TLR2 signaling that also may play a role in induction of lung inflammation (see above).

In conclusion, we here show for the first time that pneumococcal LTA induces a profound inflammatory response and activation of the coagulation and fibrinolytic pathways in the lungs in a largely TLR2 dependent manner. In addition, we report that although pneumococcal LTA activates TLR4 deficient cells as potently as WT cells *in vitro* (14, 15), TLR4 KO mice display a somewhat reduced responsiveness to LTA *in vivo*, suggesting the involvement of secondary endogenous TLR4 ligands induced by the interaction between LTA and TLR2. These results identify pneumococcal LTA containing D-alanine as a proinflammatory and procoagulant factor during respiratory tract infection by S. pneumoniae *in vivo*.

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Chapter 3

Role of Toll-like receptors 2 and 4 in pulmonary inflammation and injury induced by pneumolysin

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Abstract

Pneumolysin (PLN) is an intracellular toxin of *Streptococcus pneumoniae* that has been implicated as a major virulence factor in infections caused by this pathogen. Conserved bacterial motifs are recognized by the immune system by pattern recognition receptors among which the family of Toll-like receptors (TLRs) prominently features. Recent studies have identified TLR4 as a receptor involved in PLN signaling. The primary objective of the present study was to determine the role of TLR2 and TLR4 in lung inflammation induced by intrapulmonary delivery of PLN *in vivo*. First, we confirmed that purified PLN activates cells via TLR4 (not via TLR2) *in vitro*, using human embryonic kidney cells transfected with either TLR2 or TLR4. Intranasal administration of PLN induced an inflammatory response in the pulmonary compartment of mice *in vivo*, as reflected by influx of neutrophils, release of proinflammatory cytokines and chemokines and a rise in total protein concentrations in bronchoalveolar lavage fluid. These PLN-induced responses were dependent in part not only on TLR4 but also on TLR2, as indicated by studies using TLR deficient mice. These data suggest that although purified PLN is recognized by TLR4 *in vitro*, PLN elicits lung inflammation *in vivo* by mechanisms that may involve multiple TLRs.

Introduction

Streptococcus pneumoniae is the most frequently isolated pathogen in community acquired pneumonia (1, 2). Several pneumococcal proteins and enzymes have been implicated in the virulence of this bacterium and the pathogenesis of pneumonia (review (3)). Pneumolysin (PLN) is an intracellular peptide of *Streptococcus pneumoniae* that is present in virtually all clinical isolates (4, 5). PLN is considered to be an import virulence factor of the pneumococcus. Indeed, mice infected with a PLN-deficient strain of *S. pneumoniae* showed a reduced lethality and a diminished inflammatory response when compared to animals infected with PLN-producing *S. pneumoniae* (6, 7). PLN remains within the pneumococcus during bacterial growth, but is released when the pathogen is destroyed by the host immune system or due to antibiotic treatment (8). At sublytic doses, PLN activates the classical pathway of the complement system, induces cytokine production by macrophages and monocytes, inhibits the migration, respiratory burst and antibacterial activity of neutrophils and macrophages and affects ciliary beating of epithelial cells (9-13). At lytic doses, PLN can induce cell death; PLN interacts with cholesterol in the host-cell membrane resulting in the formation of transmembrane pores and death of the host (immune) cell (14). Our laboratory recently demonstrated that purified PLN induces neutrophil influx and the production of cytokines and chemokines in the lungs of mice (15). In addition, PLN dose dependently induced vascular permeability and pulmonary edema in mice (16, 17). Together these data suggest that PLN has a strong impact on the host response to invasion of the lower respiratory tract by *S. pneumoniae*.

Toll-like receptors (TLRs) are pattern recognition receptors that sense the presence of microorganisms by virtue of their capacity to recognize pathogen associated molecular patterns (review (18)). Recent studies have shown that PLN is recognized by TLR4 (19, 20). In addition, both PLN-induced cytokine production and PLN-induced apoptosis are mediated through TLR4 (19, 20). Although the functional interaction between PLN and TLR4 has been investigated extensively *in vitro*, the role of TLRs in PLN-induced pulmonary inflammation and injury in vivo is unkown. Here we sought to determine the roles of TLR2 and TLR4 in the pulmonary effects of purified PLN in mice *in vivo*.

Methods:

Cell cultures: Human embryonic kidney (HEK)-293 cells (21) transfected with CD14 and TLR2 or TLR4 (kindly provided by Douglas Golenbock, Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, MA) were grown in DMEM (1 mM pyruvate, 2 mM L-glutamine, penicillin, streptomycin and 10% fetal bovine serum). The murine alveolar macrophage cell line MH-S (American Type Culture Collection, Rockville, MD) was grown in RPMI 1640 (1 mM pyruvate, 2 mM L-glutamine, penicillin, streptomycin and 10% fetal bovine serum). The murine transformed ATII respiratory epithelial cell line MLE-15 (kindly provided by Jeffrey Whitsett, Division of Pulmonary Biology, Department of Pediatrics, Cincinnati Children's Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati) was grown in RPMI 1640 (5 mg/L insulin, 10mg/L transferin, 5 µg/L sodium selenite, 10 nM hydrocortisone, 10 nM B-esteradiol, 2mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 2% fetal bovine serum). In vitro stimulation was conducted in 96-well plates (Greiner, Alphen aan de Rijn, the Netherlands) at a density of 1 x 10^5 cells/ml (HEK and MH-S cells) or 5 x 10^4 cells/well (MLE-15 cells). All cell lines were allowed to adhere overnight at 37º C in a humidified atmosphere containing 5% CO_2 and stimulated the next day for 6 hours. For stimulation, HEK cells were co incubated with supernatant from MD-2-excreting HEK cells (21-23) together with either highly purified PLN (24), lipopolysaccharide (LPS from *Escherichia coli* O111:B4, Sigma Aldrich, St. Louis, MO) or lipoteichoic acid (LTA from *Staphylococcus aureus*) (25). In some experiments polymyxin B (Sigma Aldrich) was used at 10 µg/ml. Contamination of LPS in our PLN preparation was 4.6 ng/mg PLN as determined with the chromogenic Limulus Amoebocyte Lysate assay (LAL assay).

MTT assay: Supernatant of stimulated cells was removed and cells were incubated for 1-2 hours at 37º C with 100 µl 10% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich) solution (5 mg/ml) in medium. Thereafter MTT solution was replaced and cells were incubated with acetic isopropanol and firmly resuspended to dissolve violet crystals and incubated for 10 minutes. OD of 560 nm was used to measure metabolic activity and corrected for cell debris by OD 655 nm (26).

Animals: Specific pathogen free 8-10 weeks old C57BL/6 wild-type (WT) mice were purchased from Charles River (Maastricht, The Netherlands). TLR2 knockout (KO) mice and TLR4 KO mice (kindly provided by Shizuo Akira, Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Suita, Osaka, Japan) were generated as described previously (27, 28) and backcrossed six times to a C57BL/6 background. All mice were bred in the animal facility of the Academic Medical Center in Amsterdam. Age and sex matched mice were used in all experiments. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam.

Experimental design: Intranasal inoculation of PLN was performed as described earlier (15). Briefly, mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, the Netherlands) after which 50 µl of sterile phosphate-buffered saline (PBS) or PLN dissolved in PBS was administered intranasally. After 6 hours, mice were sacrificed and bronchoalveolar lavage (BAL) was performed. For this the trachea was exposed through a midline incision and cannulated with a 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). The lavage was performed by instilling two 0.5-ml aliquots of PBS. Lavage fluid was retrieved thereafter and counted using Z2 Coulter particle count and size analyzer (Beckman-Coulter Inc., Miami, FL.). Differential cell counts were determined in BAL fluid (BALF) using cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, Ill).

Assays: Mouse tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, cytokine-induced neutrophil chemoattractant (KC) and macrophages inflammatory protein (MIP)-2 and human IL-8 were measured by species specific ELISA's (R&D Systems, Minneapolis, MN). Total protein level was measured by BCA protein assay (Pierce, Rockford, IL).

Statistical analysis: Data are expressed as means ± SEM. Differences were analyzed by Mann Whitney U test or where applicable Student T-test. A value of $P < 0.05$ was considered statistically significant.

Results:

PLN-induced cytokine production *in vitro* is TLR4 dependent

Earlier studies have shown that PLN is recognized by TLR4 (19, 20). To confirm that our PLN preparation is recognized by TLR4, we incubated HEK cells transfected with either TLR2 or TLR4, with PLN. As positive controls for TLR4 and TLR2 signaling we also incubated HEK cells with LPS or LTA respectively (Figure 1). HEK cells not transfected with TLR2 or TLR4 did not respond to PLN, LPS or LTA. As expected, LTA and LPS – induced IL-8 production was dependent on the presence of TLR2 and TLR4 respectively (both $P<0.001$ versus control). PLN elicited IL-8 release by HEK-TLR4 but not by HEK-TLR2 cells. Polymyxin B fully inhibited LPS-TLR4 signaling but did not influence the effect of PLN on HEK-TLR4 cells, indicating that possible contaminating LPS can not explain the capacity of PLN to stimulate TLR4 (data not shown). Of note, according to the LAL assay, the concentration of PLN used to stimulate HEK cells contained < 1 pg LPS. HEK-TLR4 cells spontaneously produced more IL-8 compared to the other two HEK cell lines. Over expression of TLR4 in HEK cells has been shown to constitutively activate NF-κB resulting in a spontaneous activated condition (29) which could explain the elevated spontaneous release of IL-8. Overall, these data confirm earlier studies by Malley and coworkers (19), showing that PLN is recognized by TLR4.

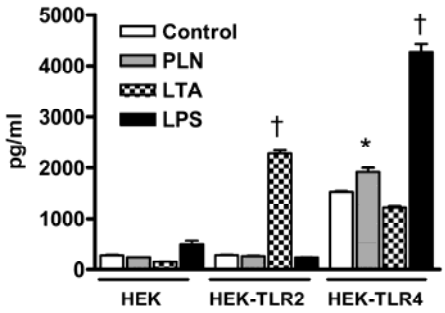

Figure 1: PLN activates HEK cells via TLR4. HEK-293 cells transfected with CD14 and either TLR2 or TLR4 were incubated with medium (control), LPS (100 ng/ml), LTA (5 µg/ml) or PLN (1 µg/ml) for 6 hours. Data are mean ± SEM (N=4 per group). * $P<0.01$ versus control, † $P<0.001$ versus control.

Dose-dependent inflammatory and lytic properties of PLN *in vitro*

Alveolar macrophages and pulmonary epithelial cells are the first cells in lungs to interact with PLN after intranasal inoculation. To investigate PLN-induced cytokine production and lysis of cells, we incubated mouse alveolar macrophage MH-S cells and mouse respiratory epithelial MLE-15 cells with increasing doses of PLN for 6

hours (corresponding with the observation period used in our *in vivo* experiments – see further). TNF-α and MIP-2 production from MH-S cells (Figure 2) and MIP-2 production from MLE-15 cells (Figure 3) increased dose dependently after incubation with PLN. PLN-induced TNF-α and MIP-2 production was not affected by the LPS inhibitor polymyxin B (data not shown). According to the LAL assay, the highest concentration of PLN used to stimulate MLE-15 and MH-S cells contained maximal 9 pg LPS. PLN is known to induce lysis of cells when incubated at high doses by inducing pores into the cell membrane (14). To further investigate the lytic properties of PLN we determined cell metabolic activities by MTT assay; a tool to measure the induction of cell death (26). Overall cell metabolic activity was reduced in MH-S and MLE-15 cells incubated with the highest PLN dose (10 µg/ml), indicative of enhanced cell death (Figure 2C; P=0.06 and Figure 3B; P<0.01 compared to control). Microscopic observations of MH-S and MLE-15 cells showed that after a 6-hour incubation with high doses of PLN, cells were ruffled and collapsed (Figure 2 and 3). Together these data suggest that PLN activates alveolar macrophages and respiratory epithelial cells to produce cytokines and/or chemokines at sublytic doses, whereas higher doses cause cell death.

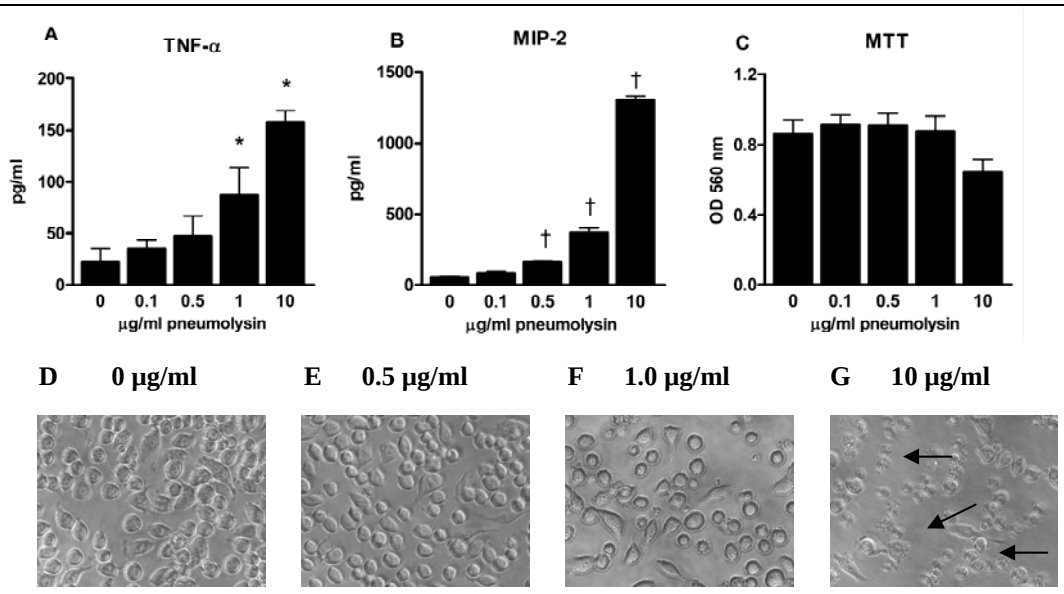

Figure 2: Inflammatory and cytolytic effects of PLN on mouse alveolar macrophage MH-S cells. MH-S cells were incubated with increasing doses of PLN for 6 hours and TNF-α (A), MIP-2 (B) and cell death (C) were determined thereafter. Cell death was measured using MTT assay as described in Methods section. Data are mean ± SEM (N= 5 per group). * P<0.05, † P<0.01 versus control. Microscopic observation of M-HS cells (D-G) stimulated with different dose of PLN for 6 hours, arrows indicate collapsed cells, magnification 10x.

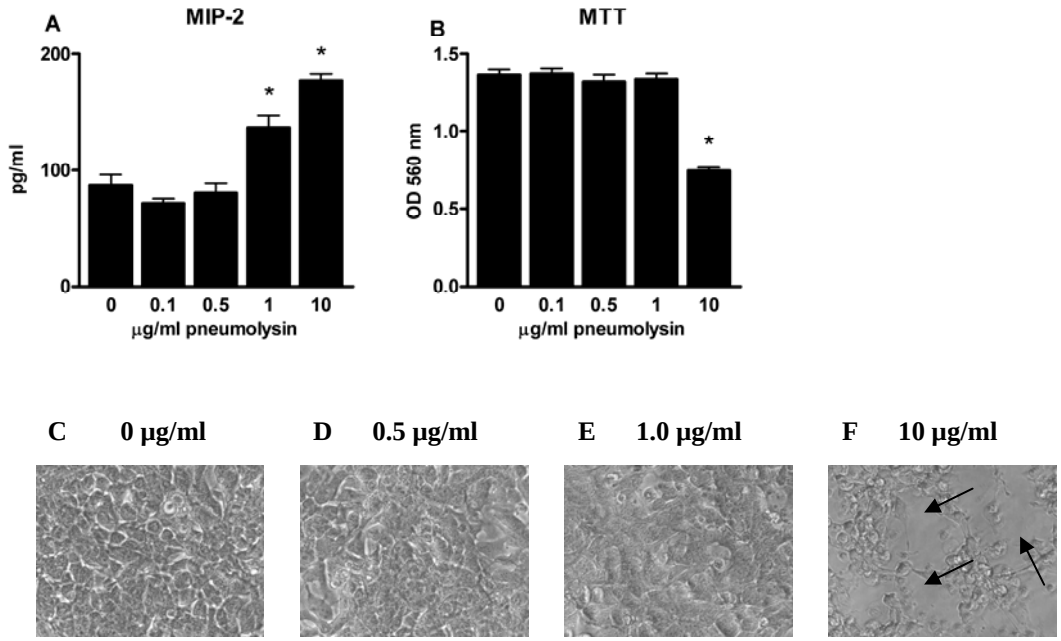

Figure 3: Inflammatory and cytolytic effects of PLN on mouse respiratory epithelial MLE-15 cells. MLE-15 cells were incubated with increasing doses of PLN for 6 hours and MIP-2 (A) and cell death (B) were determined thereafter. Cell death was measured using MTT assay as described in Methods section. Data are mean ± SEM (N= 5 per group). * P<0.01 versus control. Microscopic observation of MLE-15 cells (C-F) stimulated with different dose of PLN for 6 hours, arrows indicate disrupted monolayer, magnification 10x.

Role of TLR2 and 4 in PLN-induced lung inflammation and injury *in vivo*

Previous studies have documented the capacity of PLN to induce lung inflammation and injury in rodents *in vivo* (15, 16, 30). In preliminary experiments we first confirmed that PLN causes dose-dependent effects in the lungs of WT mice upon intranasal administration with respect to recruitment of neutrophils and release of cytokines and chemokines into the bronchoalveolar space, and with regard to pulmonary vascular leakage as determined by total protein levels in BALF (see below and data not shown). Based on these studies we investigated the roles of TLR2 and TLR4 in the effects of two PLN doses: one dose that caused modest lung inflammation and vascular leakage (25 ng/mouse, Figure 4) and one that caused profound lung inflammation and injury (500 ng/mouse, Figure 5). Notable, according to the LAL assay, the amount of LPS in the low and high dose PLN used in vivo contained respectively 0.12 pg LPS/mouse and 2 pg LPS/mouse which does not induce an inflammation (data not shown). Intranasal administration of PLN at a dose

of 25 ng induced a modest influx of leukocytes into BALF, which was caused by an increase in the number of alveolar macrophages and neutrophils ($P < 0.05$ versus PBS controls). In addition, PLN induced increases in the BALF levels of TNF-α, MIP-2 and KC (all $P < 0.05$ versus PBS controls), whereas IL-6 and IL-1β concentrations remained similar to PBS control mice (data not shown). Moreover, PLN 25 ng elicited a modest rise in BALF total protein concentrations ($P < 0.05$ versus PBS controls). These pulmonary responses to low dose PLN were unaltered in TLR2 and TLR4 KO mice with the exception of KC levels in BALF of TLR4 KO mice, which were reduced ($P<0.05$ compared to WT mice).

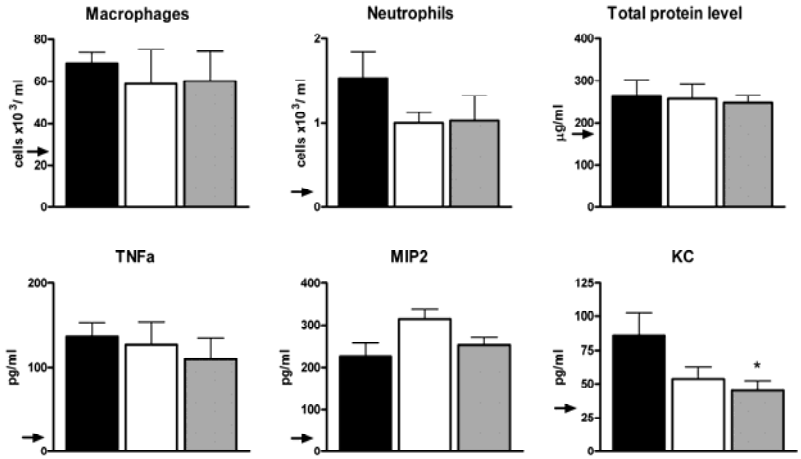

Figure 4: Roles of TLR2 and TLR4 in the lung inflammatory response to low dose PLN *in vivo*. Macrophage and neutrophil counts, total protein and TNF-α, MIP-2 and KC concentrations in BALF from WT (black bars), TLR2 KO (white bars) and TLR4 KO (grey bars) mice, 6 hours after inoculation of 25 ng /mouse (N=8 per group). Data are mean ± SEM. * $P<0.05$ versus WT mice. Arrow indicates mean value of PBS-treated WT mice.

Having established that the contribution of TLR2 and TLR4 to the lung inflammatory response to low dose PLN was neglectable, we next inoculated mice with a higher, lytic dose of PLN (500 ng/mouse). At this dose PLN elicited macrophage and neutrophil influx, release of IL-6, IL-1β, TNF-α and KC and a rise in total protein level in BALF of WT mice (all $P<0.05$ versus PBS control). MIP-2 levels remained below the detection limit. Six hours after intranasal administration of 500 ng PLN, TLR4 KO mice displayed reduced neutrophil influx, diminished IL-6, IL-1β and KC release and lower total protein levels in BALF when compared with WT mice ($P<0.05$ to $P<0.001$). Surprisingly, TLR2 KO mice also demonstrated significantly reduced

BALF levels of IL-6, KC and total protein compared to WT mice ($P < 0.05$). These observations were confirmed in a second independent experiment (data not shown). BALF TNF-α levels were similar in WT, TLR2 KO and TLR4 KO mice. Twenty-four hours after inoculation of PLN at 500 ng/mouse, the BALF cell composition was similar in WT, TLR2 KO and TLR4 KO mice and cytokine and chemokine levels were undetectable in all three mouse strains (data not shown). These data suggested that the induction of lung inflammation and injury by high dose PLN was dependent on the presence of TLR2 and TLR4.

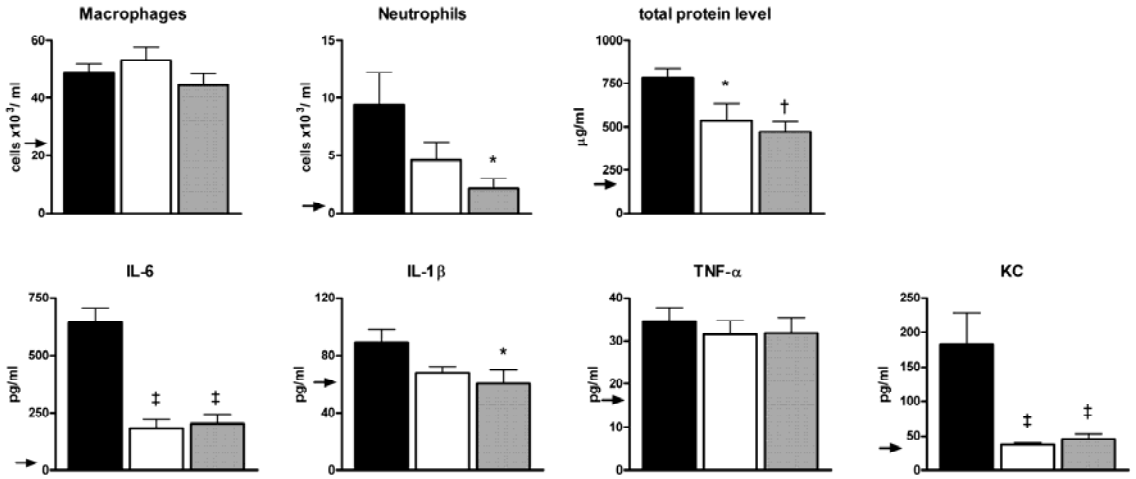

Figure 5: Roles of TLR2 and TLR4 in the lung inflammatory response to high dose PLN *in vivo*. Macrophage and neutrophil counts, total protein, IL-6, IL-1β, TNF-α and KC concentrations in BALF from WT (black bars), TLR2 KO (white bars) and TLR4 KO (grey bars) mice, 6 hours after inoculation of 500 ng/mouse (N=8 per group). Data are mean ± SEM. * P<0.05 versus WT mice, † P<0.01 versus WT mice, ‡ P<0.001 versus WT mice. Arrow indicates mean value of PBS-treated WT mice.

Discussion

The pneumococcal cell wall consists of several proteins and enzymes that contribute to the virulence of the pathogen and the pathogenesis of pneumonia (3). PLN is an intracellular toxin of *S. pneumoniae* that is present in all clinical isolates (4, 5). In a series of elegant experiments Malley et al. recently demonstrated that PLN is recognized by the immune system through a specific interaction with TLR4 (19, 20). The primary objective of the present investigation was to determine the contribution of TLR2 and TLR4 in lung inflammation and injury induced by PLN *in vivo*. First we confirmed the earlier *in vitro* findings of Malley et al. (19, 20), showing that our PLN preparation activated HEK cells via TLR4. We then revealed that intrapulmonary delivery of PLN induces an inflammatory response in the mouse lung that is dependent in part not only on TLR4, but also on TLR2. These data provide the first insight in the contribution of TLRs to the pulmonary effects of PLN *in vivo*.

Several *in vitro* studies have shown that sublytic doses of PLN, induce proinflammatory reactions in immune cells like neutrophils (12, 31), macrophages (32) and monocytes (9). Epithelial cells can detect very low concentrations of PLN (33) and this toxin can affect epithelial cell function by inhibiting the cilliary beating and disruption of tight junctions (13, 34-36). Alveolar macrophages and epithelial cells are the first cells to interact with respiratory pathogens upon invasion of the lower airways. Both cell types responded to PLN by production of cytokines and/or chemokines in a dose dependent manner, whereas high PLN doses caused enhanced cell death.

Our current findings of PLN-induced lung inflammation in WT mice confirm and extend previous studies. Two investigations reported leakage of the alveolar-endothelial barrier resulting in pulmonary edema after pulmonary instillation and aerosol delivery of PLN (17, 37). In addition, installation of PLN resulted in depletion of the alveolar macrophage pool and influx of neutrophils and monocytes; PLN-induced lung injury was associated with only a small increase in TNF-α and MIP-2 levels in BALF (37). In a study performed earlier in our laboratory, intranasal installation of PLN dose dependently induced neutrophil influx and IL-6, KC and MIP-2 production in the bronchoalveolar compartment (15). Here we utilized this

model of PLN-induced lung inflammation to determine the contribution of TLR2 and TLR4 to PLN effects *in vivo*. In line with the *in vitro* data generated by Malley et al. (19, 20), PLN responses in the lungs were (in part) TLR4 dependent: in particular KC release relied on the presence of TLR4, whereas other responses (neutrophil influx, protein leakage, cytokine release) were significantly reduced in TLR4 KO mice only after administration of high dose PLN. Remarkably, also TLR2 KO mice displayed a reduced responsiveness to PLN and this attenuated phenotype was not much different from that of TLR4 KO mice. A possible explanation could be that PLN induces endogenous ligands which may signal through TLR2 (and/or TLR4) (38, 39). One of these danger associated ligands is hyaluronan (39). However, BALF hyaluronan levels were even lower in TLR2 KO and TLR4 KO mice than in WT mice (data not shown), suggesting that hyaluronan concentrations in BALF may at least in part reflect pulmonary leakage. This however does not exclude that PLN induces other endogenous ligands which could signal through TLR2 and/or TLR4. The concept of endogenous TLR ligands amplifying host responses to inflammatory triggers is supported by our recent findings that highly purified LTA, which is an established TLR2 ligand (40-44), induces less profound lung inflammation not only in TLR2 KO mice, but also in TLR4 KO mice (M.C. Dessing et al., manuscript submitted).

The PLN used in this study was manufactured according to the method of Mitchell et al. which results in highly purified pneumococcal PLN (24). Several experiments were done to exclude that PLN-induced effects were caused by LPS contamination. First, polymyxin B, an established LPS inhibitor, did not influence PLN effects on HEK, MH-S or MLE-15 cells. Secondly, the highest PLN concentration used in vivo, contained 2 pg LPS/mouse which does not induce neutrophil influx or cytokine/chemokine release in WT mice (data not shown). Finally, heat inactivated PLN (80 oC, 60 minutes) did not induce lung inflammation in WT mice in vivo (data not shown), which - considering that LPS is heat stabile - further argues against LPS contamination. In addition, the fact that HEK-TLR2 cells did not respond to PLN argues against contaminating TLR2 ligands.

PLN is a major virulence factor in *S. pneumoniae* infections. We here show that PLN induces inflammation in the bronchoalveolar compartment of mice via mechanisms that rely in part on TLR2 and TLR4. Investigations seeking to unravel the complex

interactions between pneumococcal components and host immune cells in the lung may assist in understanding pathophysiological mechanisms at play during pneumonia caused by *S. pneumoniae*.

Acknowledgements

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Chapter 4

Toll-like receptor 2 contributes to antibacterial defense against pneumolysin-deficient pneumococci

Submitted

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Abstract

Streptococcus (S.) pneumoniae is the most common cause of community-acquired pneumonia. Toll-like receptors (TLR) are pattern recognition receptors that recognize conserved molecular patterns expressed by pathogens. Pneumolysin, an intracellular toxin found in all *S. pneumoniae* clinical isolates, is an important virulence factor of the pneumococcus that is recognized by TLR4. Although TLR2 is considered the most important receptor for gram-positive bacteria by virtue of its capacity to recognize several gram-positive cell wall components, our laboratory previously could not demonstrate a decisive role for TLR2 in host defense against pneumonia caused by a serotype 3 *S. pneumoniae*. Here we tested the hypothesis that in the absence of TLR2 *S. pneumoniae* can still be sensed by the immune system through an interaction between pneumolysin and TLR4. C57BL/6 wild type (WT) and TLR2 knockout (KO) mice were intranasally infected with either WT *S. pneumoniae* D39 (serotype 2) or the isogenic pneumolysin deficient *S. pneumoniae* strain D39 PLN. TLR2 did not contribute to antibacterial defense against WT *S. pneumoniae* D39. In contrast, pneumolysin deficient *S. pneumoniae* only grew in lungs of TLR2 KO mice, demonstrating a > 10-fold increase in the pulmonary bacterial loads between 24 and 72 hours after infection. TLR2 KO mice displayed a strongly reduced early inflammatory response in their lungs during pneumonia caused by both pneumolysin-producing and deficient pneumococci. These data suggest that pneumolysin-induced TLR4 signalling can compensate for TLR2 deficiency during respiratory tract infection with *S. pneumoniae.*

Introduction

Streptococcus (S.) pneumoniae is the most common cause of community-acquired pneumonia (1, 2). Infections caused by *S. pneumoniae* are increasingly difficult to treat due to the emergence of antibiotic resistant strains (3, 4). Increased knowledge of the first interaction between *S. pneumoniae* and host immune cells may facilitate the development of novel prophylactic and therapeutic tools to combat pneumococcal infections. In this respect Toll-like receptors (TLRs), a family of pattern recognition receptors that are capable of recognizing conserved molecular patterns expressed by pathogens, are of particular interest (5, 6).

The pneumococcal cell wall consists of several proteins and enzymes that contribute to the virulence of the pathogen and the pathogenesis of pneumonia (7). Pneumolysin is an intracellular toxin found in *S. pneumoniae* which is produced by all clinical isolates and is an important factor for the virulence of the pneumococcus (8). Indeed, mice infected with a pneumolysin-deficient strain of *S. pneumoniae* showed a reduced lethality and a diminished inflammatory response compared to mice infected with a normal, pneumolysin-producing strain (9-14). At sublytic dose, pneumolysin affects polymorphonuclear cell activity including respiratory burst, degranulation, chemotaxis and bactericidal activity (15). Furthermore, pneumolysin activates the classical pathway of complement and induces cytokine production by macrophages and monocytes (16-18). At lytic dose, pneumolysin forms ring-shaped pores in cholesterol containing cell membranes which results in cell death (19, 20). Recent work has suggested that the immune system recognizes pneumolysin through TLR4 (21, 22). Both pneumolysin-induced cytokine production and pneumolysin-induced apoptosis are mediated through TLR4 (21, 22). In a model of nasopharyngeal colonization by *S. pneumoniae*, the interaction between pneumolysin and TLR4 was found to be essential for preventing invasive disease (21). Our laboratory reported a protective role of TLR4 during infection of the lower respiratory tract by *S. pneumoniae*, demonstrating an enhanced growth of bacteria in lungs of TLR4 deficient mice (23).

Within the family of TLRs TLR2 has been implicated as the major pattern recognition receptor for ligands derived from gram-positive bacteria (5, 24-26). However, our laboratory recently demonstrated that TLR2 does not play a key role in host resistance to pneumonia caused by a serotype 3 strain of *S. pneumoniae* (27). We here hypothesized that TLR2 KO mice have an intact protective immune response against *S. pneumoniae* because they are still capable of activating their immune system through an interaction between pneumolysin and TLR4. If this assumption is true, TLR2 KO mice would display a reduced antibacterial defense against pneumolysin deficient *S. pneumoniae*, considering that these modified bacteria, devoid of a major TLR4 ligand, would primarily express TLR2 ligands. Therefore, in the present study we compared the response of TLR2 KO and WT mice during respiratory tract infection with WT and pneumolysin-deficient *S. pneumoniae*.

Material and methods:

Animals: C57BL/6 WT mice were purchased from Charles Rivers (Maastricht, The Netherlands). TLR2 KO mice (28), backcrossed to a C57BL/6 genetic background six times, were bred in the animal facility of the Academic Medical Center in Amsterdam. Sex and age matched (10-12 weeks) mice were used in all experiments. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam.

Design: The experimental procedures to induce pneumonia have been described earlier (23, 27, 29). *S. pneumoniae* serotype 2 (strain D39) and isogenic pneumolysin deficient *S. pneumoniae* (strain PLN) (9) were grown for 5 hours to mid-logarithmic phase at 37°C using Todd-Hewitt broth (Difco, Detroit, MI), harvested by centrifugation at 1500x*g* for 15 min, and washed twice in sterile isotonic saline. Fifty µl containing 5 x 10^7 colony forming units (CFU) were inoculated intranasally in mice which were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, the Netherlands). Mice were killed 6, 24 or 48 hours after infection with *S. pneumoniae* D39 or 6, 24, 48 or 72 hours after infection with *S. pneumoniae* PLN. In separate studies, survival of mice was determined during a 2-week follow up.

Measurement of bacterial loads: Lung bacterial loads were determined as described earlier (23, 27, 29). Briefly, mice were sacrificed and blood and lungs were collected.

Lungs were homogenized at 4^0C in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, OK) Serial 10-fold dilutions in sterile isotonic saline were made from these homogenates (and blood), and 50 µl volumes were plated onto sheep-blood agar plates and incubated overnight at 37^0C and 5% CO_2.

Histology: Lungs for histology were fixed in 10% formalin and embedded in paraffin. Four µm sections were stained with hematoxylin and eosin (HE) and analyzed by a pathologist who was blinded for groups. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: bronchitis, edema, interstitial inflammation, intra-alveolar inflammation, pleuritis and endothelialitis. Each parameter was graded on a scale of 0 to 4 with 0 as 'absent' and 4 as 'severe'. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 24. Granulocyte staining was done as described earlier by Ly-6G staining (29).

Assays: Lung homogenates were prepared as described earlier (27). Myeloperoxidase (MPO) was measured by ELISA (HyCult, Uden, the Netherlands). Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-10, macrophage inflammatory protein (MIP)-2 and cytokine-induced neutrophil chemoattractant (KC) were measured by ELISA (R & D Systems, Abingdon, UK).

Statistical analysis: Statistics were performed with GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego CA. All data are given as means ± SEM. Differences between groups were analyzed using Mann-Whitney U test. For survival analyses, Kaplan-Meier analysis followed by log rank test was performed. A value of $P < 0.05$ was considered statistically significant.

Results:

TLR2 does not contribute to host defense and pulmonary inflammation against pneumonia caused by WT *S. pneumoniae* D39.

We previously showed that TLR2 KO mice are indistinguishable from WT mice with regard to bacterial outgrowth and mortality after intranasal infection with a serotype 3 *S. pneumoniae* strain (27). Considering that the pneumolysin deficient strain used here is a serotype 2 (derived from *S. pneumoniae* D39), we first investigated the impact of TLR2 deficiency on the course of pneumonia caused by WT *S. pneumoniae* D39 (Figure 1). Mortality did not differ between TLR2 KO and WT mice after intranasal infection with *S. pneumoniae* D39; if anything, TLR2 KO mice displayed a slightly reduced mortality (62.5 %) although the difference with WT mice (75 % mortality) was not significant (P = 0.13; Figure 1A). We next determined bacterial loads in whole lung homogenates at 24 and 48 hours after infection, i.e. at time points just before the first mice started dying (Figure 1B). At both 24 and 48 hours, bacterial loads were identical in lungs of TLR2 KO and WT mice. Together these data extend our earlier study using a serotype 3 *S. pneumoniae* strain (27), showing that TLR2 does not contribute to a protective immune response during pneumonia caused by a serotype 2 pneumococcus.

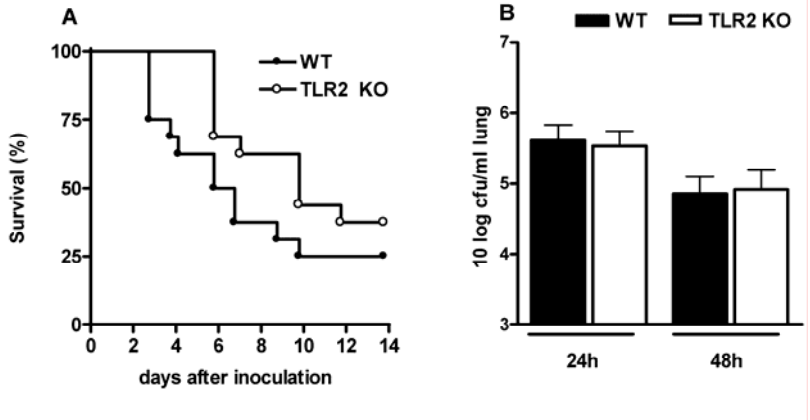

Figure 1: TLR2 does not contribute to host defense against WT *S. pneumoniae*. Survival (1A) and bacterial outgrowth (1B) of WT mice (closed symbols or bars) and TLR2 KO mice (open symbols or bars) with 5 x 10^7 CFU's *S. pneumoniae* D39. Mortality was assessed four times daily for 14 days (N= 16 per group)**.** Bacterial loads in WT mice and TLR2 KO mice were determined 24 and 48 hours after infection. Data of bacterial loads are mean ± SEM (N=7-8 per group at each timepoint).

TLR2 deficiency modestly attenuates the inflammatory response induced by WT *S. pneumoniae* D39.

Cytokines and chemokines play an important role in the antibacterial defense against bacterial pneumonia (30, 31). We therefore determined the concentrations of TNF-α, IL-1β, IL-10, MIP-2 and KC in whole lung homogenates obtained 24 and 48 hours after inoculation (Table I). Although in general the pulmonary concentrations of these mediators were lower in TLR2 KO mice, the differences with WT mice were statistically significant only for KC ($P<0.005$ at 24 and 48 hours post infection) and IL-1β ($P<0.05$ at 48 hours). To further investigate lung inflammation we determined pulmonary MPO levels, reflecting the whole organ neutrophil content, in TLR2 KO mice and WT mice (Table I). Similar to cytokine and chemokine levels, MPO concentrations were modestly lower in TLR2 KO, significantly so at 48 hours post infection ($P<0.01$). Moreover, total lung inflammation scores, determined from lung tissue slides prepared 24 and 48 hours after infection with *S. pneumoniae* D39, were similar in WT and TLR2 KO mice (Table 1). Together, these data obtained with a serotype 2 pneumococcus confirm our earlier data generated with a serotype 3 *S. pneumoniae* (27), establishing that TLR2 plays a modest role in the induction of a pulmonary inflammatory response to respiratory tract infection with WT *S. pneumoniae*.

Table I: Parameters of lung inflammation in TLR2 KO and WT mice 24 and 48 hours after infection with WT *S. pneumoniae* D39.

	T= 24 h		T=48 h	
	WT	TLR2 KO	WT	TLR2 KO
TNF-α	1229 ± 351	1026 ± 212	500 ± 92	361 ± 63
IL-1β	4029 ± 599	3248 ± 495	2874 ± 594	1462 ± 329 *
IL-10	62 ± 23	48 ± 14	123 ± 51	72 ± 13
MIP-2	5912 ± 876	5689 ± 1039	1447 ± 177	1182 ± 243
KC	5943 ± 867	2244 ± 510 ‡	3481 ± 339	695 ± 90 ‡
MPO	7668 ± 1123	6158 ± 2317	9183 ± 2365	4057 ± 578 †
TLIS	16.7 ± 0.9	16.8 ± 1.0	13.5 ± 0.6	15.5 ± 0.8

Mice were intranasally infected with 5 x 10^7 CFU's WT *S. pneumoniae* D39; whole lung homogenates were obtained 24 and 48 hours thereafter. Data are means ± SEM (N= 6 - 8 per group). * $P<0.05$ versus WT mice. † $P < 0.01$ versus WT mice. ‡ $P < 0.005$ versus WT mice. TNF-α, IL-1β, IL-10, MIP-2 and KC values are in pg/ml, MPO values are in ng/ml. TLIS = total lung inflammation score in arbitrary units.

TLR2 limits the outgrowth of pneumolysin-deficient *S. pneumoniae* PLN.

Having established that TLR2 is not essential for host defense against WT *S. pneumoniae* D39, we next infected TLR2 KO and WT mice with the isogenic mutant *S. pneumoniae* PLN (Figure 2). As expected (9), *S. pneumoniae* PLN was less virulent, in particular in WT mice. Only 23 % of WT mice died during a 2-week follow up, versus 38 % of TLR2 KO mice (not significant for the difference between mouse strains; P= 0.29; Figure 2A). Interestingly, TLR2 KO mice started to die after 3 days, whereas the first deaths among WT mice occurred after 5 days. To obtain insight in the growth of *S. pneumoniae* PLN during the infection (i.e. before the first mice started dying), we infected TLR2 KO and WT mice with *S. pneumoniae* PLN and determined bacterial loads in whole lung homogenates at 24, 48 and 72 hours thereafter (Figure 2B). Whereas the bacterial burdens were not significantly different between TLR2 KO and WT mice 24 hours post infection, at 48 and 72 hours TLR2 KO displayed significantly higher bacterial loads in their lungs than WT mice (both $P < 0.05$). Remarkably, whereas *S. pneumoniae* PLN did not further grow in the lungs of WT mice from 24 hours after infection onward, which is in line with a previous investigation (11), the bacterial load increased > 10-fold in lungs of TLR2 KO mice between 24 and 72 hours after inoculation. Hence, these data show that TLR2 serves to limit the growth of *S. pneumoniae* PLN during pneumonia.

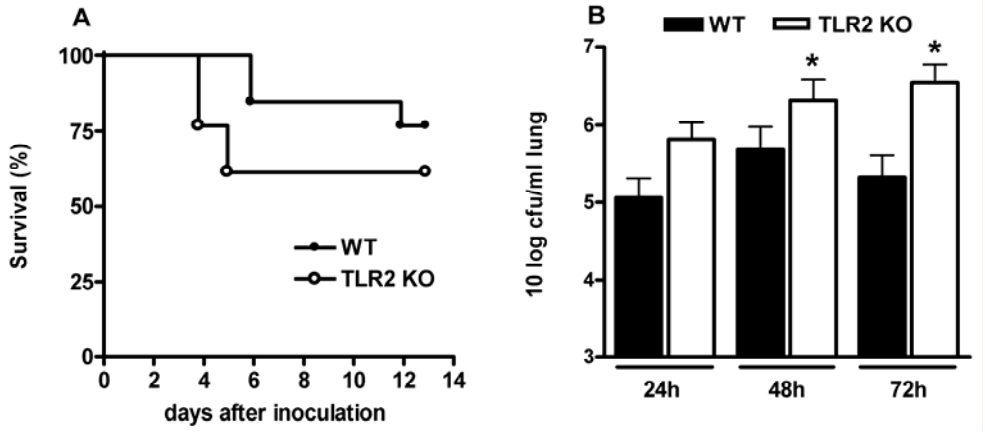

Figure 2: TLR2 limits outgrowth of pneumolysin deficient *S. pneumoniae* PLN. Survival (2A) and bacterial outgrowth (2B) of WT mice (closed symbols or bars) and TLR2 KO mice (open symbols or bars) with 5 x 10^7 CFU's *S. pneumoniae* PLN. Mortality was assessed four times daily for 14 days (N= 13 per group)**.** Bacterial loads in WT mice and TLR2 KO mice were determined 24, 48, and 72 hours after infection. Data of bacterial loads are mean ± SEM (N=7-8 per group at each timepoint) * P<0.05 versus WT mice.

TLR2 deficiency reduces lung inflammation induced by *S. pneumoniae* PLN.

To further investigate the role of TLR2 during infection with *S. pneumoniae* PLN we determined TNF-α, IL-1β, IL-10, MIP-2, KC and MPO levels in whole lung homogenates obtained 24, 48 and 72 hours after inoculation (Table II). At 24 hours post infection, pulmonary cytokine and chemokine levels were lower in TLR2 KO mice, significantly so for IL-1β ($P<0.05$). In addition, lung MPO levels were lower in TLR2 KO mice at this time point ($P<0.05$). In contrast, at 48 and 72 hours after infection, when TLR2 KO mice displayed higher bacterial burdens in their lungs, the pulmonary concentrations of cytokines, chemokines and MPO did not differ between TLR2 KO and WT mice. Histopathological analyses of lung tissue slides demonstrated reduced lung inflammation in TLR2 KO mice at 24 hours, but increased lung inflammation at 48 hours and 72 hours (Table II). Representative lung tissue slides from WT and TLR2 KO mice 24, 48 and 72 hours after infection with *S. pneumoniae* PLN are shown in Figure 3.

Table II: Parameters of lung inflammation in TLR2 KO and WT mice 24, 48 and 72 hours after infection with pneumolysin deficient *S. pneumoniae* PLN.

	T = 24 h		T = 48 h		T = 72 h	
	WT	TLR2 KO	WT	TLR2 KO	WT	TLR2 KO
TNF-α	325 ± 77	295 ± 87	195 ± 34	195 ± 34	506 ± 114	522 ± 163
IL-1β	2414 ± 519	903 ± 496 *	552 ± 215	802 ± 200	1724 ± 638	2092 ± 956
IL-10	B.D.	B.D.	B.D.	B.D.	135 ± 27	153 ± 63
MIP-2	2703 ± 1033	1956 ± 1001	1919 ± 194	2720 ± 649	1029 ± 589	1297 ± 692
KC	1781 ± 791	600 ± 158	1700 ± 980	610 ± 188	1385 ± 323	958 ± 391
MPO	3173 ± 391	1666 ± 411 *	1675 ± 424	2205 ± 226	2722 ± 251	2402 ± 500
TLIS	7.7 ± 2.0	3.1 ± 1.2	6.9 ± 1.6	11.9 ± 2.1	5.6 ± 0.9	8.7 ± 0.8 *

Mice were intranasally infected with 5 x 10^7 CFU's *S. pneumoniae* PLN; whole lung homogenates were obtained 24, 48 and 72 hours thereafter. Data are means ± SEM (N= 6 - 8 per group). * $P<0.05$ versus WT mice. † $P < 0.001$ versus WT mice. TNF-α, IL-1β, IL-10, MIP-2 and KC values are in pg/ml, MPO values are in ng/ml. B.D. = below detection limit. TLIS = total lung inflammation score in arbitrary units.

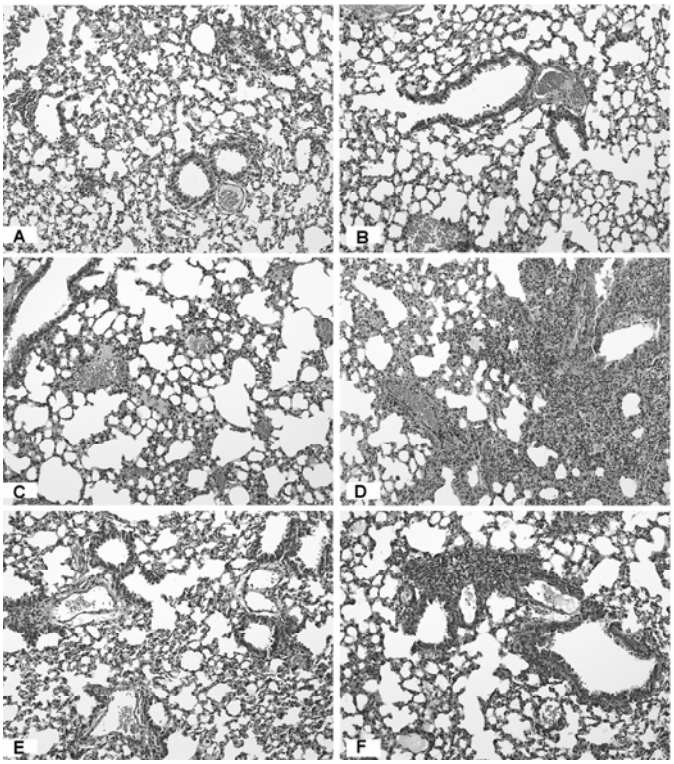

Figure 3: Lung histology in WT and TLR2 KO mice after infection with S. *pneumoniae* PLN. Representative lung tissue slides from WT mice (panel A, C and E) and TLR2 KO mice (panel B, D and F) after infection with 5 x 10^7 CFU's *S. pneumoniae* PLN. Mice were sacrificed after 24 (panel A and B), 48 (panel C and D) or 72 (panel E and F) hours. Magnification 4x. HE staining.

TLR2 deficiency strongly impairs the early inflammatory response to both *S. pneumoniae* D39 and *S. pneumoniae* PLN.

The role of TLR2 in lung inflammation later in the course of pneumonia could have been obscured by the growing bacterial load in TLR2 KO mice (that is, at 48 and 72 hours after infection TLR2 deficiency could be compensated for by the higher bacterial load, providing a more potent proinflammatory stimulus via TLR2 independent pathways). An earlier study performed at our laboratory has shown an important role for TLR2 in the early host defense against *S. pneumoniae* pneumonia using serotype 3 (27). TLR2 could also be more important for the early host inflammatory response to *S. pneumoniae* with serotype 2. Thus, we intranasally inoculated TLR2 KO and WT mice with *S. pneumoniae* D39 or *S. pneumoniae* PLN and evaluated their response to the infection 6 hours later (Table III). The bacterial loads in lungs of TLR2 KO mice and WT mice were similar at this early time point during infection with *S. pneumoniae* D39 or *S. pneumoniae* PLN. Interestingly, compared to WT mice, TLR2 KO mice showed a strongly reduced capacity to respond to both *S. pneumoniae* D39 and *S. pneumoniae* PLN; the lung concentrations of TNF-α, IL-1β, MIP-2 and KC were much lower in TLR2 KO mice ($P<0.05$ to

P<0.001) (Table III). In addition, histopathological analyses of lung tissue slides demonstrated a significantly reduced inflammation in lungs of TLR2 KO mice 6 hours after infection with *S. pneumoniae* D39 or *S. pneumoniae* PLN (Figure 4). Of note, some inflammatory responses to *S. pneumoniae* PLN were more strongly reduced in TLR2 KO mice than the inflammatory responses to *S. pneumoniae* D39. In particular, whereas in WT mice *S. pneumoniae* D39 and PLN induced a similar early TNF-α response in the lungs, the pulmonary levels of this crucially protective cytokine in the early response to pneumococcal pneumonia (32, 33), were > 4-fold lower in TLR2 KO mice after infection with *S. pneumoniae* PLN versus 2-fold lower after inoculation with *S. pneumoniae* D39. In addition, whereas overall *S. pneumoniae* PLN elicited less profound histopathological alterations in lung tissue than *S. pneumoniae* D39, the difference in total lung histology scores between TLR2 KO and WT mice was especially clear after infection with the pneumolysin deficient strain.

Table III: Role of TLR2 in the early inflammatory response in the lungs after infection with *S. pneumoniae* D39 or PLN.

	D39		PLN	
	WT	TLR2 KO	WT	TLR2 KO
CFU	$6.5 \pm 1.4 \times 10^6$	$5.5 \pm 1.3 \times 10^6$	$13.2 \pm 1.3 \times 10^6$	$10.6 \pm 1.8 \times 10^6$
TNF-α	9781 ± 780	4656 ± 375 ‡	9124 ± 1203	2270 ± 236 ‡
IL-1β	5754 ± 714	3566 ± 348 *	18963 ± 2522	8716 ± 1939 *
IL-10	31 ± 2	32 ± 3	B.D.	B.D.
MIP-2	42193 ± 2529	23855 ± 3396 †	9114 ± 1112	2643 ± 505 ‡
KC	61120 ± 2879	23266 ± 3124 †	16776 ± 2314	4783 ± 1352 ‡
MPO	76.7 ± 9.3	41.6 ± 3.4 *	15.5 ± 3.4	12.4 ± 4.0
TLIS	18.4 ± 0.5	15.1 ± 0.8 *	9.5 ± 0.9	5.3 ± 1.3 *

Mice were intranasally infected with 5×10^7 CFU's *S. pneumoniae* D39 or PLN and whole lung homogenates were obtained 6 hours later. Data are means ± SEM (N= 8 per group). * P <0.05, † P<0.01, ‡ P < 0.001 versus WT mice. TNF-α, IL-1β, IL-10, MIP-2 and KC values are in pg/ml, CFU values are in CFU/ml lung. MPO levels are in µg/ml. TLIS = total lung inflammation score in arbitrary units. B.D. = below detection limit.

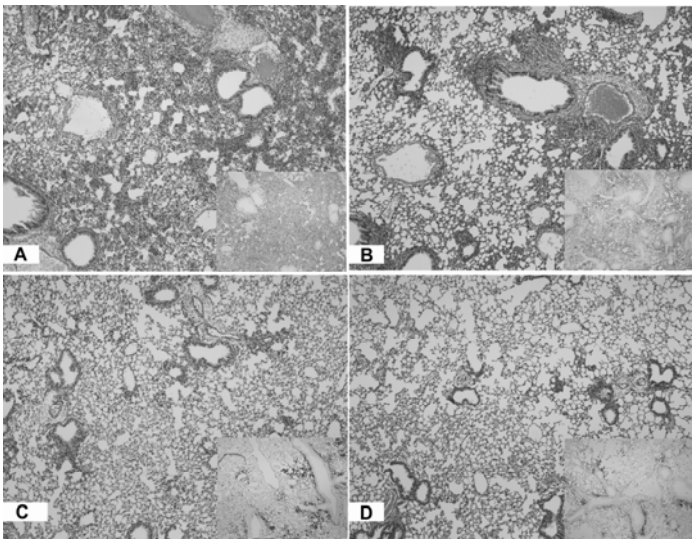

Figure 4: Reduced lung inflammation in TLR2 KO mice early after infection with *S. pneumoniae* D39 or *S. pneumoniae* PLN. Representative lung tissue slides from WT mice (panel A and C) and TLR2 KO mice (panel B and D) 6 hours after infection with 5 x 10^7 CFU's *S. pneumoniae* D39 (panel A and B) or *S. pneumoniae* PLN (panel C and D). HE staining: magnification 4x. Insets show Ly-6G staining.

Discussion

Pneumolysin is an essential virulence factor of *S. pneumoniae* (8). Recent studies have identified TLR4 as a recognition receptor for pneumolysin in the respiratory tract (21, 22). The interaction between pneumolysin and TLR4 was found to contribute to a protective immune response to *S. pneumoniae*, in particular in a model of upper airway colonization (21, 22) and to a lesser extent during experimental lower respiratory tract infection (23). Although the pneumococcus expresses several potent TLR2 ligands (24-26), our laboratory previously could not demonstrate a decisive role for TLR2 in host defense against pneumococcal pneumonia (27). We here hypothesized that in the absence of TLR2, *S. pneumoniae* could still be sensed by the immune system through an interaction between pneumolysin and TLR4. The experiments described herein support this hypothesis: whereas the growth of WT pneumococci occurred to a similar extent in TLR2 KO and WT mice, the pneumolysin deficient *S. pneumoniae* PLN strain only grew out in TLR2 KO mice. These data suggest that pneumolysin-induced TLR4 signaling can compensate for TLR2 deficiency during respiratory tract infection with *S. pneumoniae*.

In a series of elegant experiments Malley and coworkers demonstrated that pneumolysin is a ligand for TLR4 (21, 22). Purified pneumolysin was shown to activate cells via a TLR4 dependent, TLR2 independent pathway, accomplished by a

physical interaction between pneumolysin and TLR4 (21, 22). Interestingly, pneumolysin induced proinflammatory responses in primary macrophages in synergy with TLR2 ligands derived from *S. pneumoniae*, in particular peptidoglycan and whole pneumococcal cell walls (21, 22), suggesting that during infection with intact pneumococci the combined action of TLR4 and TLR2 may facilitate an optimal innate immune response. Such roles for these two distinct TLRs is further corroborated by findings that in the human embryonic kidney cell line 293 transfection of either TLR2 or TLR4 conferred responsiveness to *S. pneumoniae* (34). Thus far, the isolated roles of either TLR4 or TLR2 in host defense against *S. pneumoniae in vivo* have been investigated in a number of studies. The most dramatic phenotype was reported in the original publication by Malley et al (21, 22), showing that C3H/HeJ mice, which carry a loss-of-function *tlr4* mutation, are more susceptible to pneumococcal colonization after nasopharyngeal challenge eventually resulting in invasive infection, bacteremia and death. Our laboratory found a more modest protective role for TLR4 during lower respiratory tract infection by *S. pneumoniae*, as reflected by a reduced survival and a slightly enhanced bacterial outgrowth after intranasal infection of C3H/HeJ mice with a relatively low infectious dose (23). TLR2 KO mice demonstrated an increased disease severity together with a moderately enhanced bacterial growth in the central nervous system during meningitis induced by intracisternal injection of pneumococci (34, 35). In contrast, our group could not demonstrate a protective role for TLR2 in pneumonia caused by *S. pneumoniae*, showing similar bacterial multiplication and lethality after intranasal infection of TLR2 KO and WT mice (27). A limited role for TLR2 during infection with WT *S. pneumoniae* is further supported by a recent study in which intact pneumococci were administered intraperitoneally (36), although TLR2 KO mice displayed a modestly slower clearance of *S. pneumoniae* from their nasopharynx in another investigation (37). Altogether these studies suggest that TLR2 at best plays a modest role in host defense against *S. pneumoniae* airway infection and led us to hypothesize that intact TLR4 signaling through pneumolysin may balance the lack of TLR2 signaling. We tested this hypothesis by infecting TLR2 KO mice with pneumolysin deficient *S. pneumoniae* arguing that these bacteria, devoid of a major TLR4 ligand, predominantly express TLR2 ligands. Indeed, whereas antibacterial defense in TLR2 KO mice was unimpaired during infection with *S. pneumoniae* D39, infection with *S.*

pneumoniae PLN resulted in enhanced outgrowth in these mice. If our hypothesis is correct, inoculation of WT *S. pneumoniae* D39 in TLR2x4 double KO mice should result in a comparable setting as pneumolysin-deficient *S. pneumoniae* in TLR2 KO mice, i.e. absence of TLR2 and TLR4 signaling. Our first preliminary results show that indeed this is the case: growth of WT *S. pneumoniae* D39 was significantly higher in the lungs of TLR2x4 double KO mice compared to WT mice 48 hours after inoculation (data not shown). In line, Albiger *et al.* recently showed that mice deficient of the TLR2 and TLR4-common intracellular adaptor molecule MyD88 also displayed an enhanced bacterial outgrowth in MyD88 KO mice compared to WT mice (38).

The early inflammatory response is an essential component of host defense in this model of pneumococcal pneumonia, as documented by previous studies in which the early cytokine response was inhibited (32, 33). Although TLR2 KO mice displayed a reduced inflammatory response 6 hours after infection with either *S. pneumoniae* D39 or PLN, some responses were more strongly diminished after infection with the pneumolysin deficient strain. This was in particular true for the early TNF-α response. Considering that especially low TNF-α concentrations in the lungs early after induction of pneumococcal pneumonia are important for limiting the growth of *S. pneumoniae* (32, 33), this differential response may have contributed to the enhanced growth of *S. pneumoniae* PLN in TLR2 KO mice. In addition, mediators other than measured in this study could contribute to this finding. Of note, TLR2 KO mice still display an induction of cytokines and chemokines when infected with pneumolysin deficient *S. pneumoniae*, suggesting that other pattern recognition receptors contribute to this response, In this respect the recent finding that TLR9 can recognize pneumococcall DNA is of relevance (39). Moreover, although histopathological analysis of lung tissue showed diminished lung inflammation in TLR2 KO mice during the early course of infection with *S. pneumoniae* PLN, which is in line with a TLR2 dependent immune response, during the later phase of pneumonia lung inflammation of TLR2 KO mice was enhanced compared to WT mice, which corresponded with the higher bacterial loads. This finding suggests that in the presence of a high bacterial burden *S. pneumoniae* PLN is able to elicit significant lung inflammation via a TLR2 independent route.

Our results exemplify the complex interactions at play during the first encounter between the host, expressing multiple pattern recognition receptors, and an intact pathogen, expressing multiple virulence factors and pathogen associated molecular patterns. Whereas during infection of TLR2 KO mice with WT pneumococci the interaction between TLR4 and pneumolysin apparently is sufficient to maintain an adequate immune response, during infection of TLR2 KO mice with pneumolysin deficient *S. pneumoniae* the absence of the interaction between pneumococcal TLR2 ligands such as lipoteichoic acid and peptidoglycan can not be compensated for by the TLR4-pneumolysin mediated immune response. As such, our data demonstrate redundancy at both the microbial site and the site of the host during airway infection by *S. pneumoniae*.

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Chapter 5

CD14 facilitates invasive respiratory tract infection by *Streptococcus pneumoniae*

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Abstract

CD14 is a pattern recognition receptor that can interact with a variety of bacterial ligands. During Gram-negative infection CD14 plays an important role in the induction of a protective immune response by virtue of its capacity to recognize lipopolysaccharide in the bacterial cell wall. Knowledge of the contribution of CD14 to host defense against Gram-positive infections is limited. We therefore studied the role of CD14 in Gram-positive bacterial pneumonia. CD14 knockout (KO) and normal wild-type (WT) mice were intranasally infected with *Streptococcus (S.) pneumoniae*. CD14 KO mice demonstrated a strongly reduced lethality, which was accompanied by a more than 10-fold lower bacterial load in lung homogenates but not in bronchoalveolar lavage fluid at 48 hours after infection. Strikingly, CD14 KO mice failed to develop positive blood cultures, whereas WT mice had positive blood cultures from 24 hours onward and eventually invariably had evidence of systemic infection. Lung inflammation was attenuated in CD14 KO mice at 48 hours after infection, as evaluated by histopathology and cytokine and chemokine levels. Intrapulmonary delivery of recombinant soluble CD14 to CD14 KO mice rendered them equally susceptible to *S. pneumoniae* as WT mice, resulting in enhanced bacterial growth in lung homogenates and bacteremia, indicating that the presence of soluble CD14 in the bronchoalveolar compartment is sufficient to cause invasive pneumococcal disease. These data suggest that *S. pneumoniae* uses (soluble) CD14 present in the bronchoalveolar space to cause invasive respiratory tract infection.

Introduction

CD14 is a glycosyl phosphatidylinositol surface anchored molecule expressed by myeloid cells, in particular monocytes/macrophages and to a lesser extent neutrophils (1, 2)(review (3)). CD14 is a pattern recognition receptor for several conserved bacterial motifs, including lipopolysaccharide (LPS), the toxic moiety in the outer membrane of Gram-negative bacteria, and peptidoglycan and lipoteichoic acid, both major components of the Gram-positive bacterial cell wall (4-6). Membrane bound CD14 lacks an intracellular domain and requires interaction with other receptors for signal transduction (7). As such the role of CD14 as the ligand binding portion of the LPS receptor complex, further consisting of Toll-like receptor (TLR) 4 and the extracellular protein MD-2, has been widely documented (8, 9). Besides as a membrane bound receptor, CD14 can exist as a soluble protein. Two isoforms of this soluble CD14 have been identified: one that is formed by shedding from the cell surface and one that is released from cells before addition of the glycosyl phosphatidylinositol anchor (2, 10-14).

Investigations on the role of CD14 during inflammation and infection *in vivo* have almost exclusively focused on LPS and Gram-negative bacterial infections (15-21). These studies have established that CD14 plays a pivotal part in systemic and pulmonary inflammation induced by LPS. The recognition of LPS by CD14, resulting in a rapid induction of an innate immune response via TLR4, contributes to an effective host defense against intact Gram-negative bacteria. Indeed, elimination or inhibition of CD14 has been found to facilitate the outgrowth of several Gram-negative pathogens *in vivo* (19-21). In this respect, our laboratory recently documented a clear role for CD14 in improving the clearance of clinically relevant pathogens such as *Haemophilus influenzae* (22) and *Acinetobacter baumannii* (23) from the mouse respiratory tract. In contrast to this abundant data on the contribution of CD14 in Gram-negative infections, knowledge of the role of this receptor in host defense against Gram-positive bacteria is limited. In a model of severe sepsis induced by intravenous or intraperitoneal injection of *Staphylococcus (S.) aureus*, CD14 knockout (KO) mice displayed unaltered bacterial loads and survival when compared to normal wild-type (WT) mice (24). More recently, CD14 KO mice were found to be more susceptible to meningitis induced by intrathecal administration of *Streptococcus (S.) pneumoniae*, as reflected by higher disease severity

scores and an accelerated mortality (25). *S. pneumoniae* is the most prevalent microorganism in community-acquired pneumonia responsible for more than half a million cases each year in the United States alone, bearing a fatality rate of 5-7% (26, 27). Bacteremia with *S. pneumoniae* originates in almost 90% of cases from the lungs. In addition, in recent sepsis trials the pneumococcus was an important causative pathogen especially in the context of pneumonia (28). We here sought to determine the role of CD14 in the host response to respiratory tract infection caused by *S. pneumoniae.*

Materials and Methods:

Animals: C57BL/6 WT mice were purchased from Charles River (Maastricht, The Netherlands). CD14 KO mice, backcrossed to a C57BL/6 genetic background, were obtained from Jackson Laboratory (Bar Harbor, Maine) and bred in the animal facility of the Academic Medical Center in Amsterdam. Sex and age matched (10-12 weeks) mice were used. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam.

Design: Pneumonia was induced as described earlier (29-31). Mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, The Netherlands) and 50 µl containing 1-5 x 10^4 CFU *S. pneumoniae* serotype 3 (American Type Culture Collection, ATCC 6303, Rockville, MD) was inoculated intranasally. In these experiments mice were killed at 5, 24 or 48 hours after infection or followed for 2 weeks. In a separate experiment mice infected with *S. pneumoniae* received either saline or recombinant mouse soluble CD14 (1 µg; Biometec, Greifswald, Germany) intranasally at 0 and 24 hours relative to the time of infection ; mice were killed 48 hours after infection. In an additional survival experiment mice received either saline or sCD14 at 0, 24 and 48 hours relative to the time of infection.

Measurement of bacterial loads: Lung bacterial loads were determined as described earlier (29-31). Briefly, mice were anesthetized with Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Meidrecht, the Netherlands), and blood and lungs were collected. Lungs were homogenized at 4^0C in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, OK) Serial 10-fold dilutions in sterile isotonic saline were made from these homogenates (and blood), and

50 µl volumes were plated onto sheep-blood agar plates and incubated overnight at 37^0C and 5% CO_2.

Bronchoalveolar lavage: Bronchoalveolar lavage fluid (BALF) was obtained as described earlier (32) Briefly, the trachea was exposed through a midline incision and BALF was harvested by instilling and retrieving two 0.5 ml aliquots of sterile isotonic saline. Total cell numbers were counted using Z2 Coulter particle count and size analyzer (Beckman-Coulter Inc., Miami, FL.). BALF differential cell counts were carried out on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, Ill).

Histology: Lungs for histology were prepared and analyzed as described earlier (30) Parameters: bronchitis, edema, interstitial inflammation, intra-alveolar inflammation, pleuritis and endothelialitis were graded on a scale of 0 to 4 with 0 as 'absent' and 4 as 'severe'. The total 'lung inflammation score' was expressed as the sum of the scores for each parameter, the maximum being 24. Granulocyte staining was done using FITC-labelled rat anti-mouse Ly-6G mAb (Pharmingen, San Diego, CA) exactly as described (30).

Assays: Lung homogenates were prepared as described earlier (30) TNF-α, IL-6, IL-10 and MCP-1 were measured by cytometric beads array (CBA) multiplex assay (BD Biosciences, San Jose, CA). IL-1β, MIP-2 and KC were measured by ELISA (R & D Systems, Abingdon, UK). Total protein concentrations were measured in BALF using the BCA protein kit (Pierce, Rockford, IL). Soluble CD14 was measured by ELISA (Biometec, Greifswald, Germany).

Statistical analysis: All data are given as means ± SEM and were analyzed using Graphpad prism 4 (GraphPad Prism v. 4 for Windows, GraphPad Software, San Diego California USA). Differences between groups were analyzed using Mann-Whitney U test or Kruskal-Wallis analysis where appropriate. For survival analyses, Kaplan-Meier analysis followed by log rank test or Cox regression analysis was performed where appropriate. A value of $P < 0.05$ was considered statistically significant.

Results:

CD14 KO mice are protected against lethality during pneumococcal pneumonia

To investigate the role of CD14 in the outcome of pneumococcal pneumonia, WT and CD14 KO mice were inoculated with *S. pneumoniae* (either 1 or 5 x 10^4 colony forming units (CFU) in two independent experiments) and monitored for 14 days (Fig. 1A and B). After infection with the lower dose, WT mice started dying after 2 days and 93 % had died by day 7. In contrast, the first CD14 KO mice died after 4 days and only 21 % had died at the end of the observation period ($P < 0.0001$ for the difference between groups). After infection with the higher dose, the vast majority of WT mice died shortly after the second day and all animals were dead at day 6; CD14 KO mice displayed a delayed mortality and 16% survived ($P < 0.005$ for the difference between groups). These data suggested that CD14 contributes to lethality during *S. pneumoniae* pneumonia.

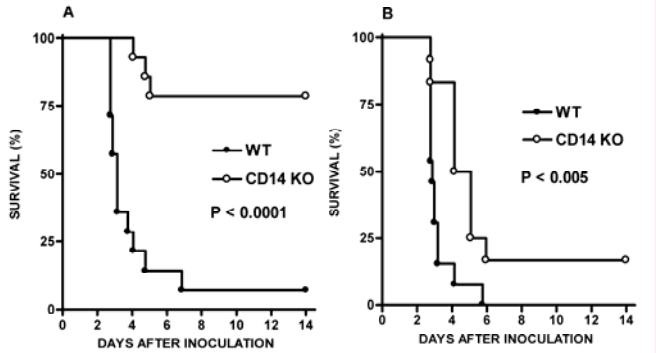


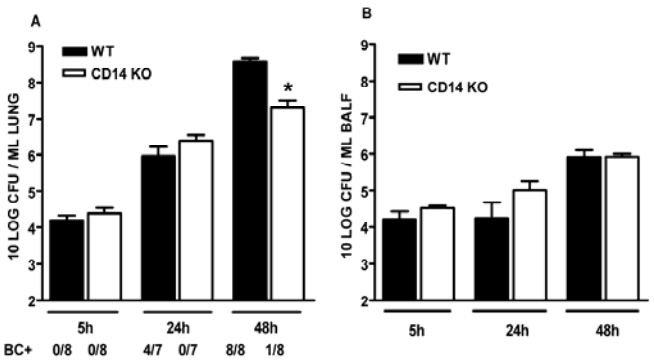
Figure 1: CD14 KO mice are protected against pneumococcal pneumonia. Survival after intranasal infection with 1x10^4 CFU (A) or 5x10^4 CFU (B) *S. pneumoniae*. Mortality was assessed four times daily for 14 days (N = 13-14 per group in each experiment).

CD14 KO mice display diminished invasiveness and dissemination of the infection

To obtain insight in the involvement of CD14 during the early phase of host defense against pneumococcal pneumonia bacterial loads were determined in lung homogenates and blood obtained from WT and CD14 KO mice 5, 24 or 48 hours after infection, *i.e.* at time points before the first WT mice started dying. Whereas at the first two time points the number of *S. pneumoniae* CFU recovered from lung homogenates was similar in WT and CD14 KO mice, at 48 hours after infection the bacterial load in lungs of CD14 KO mice was more than 10-fold lower than in the lungs of WT mice ($P < 0.001$, Fig. 2A). Strikingly, WT mice had positive blood cultures from 24 hours onward (24 hours: 4/7; 48 hours: 8/8), whereas no CD14 KO mouse had a positive blood culture at 24 hours and from only 1/8 CD14 KO mice *S. pneumoniae* could be cultured

from blood at 48 hours (Fig. 2A). This latter finding, which was reproduced in 3 independent experiments, suggested that CD14 contributes to the invasion of pneumococci from the alveolar compartment (the primary site of the infection) into the blood stream. This prompted us to perform a next series of experiments to obtain insight into the bacterial loads in the bronchoalveolar compartment of WT and CD14 KO mice after intranasal instillation of *S. pneumoniae.* For this, the alveolar space was gently lavaged 5, 24 or 48 hours after infection and the number of *S. pneumoniae* CFU was counted in bronchoalveolar lavage fluid (BALF) obtained. In contrast to the differences in bacterial burdens in whole lung homogenates (and blood), BALF of WT and CD14 KO mice contained equal numbers of *S. pneumoniae* at all time points (Fig. 2B). To obtain insight in the location of bacteria in the lungs of CD14 KO and WT mice we performed gram-stainings on lung tissue slides. *S. pneumoniae* was visualized primarily extracellularly throughout infected areas in the lungs without apparent differences between the two mouse strains (see Figure E1 in online supplement). Together these data suggested that CD14 contributes to the invasion of *S. pneumoniae* from the alveolar space into lung tissue and the circulation.

Figure 2: CD14 KO mice demonstrate reduced invasiveness and dissemination of the infection.



Bacterial loads in lungs (A) and bronchoalveolar lavage fluid (B) at 5, 24 and 48 hours after infection with 5 x 10^4 CFU *S. pneumoniae*. BC+ indicate the number of positive blood cultures. Data are means ± SEM (N = 7-8 mice per group at each time point).

* $P < 0.001$ versus WT mice.

CD14 KO mice demonstrate reduced lung inflammation

To determine the role of CD14 in the induction of pulmonary inflammation in response to *S. pneumoniae* infection lung tissue slides were prepared from WT and CD14 KO mice at 5, 24 or 48 hours after infection. Whereas at 5 hours the extent of lung inflammation did not differ between the two mouse strains, pulmonary inflammation was clearly less pronounced in CD14 KO mice, as determined by the semi-quantitative scoring system described in the Methods section, at both 24 hours ($P < 0.05$) and 48

hours ($P < 0.005$) after inoculation (Table I). Representative slides of lung tissue from WT and CD14 KO mice 24 and 48 hours after inoculation of *S. pneumoniae* are presented in Figure 3. In addition, CD14 KO mice demonstrated a reduced accumulation of neutrophils in lung tissue at 24 and 48 hours after infection, as visualized by Ly-6G staining (not shown). The reduced lung inflammation in CD14 KO mice was accompanied by an attenuated leak of protein into BALF at 48 hours ($P<0.05$ versus WT mice, Table I). Of note, uninfected CD14 KO and WT mice displayed similar leukocyte differentiation in peripheral blood (data not shown).

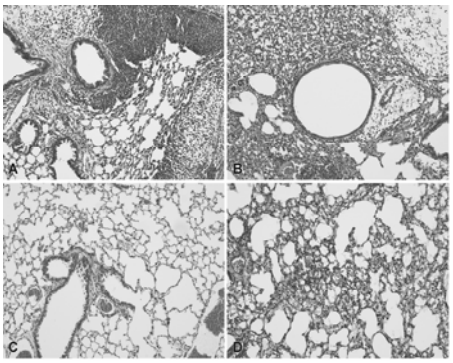


Figure 3: CD14 KO mice display reduced lung inflammation. Representative lung slides of WT (panels A and B) and CD14 KO (panels C and D) mice 24 hours (panels A and C) and 48 hours (panels B and D) after infection with 5 x 10^4 CFU *S. pneumoniae*. H&E staining: magnification x 4.

CD14 KO mice show a reduced early neutrophil migration into BALF

The histological analyses indicated that CD14 deficiency resulted in a reduced lung inflammatory response to *S. pneumoniae* including a diminished influx of neutrophils into lung tissue. Considering that neutrophils play an important role in the immune response to bacterial pneumonia (33), we next sought to evaluate the extent of neutrophil recruitment into the bronchoalveolar space. At 5 and 24 hours after infection, the number of neutrophils in BALF of CD14 KO mice was less than that in BALF of WT mice ($P = 0.05$ and $P <0.05$ respectively, Table I). Forty-eight hours after infection, neutrophil counts were equal in BALF of both mouse strains.

CD14 KO mice have decreased cytokine and chemokine levels in lung and blood

Cytokines and chemokines play an important role in host defense against bacterial pneumonia (34). Thus, we determined the concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-2 and cytokine-induced neutrophil chemoattractant (KC) in lung homogenates obtained 5, 24 and 48 hours after infection (Table I). Except for increased IL-10 production in lungs of CD14 KO mice 24 hours

after inoculation (P<0.05) the levels of these mediators did not differ between the two mouse strains at 5 and 24 hours after inoculation. At 48 hours CD14 KO mice demonstrated reduced concentrations of all mediators except TNF-α (Table I). Twenty-four hours after inoculation IL-6 and MCP-1 levels in plasma were lower compared to WT mice; TNF-α, MCP-1 and IL-6 levels were also lower in CD14 KO mice 48 hours after inoculation (Table I).

Table I: CD14 KO mice display reduced inflammation and immune response compared to WT mice.

	T = 5h		T = 24h		T = 48h	
	WT	CD14 KO	WT	CD14 KO	WT	CD14 KO
Total lung score (AU)	n.d.	n.d.	7.0 ± 1.5	3.0 ± 0.8 *	12.8 ± 1.9	5.3 ± 1.6 ‡
Total protein level BAL (µg/ml)	249 ± 8	254 ± 12	278 ± 24	308 ± 25	743 ± 123	362 ± 47 *
Neutrophil count BAL (x10^3 / ml)	5 ± 2	1 ± 0.2 *	48 ± 12	16 ± 3 *	100 ± 14	97 ± 24
Cytokine and chemokine production in lung homogenate (pg/ml)						
TNF-α	34 ± 9	29 ± 8	108 ± 54	267 ± 75	421 ± 82	528 ± 229
IL-1β	b.d.	b.d.	2960 ± 1390	4772 ± 1609	12596 ± 865	4275 ± 2872*
IL-6	20 ± 8	30 ± 11	368 ± 142	546 ± 181	2972 ± 542	479 ± 192 †
IL-10	218 ± 91	199 ± 26	675 ± 55	1067 ± 127 *	2038 ± 423	566 ± 17 ‡
MCP-1	226 ± 35	230 ± 34	1219 ± 409	776 ± 200	7499 ± 955	1568 ± 459 †
MIP-2	272 ± 30	238 ± 29	3112 ± 355	3991 ± 416	19131 ± 4928	7591 ± 3166*
KC	179 ± 18	320 ± 61	2346 ± 515	2391 ± 575	5922 ± 748	2352 ± 459 ‡
Cytokine and chemokine production in plasma (pg/ml)						
TNF-α	20 ± 8	30 ± 11	368 ± 142	546 ± 181	2972 ± 542	479 ± 192 †
IL-6	4 ± 3	1 ± 0.3	196 ± 71	36 ± 9 *	6281 ± 1789	86 ± 16 ‡
MCP-1	15 ± 3	11 ± 1	49 ± 14	14 ± 3 ‡	1224 ± 406	31 ± 5 ‡

Mice were intranasally infected with 5x10^4 CFU *S. pneumoniae* and lung homogenates were prepared 5, 24 or 48 hours later. Data are means ± SEM (N=8 per group). N.d. = not determined, b.d. = below detection limit. * P <0.05 † P<0.001 ‡ P<0.0005.

Intrapulmonary delivery of soluble CD14 results in invasive infection in CD14 KO mice with increased lethality

We next wished to determine whether soluble (s) CD14 could compensate for CD14 gene deficiency during *S. pneumoniae* pneumonia. First we measured sCD14

concentrations in BALF harvested from WT mice before and 5, 24 or 48 hours after infection. sCD14 was readily detectable in normal BALF and significantly increased during the course of pneumonia ($P < 0.05$, Fig. 4A). Intranasal administration of recombinant mouse sCD14 to CD14 KO mice changed this mouse strain into a WT phenotype during pneumonia. Indeed, whereas CD14 KO mice were protected against lethality when compared with WT mice (confirming the data presented in Fig. 1), CD14 KO mice treated with sCD14 showed accelerated and increased lethality similar to WT mice (Fig. 4B). In addition, whereas CD14 KO mice displayed more than 10-fold lower bacterial loads in lung homogenates than WT mice at 48 hours post infection and whereas only 1/8 CD14 KO mice had a positive blood culture at this time point versus 8/8 WT mice (confirming the data presented in Fig. 2A), CD14 KO mice that had received sCD14 demonstrated similar bacterial loads when compared to WT mice and 7/8 of CD14 KO mice treated with sCD14 had positive blood cultures (Fig. 4C).

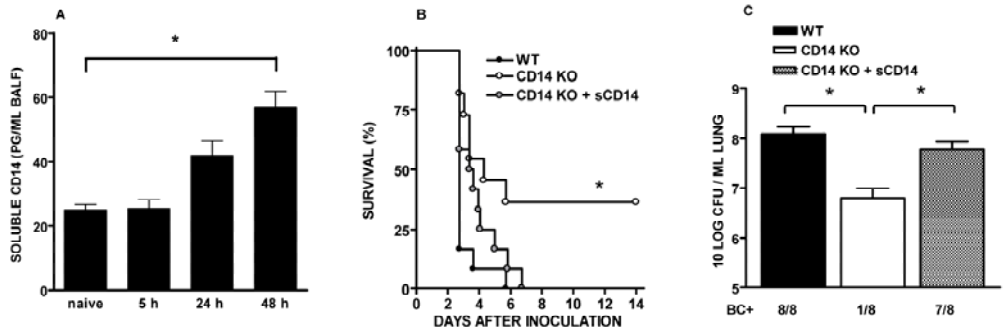


Figure 4: Treatment with recombinant soluble CD14 results in invasive infection in CD14 KO mice. (A) Soluble CD14 concentrations in BALF of WT mice infected with 5 x 10^4 CFU *S. pneumoniae*. Data are means ± SEM (N = 7-8 mice per group at each time point) * $P < 0.05$ versus naïve mice (B) Survival of WT and CD14 KO mice which received either saline or sCD14 intranasally (1 µg; 0, 24 and 48 hours). * P <0.05 versus WT (N= 11-12 mice per group). (C) Bacterial loads in lungs 48 hours after infection with 5 x 10^4 CFU *S. pneumoniae*. BC+ indicate the number of positive blood cultures. CD14 KO + sCD14: CD14 KO mice treated with sCD14 (1 µg; 0 and 24 hours). Data are means ± SEM (N = 7-8 mice per group at each time point) * $P < 0.05$ versus CD14 KO mice.

Moreover, administration of sCD14 to CD14 KO mice enhanced the pulmonary inflammatory response that again was clearly reduced in CD14 KO mice not receiving sCD14, to an extent observed in WT mice, as indicated by the semi-quantitative scoring system described in the Methods (Table II). Moreover, CD14 KO mice treated with

sCD14 demonstrated an increased inflammation and accumulation of neutrophils in lung tissue slides compared to CD14 KO mice as visualized by HE and respectively Ly-6G staining (Fig 5). In line, the lung and plasma concentrations of cytokines and chemokines were increased by sCD14 administration to CD14 KO mice (Table II). Together these data indicate that the presence of sCD14 in the bronchoalveolar compartment of CD14 KO mice can fully compensate for CD14 gene deficiency.

Table II: soluble CD14 enhances inflammation and inflammatory response in CD14 KO mice.

	WT	CD14 KO	CD14 KO + sCD14
Total lung score (AU)	15 ± 2	4 ± 2 *,†	17 ± 2
Cytokine and chemokine production in lung homogenate (pg/ml)			
TNF-α	794 ± 193	523 ± 222 †	1816 ± 322 *
IL-6	2270 ± 898	465 ± 119 *,†	1447 ± 310
MCP-1	6296 ± 1654	941 ± 407 *,†	4576 ± 829
Cytokine and chemokine production in plasma (pg/ml)			
TNF-α	623 ± 394	92 ± 10	310 ± 149
IL-6	8675 ± 5063	78 ± 16 ‡,†	7291 ± 6998
MCP-1	892 ± 361	143 ± 8 ‡	827 ± 635

Mice were intranasally infected with $5x10^4$ CFU *S. pneumoniae* and treated with either saline or sCD14 (1 µg; 0 and 24 hours). Lung homogenates were prepared on day 2. Data are means ± SEM (N=8 per group). * $P<0.05$ vs WT, ‡ $P<0.01$ vs WT, † $P<0.01$ vs CD14 KO + sCD14.

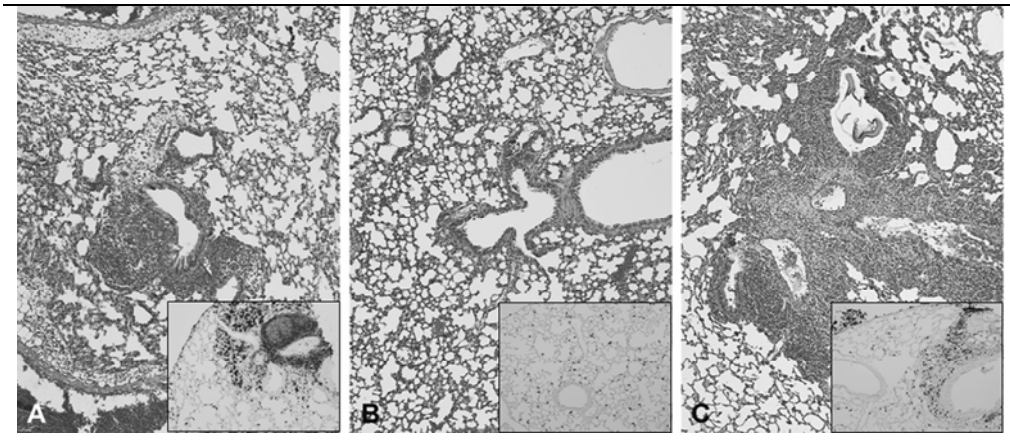


Figure 5: Treatment with recombinant soluble CD14 enhances lung inflammation in CD14 KO mice. Representative lung slides of WT (panel A) and CD14 KO treated with saline (panel B) or sCD14 (panel C). Mice were sacrificed 48 hours after infection with 5 x 10^4 CFU *S. pneumoniae*. H&E staining: magnification x 4. Insets show Ly-6G staining, magnification x4.

Discussion

CD14 is a pattern recognition receptor that has been shown to interact with a variety of bacterial components including LPS, peptidoglycan and lipoteichoic acid (4-6). Several studies have indicated that the early recognition of LPS by CD14 is important for mounting an effective innate immune response against Gram-negative infections (19, 35). We here report that, very much unlike the protective role of CD14 during Gram-negative respiratory tract infection (22, 23), CD14 facilitates the outgrowth and in particular the dissemination of bacteria during pneumonia caused by *S. pneumoniae*. Experiments in which sCD14 was administered into the airways of CD14 KO mice established that sCD14 present in the bronchoalveolar compartment is sufficient to render the host more susceptible to pneumococcal pneumonia.

To our knowledge only two previous studies examined the role of CD14 in host defense against a Gram-positive infection. In a model of Gram-positive septic shock induced by *S. aureus*, Haziot *et al.* did not detect a significant part for CD14 in survival or bacterial clearance (24). More recently, Echchannaoui *et al*. reported that CD14 KO mice showed more rapid and more severe signs of disease together with an accelerated lethality in a model of *S. pneumoniae* meningitis induced by direct intrathecal injection of live bacteria (25). The adverse outcome of CD14 KO mice was accompanied by an enhanced inflammatory response in the central nervous system. In addition, CD14 KO mice demonstrated a transiently enhanced outgrowth of pneumococci in their brains, as reflected by elevated bacterial loads at 24 hours but not at 48 hours. These two earlier studies contrast with our present findings in pneumonia caused by *S. pneumoniae*. The results of Haziot *et al*. do not necessarily conflict with our current data considering that these authors used a different Gram-positive pathogen and a model that due to its direct systemic nature likely relies less on local antibacterial effector mechanisms (24). The model of *S. pneumoniae* central nervous system infection used by Echchannaoui *et al*. differs significantly from the model of *S. pneumoniae* pneumonia used here. Indeed, in the former study pneumococci were injected directly into the brain, thereby circumventing normal anatomical barriers, in particular the blood-brain barrier (25). In our pneumonia model, the number of *S. pneumoniae* CFU remained similar in BALF of CD14 KO and WT mice throughout the course of infection but the bacterial loads in whole lung homogenates were more than 10-fold lower in the former strain at 48 hours

post infection. More strikingly, blood cultures remained negative in CD14 KO mice with a single exception whereas WT mice developed positive blood cultures from 24 hours onward and invariably had systemic infection at 48 hours. Treatment of CD14 KO mice with recombinant mouse sCD14 via the airways made them fully susceptible to invasive pneumococcal disease, not only confirming that the phenotype of CD14 KO mice in this model is CD14 dependent but also demonstrating that soluble CD14 is sufficient to reproduce the WT phenotype. Hence, in the bronchoaveolar compartment (soluble) CD14 is used by *S. pneumoniae* to cause a full-blown and invasive infection.

CD14 KO mice demonstrated less lung inflammation in particular at 48 hours after infection, as reflected by histopathology and cytokine and chemokine levels. Of note, whereas granulocyte staining of lung sections revealed a reduced neutrophil recruitment into lung tissues of CD14 KO mice at 48 hours, BALF of CD14 KO and WT mice contained equal neutrophil numbers at this time point. Possibly, this finding is related to the reduced bacterial load in lung tissue but not in BALF of CD14 KO mice (i.e. the reduced bacterial load in lung tissue may provide a less potent stimulus for the influx of neutrophils). CD14 KO mice were previously reported to have elevated TNF-α and IL-6 levels in blood during *S. aureus* induced sepsis (24) and elevated TNF-α and MIP-2 levels in brain homogenates during *S. pneumoniae* induced meningitis (25). In the present study, TNF-α in lungs was the only cytokine that was not affected by CD14 deficiency; all other mediators measured in lungs, including IL-6 and MIP-2, were lower in CD14 KO mice. This finding could be explained in two mutually non exclusive ways. First, CD14 could play a direct role in the responsiveness of cytokine producing cells to *S. pneumoniae*. In support of this possibility we found that alveolar macrophages obtained from CD14 KO mice produced less TNF-α and IL-6 upon stimulation with heat-killed *S. pneumoniae* (data not shown). Second, CD14 KO mice had a lower bacterial load in their lungs at 48 hours after infection, and thus were exposed to a less potent proinflammatory stimulus. In line, earlier studies have demonstrated a clear correlation between the pulmonary bacterial load and the extent of lung inflammation including cytokine levels during experimentally induced *S. pneumoniae* pneumonia (34).

The pneumococcal cell wall contains phosphoryl choline that can specifically bind the platelet activating factor receptor (PAFR), an interaction that facilitates bacterial entry

into these cells (36-38). Furthermore, the capacity of *S. pneumoniae* to transcytose to the basal surface of rat and human endothelial cells is dependent on the PAFR. Our laboratory recently provided evidence that this mechanism is important for the virulence of *S. pneumoniae* during murine respiratory tract infection *in vivo* (29). Using PAFR KO mice we demonstrated that the PAFR is used by *S. pneumoniae* to induce lethal pneumonia, as reflected by a strongly reduced mortality, an attenuated bacterial outgrowth in the lungs and a diminished dissemination of the infection in PAFR KO mice. As such, the phenotype of PAFR KO mice strongly resembles the phenotype of CD14 KO mice in this model. It is tempting to speculate that (soluble or surface) CD14 is involved in the presentation (of components) of *S. pneumoniae* to the PAFR so that the phosphoryl – PAFR mediated invasion is facilitated. The possibility exists that (soluble or surface) CD14 serves as a chaperone that facilitates internalization and thus invasiveness of *S. pneumoniae*. CD14 itself is known to bind LPS and rapidly traffic between the cell membrane and intracellular compartments (39, 40). The more recent observation that binding and internalization of polyinosine-polycytidylic acid (pIpC) depends on CD14 illustrates that this property of CD14 is not restricted to LPS (41). Although we were not able to verify direct binding of CD14 to *S. pneumoniae* using *in vitro* binding assays or fluorescence microscopy (data not shown), we certainly cannot exclude the possibility of CD14-mediated internalization of bacteria *in vivo*.

An interaction between CD14 and TLRs is unlikely to explain our observations. Indeed, although CD14 can facilitate the presentation of several bacterial components to either TLR2 or TLR4, the presence of neither of these pattern recognition receptors facilitates invasive pneumococcal infection: both TLR2 and TLR4 have no/little contribution to host defense against pneumococcal pneumonia (30, 31, 42). Moreover, mice deficient for the common TLR adaptor protein MyD88 displayed a strongly reduced resistance against nasopharyngeal infection with *S. pneumoniae* (43). Thus, if the role of CD14 observed here would rely on TLRs, one would expect that CD14 KO mice would have been more susceptible rather than protected against pneumococcal pneumonia.

Our study is the first to identify a detrimental role for CD14 in host defense against a common bacterial infection. We show that (soluble) CD14 is required for the development of severe invasive pneumonia upon infection of the lower airways by *S. pneumoniae*. Our current data strongly suggest that *S. pneumoniae* specifically uses

(soluble) CD14 in the bronchoalveolar compartment to cause invasive disease by a TLR independent mechanism.

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Chapter 6

Monocyte chemoattractant protein 1 does not contribute to protective immunity against pneumococcal pneumonia

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Abstract

To determine the role of monocyte chemoatractant protein (MCP)-1 during pneumococcal pneumonia, MCP-1 knockout and wild-type mice were infected with *Streptococcus pneumoniae*. Pulmonary MCP-1 levels were strongly correlated to bacterial loads in wild-type mice. However, MCP-1 knockout and wild-type mice were indistinguishable with respect to bacterial growth, inflammatory responses and lethality.

Text

Streptococcus (S.) pneumoniae is the most frequently isolated causative pathogen in community-acquired pneumonia (1, 2). Previous studies examined the role of several cytokines in host defense against pneumococcal pneumonia (3-7), but knowledge of the role of chemokines is limited. Monocyte chemoattractant protein (MCP)-1 is a chemokine which primarily attracts monocytes and memory T cells (8), but during severe bacterial infection may also contribute to neutrophil recruitment (9, 10). In addition, MCP-1 has been found to exert anti-inflammatory effects during murine endotoxemia (11). In a model of acute non lethal pneumonia caused by *Pseudomonas (P.) aeruginosa* treatment with anti-MCP-1 resulted in increased neutrophil influx into the lungs and enhanced lung injury without influencing the clearance of *Pseudomonas* (12). In a lethal pneumococcal pneumonia model anti-MCP-1 treatment did not influence the accumulation of either neutrophils or macrophages in the lungs; the impact on the growth of pneumococci or lethality was not reported (13).

To further investigate the role of MCP-1 in pneumococcal pneumonia we infected 10-11 weeks old MCP-1 knockout (KO) C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine, USA) and sex and aged matched C57BL/6 wild-type (WT) mice (Charles Rivers, Maastricht, the Netherlands) with various doses of *S. pneumoniae* serotype 3 (American Type Culture Collection ATCC 6303, Rockville, MD). All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, the Netherlands). Mice were inoculated intranasally with 50 µl containing 4-50 x 10^3 colony forming units (CFU) *S. pneumoniae* as described earlier (3, 6)**.** Blood and lungs were obtained and processed for immunoassays and quantitative cultures as described (3, 6)**.** MCP-1, tumor necrosis factor (TNF)-α and interleukin (IL)-6 were measured by cytometric beads array multiplex assay (BD Biosciences, San Jose, CA). Macrophage inflammatory protein (MIP)-2 and cytokine-induced neutrophil chemoattractant (KC) were measured by ELISA (R & D Systems, Abingdon, UK). Myeloperoxidase (MPO) was measured by ELISA (HyCult, Uden, the Netherlands). Hemotoxylin and eosin stained lung slides were analyzed for bronchitis, edema, interstitial inflammation, pleuritis, endothelialitis and intra-alveolar inflammation. Each parameter was graded on a scale from 0 to 4 with 0 as 'absent'

and 4 as 'severe'. The total "lung inflammation score" was expressed as the sum of the scores for each parameter. MLE-12 mouse alveolar epithelial cells (10^5/ml in RPMI 1640 supplemented with 5 mg/L insulin, 10mg/L transferin, 5 μg/L sodium selenite, 10 nM hydrocortisone, 10 nM B-esteradiol, 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 2% fetal bovine serum, Sigma) and primary mouse peritoneal macrophages (10^5/ml in RPMI 1640 supplemented with 1 mM pyruvate, 2 mM L-glutamine, penicillin, streptomycin and 10% fetal bovine serum) were incubated overnight with $1x10^7$ CFU heat killed *S. pneumoniae* (HKSP, 30 minutes at 70º C) or medium alone and MCP-1 was measured in the supernatant. Statistics were analyzed by using Mann-Whitney U test. Difference in positive blood culture between groups was analyzed by Chi-square test. For survival analyses, Kaplan-Meier analysis followed by log rank test was performed. Correlations between pulmonary bacterial load and MCP-1 concentrations were calculated by Spearman's rank correlation test. Values are expressed as mean ± SEM. A value of $p \leq 0.05$ was considered statistically significant.

At 48 hours after infection of WT mice with various doses ($4\text{-}50x10^3$ CFU) of *S. pneumoniae* lung MCP-1 levels were strongly correlated with the pulmonary bacterial load (Figure 1A; $P<0.0001$, $R^2= 0.8228$). Mice with positive blood cultures had significantly higher levels than non-bacteremic mice (972 ± 312 vs. 56 ± 15 pg/ml respectively $P<0.0001$). To further investigate which cell types produce MCP-1 during pneumococcal pneumonia we stimulated MLE-12 alveolar epithelial cells and primary macrophages with HKSP. Stimulation with HKSP significantly increased MCP-1 production in both cell types (Figure 1B).

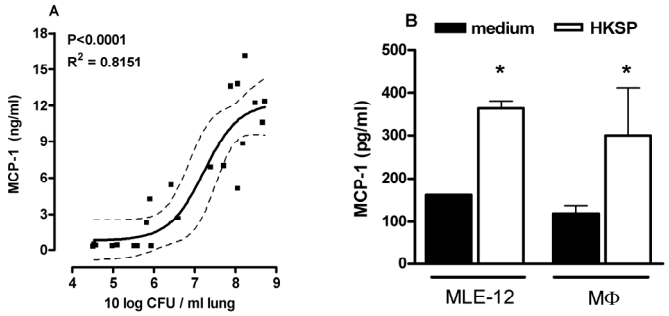

Figure 1: MCP-1 production. (A) Correlation between pulmonary MCP-1 levels and bacterial load during pneumococcal pneumonia. MCP-1 levels and bacterial loads in whole lung homogenates from WT mice 48 hours after inoculation with 4-50x10^3 *S. pneumoniae* CFU. Closed line represents curve fit, dashed line represents 95% confidence band. Goodness of fit is presented as R^2. (B) MCP-1 production in MLE-12 cell line and mouse macrophages. MLE-12 and mouse macrophages (MΦ) incubated with either medium (black bars) or HKSP (white bars). * P< 0.05. Data are mean ± SEM (N=4-5 per group).

To evaluate the role of MCP-1 in host defense against pneumococcal pneumonia, we determined the bacterial load in lung homogenates prepared 5, 24 and 48 hours after infection with 5 x 10^4 *S. pneumoniae* CFU. MCP-1 KO and WT mice displayed similar bacterial outgrowth and occurrence of bacteremia (Figure 2A). Also during less overwhelming infection (10^4 or 4 x 10^3 CFU, 48 hours) no significant differences in bacterial outgrowth in the lungs of MCP-1 KO and WT mice were observed (Figure 2B and 2C). After infection with 10^4 *S. pneumoniae* CFU, more MCP-1 KO than WT mice had a positive blood culture at 48 hours suggesting that MCP-1 may reduce the systemic spread of pneumococci (P=0.05); however, such an effect was not seen after infection with 5 x 10^4 or 4 x 10^3 CFU. To determine whether this difference was of biological relevance, we repeated these experiments but found no significant difference in mortality (Figure 2D and E). Lung inflammation scores, determined 48 hours after infection with 5 x 10^4 or 10^4 *S. pneumoniae* CFU, were similar in WT and MCP-1 KO mice (5 x 10^4 CFU: 12.4 ± 2.6 versus 9.4 ± 1.3 respectively, P=0.57; 10^4 CFU: 3.7 ± 1.5 versus 4.8 ± 0.5 respectively, P=0.10). In addition, histopathologic analysis and pulmonary MPO levels, revealed similar granulocyte influx in WT and MCP-1 KO mice (data not shown).

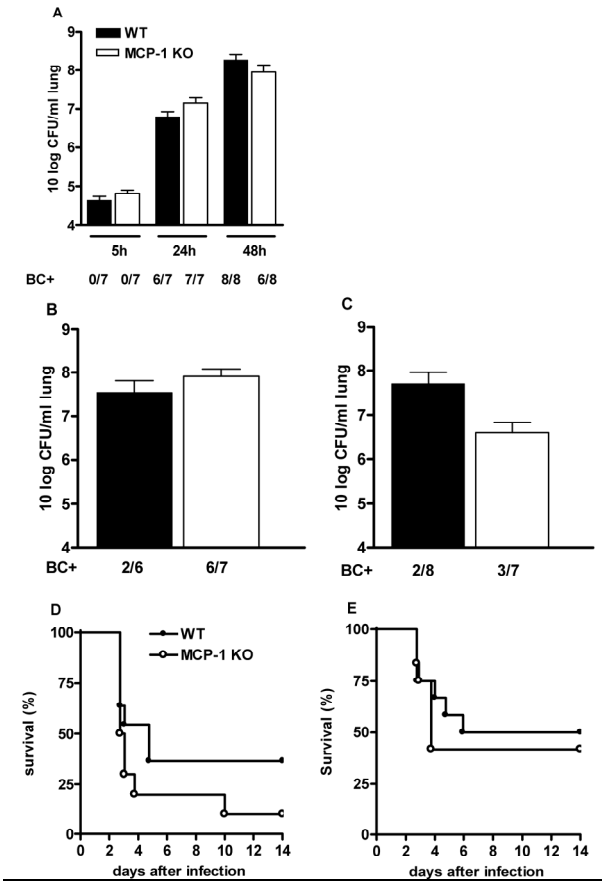

Figure 2: MCP-1 deficiency does not influence bacterial growth or survival during pneumococcal pneumonia. (A) Bacterial loads in whole lung homogenates 5, 24 and 48 hours after inoculation with $5x10^4$ *S. pneumoniae* CFU in WT (black bars) and MCP-1 KO mice (open bars). Bacterial loads in whole lung homogenates (B and C) and survival (D and E) of WT (black bars or symbols) and MCP-1 KO mice (open bars or symbols) inoculated with 10^4 CFU (B and D) or $4x10^3$ CFU (C and E). BC+ indicates the number of positive blood cultures. Data are mean ± SEM. (A, B and C: N= 6 – 8 per group; D and E: N = 10-12 per group).

Cytokines and chemokines play an import role in an adequate antibacterial defense in bacterial infections (14, 15). Thus, we determined the levels of the cytokines TNF-α and IL-6 and the chemokines MIP-2 and KC in whole lung homogenates and cytokine concentrations in plasma obtained from WT and MCP-1 KO mice after infection with 5 x 10^4 *S. pneumoniae* CFU (Figure 3). Although several pulmonary cytokine and chemokine concentrations tended to be lower in MCP-1 KO mice, especially at 48 hours after infection, differences were not significantly different (P>0.25). Similarly, no differences between WT and MCP-1 KO mice were detected after infection with the two lower bacterial doses (data not shown).

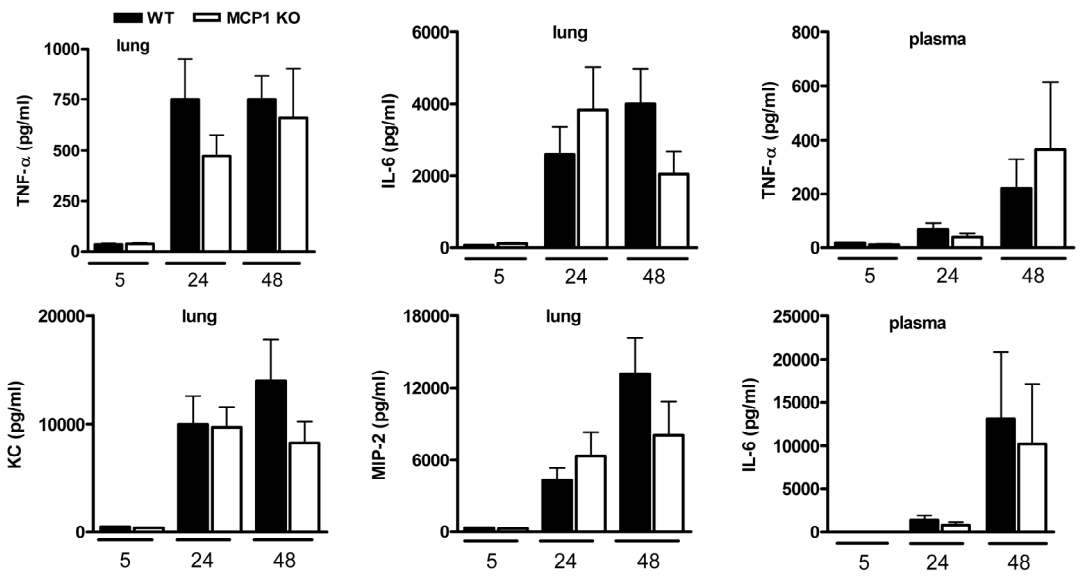

Figure 3: MCP-1 deficiency does not influence cytokine or chemokine concentrations. Levels of cytokines and chemokines in whole lung homogenates and cytokine concentrations in plasma obtained from WT (black bars) and MCP-1 KO (white bars) mice after infection with 5 x 10^4 *S. pneumoniae* CFU. Data are mean ± SEM (N= 6-8 per group).

In conclusion, we demonstrate that pulmonary MCP-1 production is correlated to the bacterial growth during pneumonia caused by *S. pneumoniae.* MCP-1 deficiency did not influence the host response after infection with several doses of *S. pneumoniae*, suggesting that endogenous MCP-1 does not play a major role in the pathogenesis of pneumococcal pneumonia. Of note, this conclusion only applies for the specific (serotype 3) bacterial strain and the model used here. An earlier study showed that administration of an anti-MCP-1 antibody did not impact on leukocyte recruitment to the lungs after infection with *S. pneumoniae,* whereas the combined treatment with antibodies directed against MCP-1, MIP-1α and RANTES reduced the influx of macrophages/monocytes (13). Together with our current results, these data suggest that during pneumococcal pneumonia the lack of MCP-1 may be compensated for by other mediators.

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Part II

Viral pneumonia

Chapter 7

Monocyte chemoattractant protein 1 contributes to an adequate immune response in influenza pneumonia

Submitted

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Abstract

Monocyte chemoattractant protein 1 (MCP-1) and its receptor CCR2 have been shown to play an import role in leukocyte recruitment to sites of infection and inflammation. To investigate the role of MCP-1 during infection with influenza we inoculated wild type (WT) and MCP-1 knockout (KO) mice with a non-lethal dose of a mouse adapted strain of influenza A. Influenza infection of WT mice resulted in a profound increase in pulmonary MCP-1 levels. MCP-1 KO mice had enhanced weight loss and did not fully regain their body weight during the 14-day observation period. In addition, MCP-1 KO mice demonstrated elevated viral loads 8 days after infection, which was accompanied by reduced leukocyte recruitment into the infected lungs, primarily caused by a diminished influx of macrophages and granulocytes. The pulmonary concentrations of tumor necrosis factor-α, interleukin-6, macrophage-inflammatory protein-2 and interferon-γ were higher in MCP-1 KO mice. This study shows that MCP-1 contributes to an adequate protective immune response against influenza infection.

Introduction

Influenza virus is an enveloped single-strained RNA virus and a member of the *Orthomyxoviridae* family. During infection of the upper respiratory tract influenza A is associated with fever, sneezing, chills, cough, soar throat and general malaise (1, 2). In severe cases influenza infection may lead to pneumonia. In the United States, an average of about 36,000 people per year die from influenza, and 114,000 per year are admitted to a hospital as a result of influenza. Between 250,000 and 500,000 die from influenza infection each year worldwide according to the World Health organization. Influenza virus infect mainly epithelial cells but can also infect monocytes/macrophages which produce inflammatory cytokines and chemokines to facilitate the cellular immune response (3)[review (4)).

Several studies have examined the role of cytokines, chemokines and chemokine-receptors in the immune response against influenza pneumonia (5-17). Chemokines are members of the family of small inducible peptides which attract leucocytes. Monocyte chemoattractant protein 1 (MCP-1) is a member of the CC chemokine family with pleiotropic activities and a ligand for the chemokine receptor CCR2 (18). MCP-1 can be produced by several cells like monocytes, macrophages, epithelial cells, endothelial cells and fibroblasts after stimulation with cytokines or microbacterial product (19, 20). MCP-1 primarily attracts monocytes and T-cells (21) but also contributes to neutrophil recruitment during severe bacterial infections (22, 23). During infection with *Listeria monocytogenes* (24) CCR2 knockout (KO) mice were unable to clear the pathogen and were flawed in both delayed-type hypersensitivity response and production of Th1-type cytokines (25). In addition, MCP-1 played an important role in the T-cell dependent immune response against respiratory tract infection with *Cryptococcus neoformans*; treatment with an antibody against MCP-1 reduced leukocyte infiltration in the bronchoalveolar space and inhibited clearance of the pathogen (26). T-cell mediated immune response is also important in protective immunity against influenza virus (27). Mice lacking CCR2, displayed an increased viral load and delayed pulmonary leukocyte recruitment, but were less susceptible to death due to influenza infection (5). These data suggest a vital role for MCP-1 during T-cell mediated immune response in pulmonary infection.

Besides MCP-1 other chemokines like MCP-3 to MCP-5 are ligands for CCR2 (28-32). To investigate the specific role of MCP-1 during influenza pneumonia we inoculated MCP-1 KO and wild-type (WT) mice with the mouse adapted strain of influenza A and compared viral load, cytokine and chemokine production and T-cell activation in the lungs. We show a significant role for MCP-1 during influenza infection: MCP-1 KO mice displayed enhanced progression of influenza infection which was accompanied by elevated cytokine levels in the lungs and decreased leukocyte recruitment into the pulmonary compartment.

Materials and Methods

Animals: Specific pathogen free 8-10 weeks old C57BL/6 mice (WT) were purchased from Charles River (Maastricht, The Netherlands). MCP-1 KO mice, backcrossed to a C57BL/6 genetic background, were obtained from Jackson Laboratory (Bar Harbor, Maine) and bred in the animal facility of the Academic Medical Center in Amsterdam. Age and sex matched mice were used in all experiments. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, the Netherlands).

Viral infection: The model of influenza pneumonia has been described earlier (13, 15). Briefly, mice were anesthesized by inhalation of isoflurane (Abbott Laboratories, Kent, UK) and inoculated intranasally with 50 µl phosphate buffered saline (PBS) containing 4000 viral copies of influenza A/PR/8/34 (ATCC VR-95, Rockville, MD).

Measurement of viral load: Mice were anesthetized with Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Meidrecht, the Netherlands). Lungs were harvested and homogenized at 4°C. in 4 volumes of sterile isotonic saline with tissue homogenizer (Biospect Products, Bartlesville, UK). Hundred µl of lung homogenate was dissolved in TRIzol (Invitrogen, Breda, the Netherlands) and RNA was prepared according to manufacturer´s protocol. Next, cDNA synthesis was performed and viral loads in lungs obtained 2, 8 and 14 days after infection were determined using real-time quantitative polymerase chain reaction (PCR) (33). The detection limit of this PCR is approximately 300 viral particles per lung.

Assays: Lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, and 20 ng/ml Pepstatin A, Leupeptin and Aprotinin, pH 7.4 and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g at 4°C for 15 minutes, and supernatants were stored at -20°C until assays were performed. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, IL12p70, monocyte chemoattractant protein (MCP)-1 and interferon (IFN)-γ were measured by cytometric beads array (CBA) multiplex assay (BD Biosciences, San Jose, CA). The detection limit of these measurements is 2.5 pg/ml for each cytokine/chemokine. Macrophage-inflammatory protein (MIP)-2 was measured by ELISA (R&D systems, Abingdon, UK).

Histopathological analysis: Lungs were fixed in 10% formalin and embedded in paraffin. Four μm lung sections were stained with hemotoxylin and eosin (HE) and analyzed by a pathologist who was blinded for the groups. A semi-quantitative scoring system was used to score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: pleuritis, bronchitis, edema, interstitial inflammation, intra-alveolar inflammation and endothelialitis. Each parameter was graded on a scale of 0 to 4 with 0 as 'absent' and 4 as 'severe'. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 24.

Flow cytometry: Pulmonary cell suspension were obtained by dispersing tissue through 40-μm cell strainer (Becton Dickinson, Franklin lakes, NJ) and collected in FACS staining buffer (PBS with 0.5% (w/v) bovine serum albumine). Erythrocytes were lysed with ice-cold isotonic lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4); the remaining cells were washed twice with FACS staining buffer. Cells ($1x10^6$) were stained for 15 minutes at 4ºC. with either: 1) anti-CD3-PE, anti-CD4-APC, anti-CD8-PerCP, anti-CD25-FITC or CD69-FITC, 2) GR-1-FITC, CD11b-PE, CD62L-APC, 3) NK1.1-APC or 4) F4/80-FITC. All antibodies were used in concentrations recommended by the manufacturer (Pharmingen, San Diego, CA). FACS analysis was performed on a FACS caliber with Cell Quest software (Becton Dickinson, San Jose, CA).

Statistical analysis: Data are expressed as means ± SEM. Changes in MCP-1 concentrations in time were analyzed by one-way analysis of variance. Differences

between WT and MCP-1 KO mice were analyzed by Mann Whitney U test. A value of $P < 0.05$ was considered statistically significant.

Results:

MCP-1 is highly expressed during influenza pneumonia

We first determined MCP-1 levels in lung homogenates from uninfected WT mice and WT mice 2, 8 and 14 days after influenza infection. Two and 8 days after infection pulmonary MCP-1 levels were significantly increased compared to uninfected mice (Figure 1: 2 days; $P<0.05$, 8 days; $P<0.001$). Peak MCP-1 levels, reached 8 days after infection, were approximately 16-fold higher than in uninfected mice. Fourteen days after infection MCP-1 levels had returned to basline values. MCP-1 remained undetectable in MCP-1 KO mice.

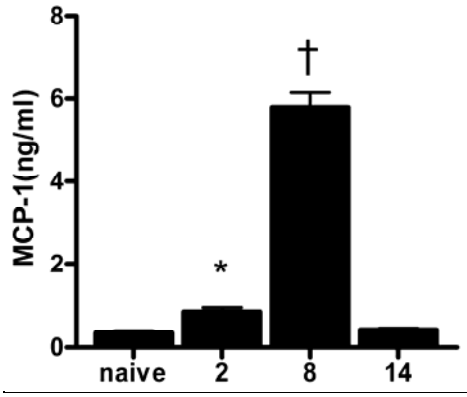

Figure 1: MCP-1 is highly expressed during influenza infection. Pulmonary MCP-1 levels from naïve or influenza-infected mice 2, 8 or 14 days after infection. Data are mean ± SEM (N=5-8 per group) and analyzed with One-way ANOVA. * $P<0.05$ † $P<0.001$ versus uninfected WT mice (0 days).

MCP-1 limits the outgrowth of influenza infection and weight loss

To investigate the role of MCP-1 during influenza infection we intranasally inoculated WT and MCP-1 KO mice with influenza and measured bodyweights and viral loads 2, 8 and 14 days after infection. MCP-1 KO mice lost significantly more weigh compared to WT mice at all time points measured (Figure 2A: 2 and 8 days $P<0.01$; 14 days $P<0.005$). While WT mice had recovered at day 14, i.e. weight was similar compared to day 0, MCP-1 KO mice had not recovered completely. To further investigate the difference seen in weight loss and recovery between MCP1-KO and WT mice we determined viral loads in lung homogenates. Viral loads in MCP-1 KO mice were significantly increased compared to WT mice 8 days after infection (Figure

2B: 8 days; P<0.05). Fourteen days after infection both mouse strains had similar viral loads in the lungs which were near the detection limit.

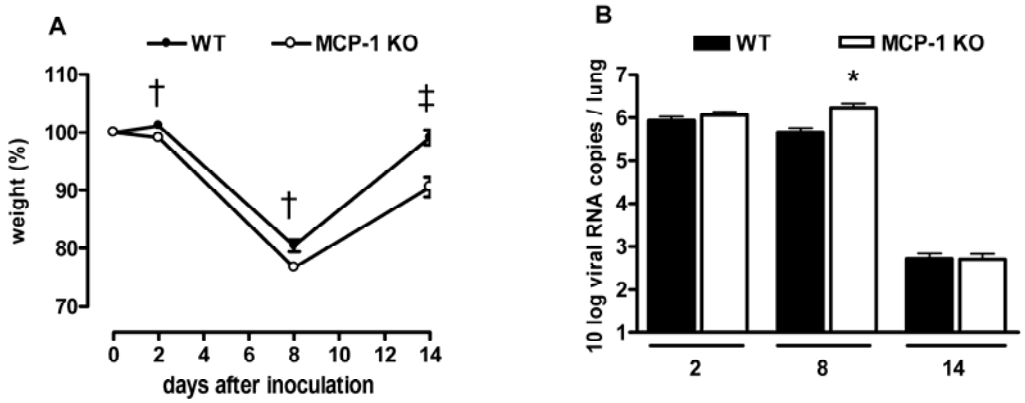

Figure 2: Increased weight loss and enhanced viral loads in MCP-1 KO mice. Bodyweight (2A) and viral load (2B) in WT (black symbols or bars) and MCP-1 KO mice (white symbols or bars) 2, 8 and 14 days after influenza infection. Data are mean ± SEM (N=7-8 per group) and analyzed with Mann-Whitney U test. * P<0.05, † P<0.01 ‡ P<0.005 versus WT mice.

Histopathology

To further investigate the difference seen in viral loads and weight recovery between MCP-1 KO and WT mice we performed histopathological analysis of lung tissue slides 2, 8 and 14 days after viral infection. The total lung scores, as outlined in the Materials and Methods section, of MCP-1 KO mice and WT mice were similar at day 2 and 8 but tended to be higher in MCP-1 KO mice at day 14 compared to WT mice (table 1: day 14; P=0.13). Analyzing the parameters of the total lung score individually showed that interstitial inflammation, pleuritis and edema all tended to be more pronounced in MCP-1 KO mice 14 days after infection compared to WT mice (table 1, P=0.08, P=0.05 and P=0.13 respectively). Figure 3 shows representative lung tissue slides of MCP-1 KO and WT mice obtained 14 days after infection.

Table 1: Histopathological analysis

	T=2 days		T=8 days		T=14 days	
	WT	MCP-1 KO	WT	MCP-1 KO	WT	MCP-1 KO
Total lung score	5.5 ± 0.4	5.0 ± 0.7	17.9 ± 1.2	19.0 ± 0.5	10.6 ± 1.8	14.5 ± 1.2 P=0.13
Interstitial inflammation	2.0 ± 0.0	2.1 ± .01	3.3 ± 0.4	3.1 ± .04	1.6 ± 0.4	2.7 ± 0.2 P=0.08
Endothelialitis	1.3 ± 0.3	0.9 ± 0.3	3.1 ± 0.3	3.5 ± 0.2	2.2 ± 0.4	2.5 ± 0.2
Bronchitis	1.3 ± 0.3	0.6 ± 0.2	3.6 ± 0.2	3.3 ± 0.3	2.8 ± 0.4	2.7 ± 0.2
Pleuritis	0.4 ± 0.2	0.6 ± 0.2	2.6 ± 0.3	2.9 ± 0.1	1.2 ± 0.4	2.3 ± 0.2 P=0.05
Edema	0.6 ± 0.2	0.9 ± 0.1	2.1 ± 0.2	2.8 ± 0.3	0.2 ± 0.2	1.3 ± 0.5 P=0.13
Intra alveolar inflammation	0	0	3.5 ± 0.2	3.4 ± 0.3	2.6 ± 0.4	3.0 ± 0.3

Histopathological analysis of WT and MCP-1 KO mice 2, 8 and 14 days after infection with influenza. Data are mean ± SEM (N=7-8 per group) and analyzed with Mann-Whitney U test. P value versus WT mice.

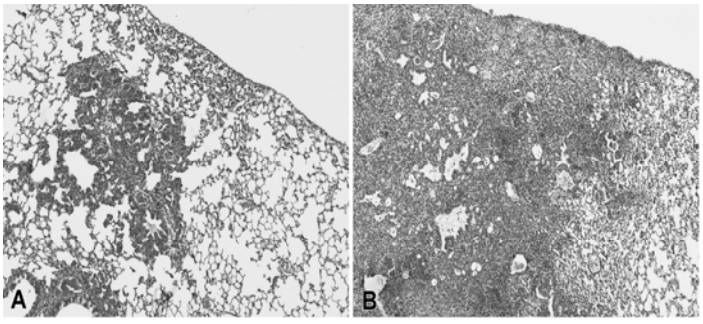

Figure 3: Histopathology.
Representative lung tissue slides of WT (A) and MCP-1 KO (B) mice obtained 14 days after infection with influenza. H & E staining.
Original magnification 4 x.

MCP-1 KO mice display reduced macrophage and neutrophil recruitment to the lungs

Considering that MCP-1 has been implicated to play an important role in leukocyte trafficking during infection and inflammation (18), we decided to characterize the inflammatory cell infiltrates in lung tissues of MCP-1 KO and WT mice at 8 days after infection by FACS analysis, i.e. at the time of maximal pathology scores. MCP-1 KO mice displayed significantly reduced total cell counts in whole lung cell suspensions as compared with WT mice (table 2, $P<0.005$). In addition, the cell composition in whole lung cell suspensions differed between the two mouse strains: the percentages of macrophages and granulocytes were diminished in MCP-1 KO

mice (table 2, both P<0.05). Granulocyte activation markers CD11b and CD62L were equally expressed between MCP-1 KO and WT GR1 positive granulocytes (data not shown). To determine whether the cell composition in whole lungs differed with respect to T cell subsets between MCP-1 KO and WT mice, we analyzed CD3 positive lymphocytes for the expression of CD4 and CD8 (table 2). This revealed that the percentage of CD4 and CD8 positive lymphocytes within the CD3 positive population was similar in both strains. T-cell activation markers CD25 and CD69 were equally expressed on CD4 and CD8 positive lymphocytes from WT and MCP-1 KO mice (data not shown). The percentage of natural killer cells was similar between the two mouse strains.

Table 2: Cell counts in whole lung cell suspensions

	WT	MCP-1 KO
Total cell count	32 ± 2 x 10^6 cells/ml	23 ± 2 x 10^6 cells/ml †
Macrophages	3.5 ± 0.4 %	2.6 ± 0.1 % *
Natural killer cells	1.6 ± 0.2 %	1.8 ± 0.4 %
Granulocytes	44.2 ± 2.0 %	33.0 ± 2.6 % *
Lymphocyte $CD4^+$	9.5 ± 0.8 %	10.7 ± 2.3 %
Lymphocyte $CD8^+$	21.9 ± 1.4 %	24.1 ± 3.0 %

Cell count in whole lung cell suspension from WT and MCP-1 KO mice 8 days after infection with influenza. Data are mean ± SEM (N=6-7 per group) and analyzed with Mann-Whitney U test. * P<0.05, † P<0.005 versus WT mice.

MCP-1 KO mice demonstrate elevated lung cytokine concentrations

Finally, we measured TNF-α, IL-6, IL-10, IL12p70, IFN-γ and chemokine MIP-2 in lung homogenates from MCP-1 KO and WT mice. At 2 days after infection, no differences were observed between the two mouse strains. The highest cytokine levels were detected 8 days after infection; at this time point the pulmonary levels of TNF-α, IFN-γ and especially IL-6 and MIP-2 were significantly increased in MCP-1 KO mice compared to WT mice (Figure 4: TNF-α and IFN-γ; P<0.05, IL-6 and MIP-2; P<0.0005). IL-6 levels in the lungs were still significantly higher in MCP-1 KO mice compared to WT mice 14 days after influenza infection (P<0.05).

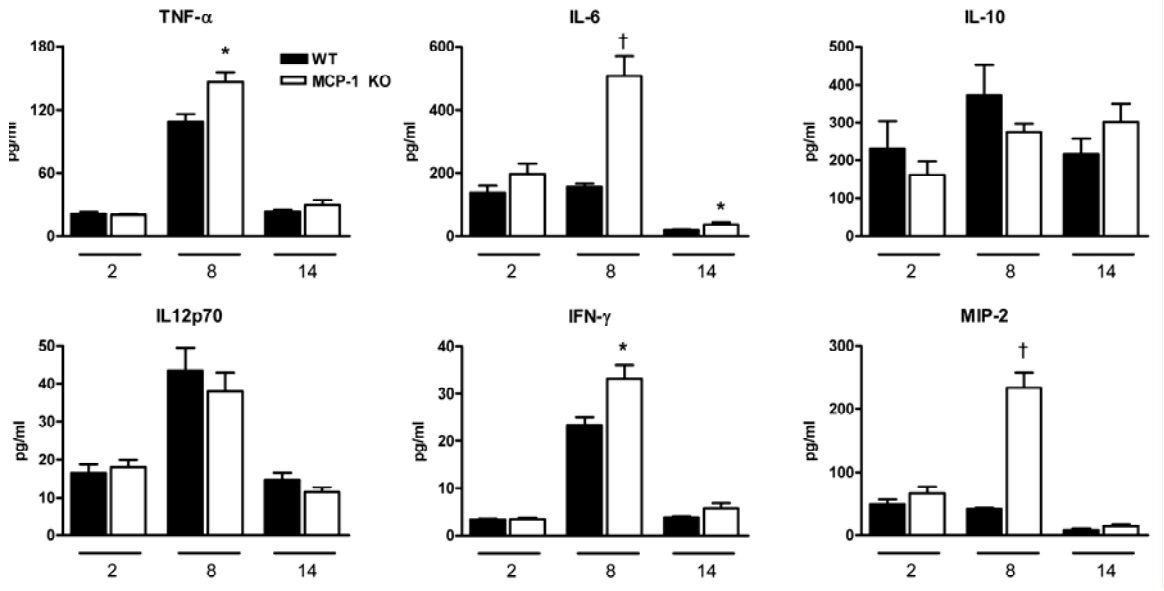

Figure 4: Elevated lung cytokine and chemokine levels in MCP-1 KO mice. Pulmonary cytokine and chemokine levels from WT (black bars) and MCP-1 KO mice (white bars) 2, 8 and 14 days after infection with influenza. Data are mean ± SEM (N=7-8 per group) and analyzed with Mann-Whitney U test. * $P<0.05$, † $P<0.0005$ versus WT mice.

Discussion

MCP-1 is a chemokine with pleiotropic properties and a chemoattractant for several leukocytes. MCP-1 and its receptor CCR2 have been shown to play an important role in T-cell mediated immune response against intracellular pathogens by controlling leukocyte recruitment to the site of infection and clearance of the pathogen (5, 24, 26). A T-cell mediated immune response is of importance in the immune response against influenza infection (27). In this study we investigated the role of MCP-1 during pulmonary infection with influenza A. We demonstrate that pulmonary MCP-1 concentrations increased during the course of infection. MCP-1 KO mice showed enhanced weight loss and in contrast to WT mice, did not fully regain their body weights during the 14-day observation period. Eight days after infection MCP-1 KO mice had increased viral load compared to WT mice which was accompanied by reduced leukocyte recruitment into the lung and higher pulmonary concentrations of TNF-α, IL-6, IFN-γ and MIP-2. Together these data indicate that MCP-1 contributes to an adequate immune response against influenza infection

Several studies have investigated the contribution of cytokines, chemokines and chemokine-receptors during a T-cell mediated immune response against intracellular pathogens (5-17, 24-26, 34-38). A comparable study performed by Dawson et al. (5) showed that CCR2 KO mice infected with influenza had a higher pulmonary viral load compared to WT mice. Histological examination showed that inflammatory cell infiltration and tissue damage was diminished in the lungs of CCR2 KO mice. Differential leukocyte counts of bronchoalveolar lavage showed that CCR2 KO mice had less monocyte/macrophage influx but increased neutrophil influx. Surprisingly, influenza infected CCR-2 KO mice demonstrated a decreased mortality. The authors concluded that the inability of macrophages to recruit to the pulmonary compartment may delay the pathogenesis of infection which increased survival rate of CCR-2 KO mice, regardless of the increased viral load (5). Although our model is a non-lethal infection model and we used mice deficient of one CCR-2 ligand (MCP-1), the differences seen in the antiviral mechanism and macrophage recruitment in our study is comparable to observations seen in the study with CCR2 KO mice (5): we observed increased viral loads in MCP-1 KO mice with a reduced leukocyte recruitment into the infected lungs of MCP-1 KO mice, primarily caused by diminished macrophage influx. In contrast to the study performed by Dawson et al. (5) we observed reduced neutrophil recruitment into the lungs of MCP-1 KO mice rather than increased neutrophil recruitment. Of note, we did not measure cell influx in bronchoalveolar lavage from influenza infected MCP-1 KO and WT mice but in whole lung cell suspensions.

Several studies have focused on the role of specific immune cells during viral airway infection. Both CD4+ and CD8+ T cells have been implicated in host defense against influenza virus. The CD4+ T cell subset has been implicated as the primary inducer of inflammatory processes during influenza. However, depletion of CD4+ T cells in normal mice had little effect on the clearance of influenza or the cell composition and the localization of CD8+ T cells in the lungs of mice (39). In contrast, depletion of CD8+ T cells did have a marked effect on influenza titers in the pulmonary compartment. CD8+ T cells are therefore considered as a primary effector cells involved in the clearance of influenza A virus [48, 49]. MCP-1 KO mice did not show any differences in the relative number and activation status of both CD4+ and CD8+ T cells, indicating that the higher viral loads on day 8 are not likely caused by

impaired T cell recruitment to the lungs. Instead, we did observe differences in the number of macrophages and granulocytes. Wijburg et al. showed that depletion of alveolar macrophages prior to influenza infection, affected pulmonary viral load 4 days after infection but viral clearance as a function of time was not affected and both treated and non-treated mice cleared the influenza virus similarly (40). In a comparable study performed by Tumpey et al. (41) depletion of macrophages and/or neutrophils prior to infection with influenza reduced the pulmonary cytokine and chemokine production and increased viral load and mortality compared to non treated mice (41). In this latter study, depletion of alveolar macrophages had a more pronounced effect on viral load at a late time-point (i.e. day 6) than depletion of granulocytes. It should be noted that both studies focused on depletion of resident alveolar macrophages, whereas we determined the number of total lung macrophages.

MCP-1 KO mice showed increased pulmonary cytokine levels of TNF-α, IFN-γ and especially IL-6 and MIP-2. These cytokines and chemokine are not likely to originate from monocytes/macrophages and/or granulocytes, since the number of these cells were diminished in MCP-1 KO mice. These cytokines are likely to originate from other cells like epithelial (42, 43) or dendritic cells (44, 45) as a consequence of the higher viral load. The lower number of leukocytes in MCP-1 KO mice during infection could at least in part explain the increased viral loads resulting in enhanced weight-loss, a marker for influenza severity frequently used in murine influenza infection models (46). Reduced weight recovery of MCP-1 KO mice at day 14 is in line with a delayed recovery of lung inflammation as observed from the pathology analysis of lung tissue slides at day 14. Of note, MCP-1 KO mice eventually did recover from the infection, did show leukocyte recruitment, and had similar viral loads on day 14. This shows that MCP-1 is not the primary key player in resolving the viral infection and absence of MCP-1 does not lead to a defective- but rather a slower clearance of the virus.

In conclusion, we here demonstrate that the MCP-1 KO mice have a reduced antiviral clearance together with reduced leukocyte recruitment into the lungs during infection with influenza A. Hence, MCP-1 contributes to a protective immune response during infection.

Acknowledgment

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Chapter 8

CD14 plays a limited role during influenza A virus infection *in vivo*

Submitted

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Abstract

Influenza A is a single stranded (ss)RNA virus that can cause upper respiratory tract infections that in rare cases may progress to pneumonia. Toll-like receptors (TLRs) and CD14 are receptors which recognize viral proteins and nucleic acid of several viruses. CD14 is required for influenza-induced cytokine production during infection of mouse macrophages. In addition, CD14 was shown to bind ssRNA, suggesting an important role for CD14 during infection with influenza. To investigate the role of CD14 during influenza pneumonia we inoculated WT and CD14 KO mice with a non-lethal dose of a mouse adapted strain of influenza A. CD14 KO mice displayed a reduced viral load in the lungs, 2 and 14 days after infection with influenza. Pulmonary cytokine production in CD14 KO mice was reduced at day 2 and elevated at day 8 compared to WT mice. CD14 deficiency did not influence lymphocyte recruitment or lymphocyte activation in lungs and draining lymph nodes 8 days after infection. These data show that CD14 plays a limited role in host defense against infection with influenza.

Introduction

Influenza A is a single stranded (ss)RNA virus that belongs to the family of *Orthomyxoviridae*. Respiratory tract infection by this virus is associated with fever, chills, cough, soar throat and general malaise and may also lead to pneumonia (1, 2). Especially in young children, the elderly and immuno-compromised individuals, influenza infection may lead to a more severe outcome of the disease (3). Antigen-presentation by macrophages and dendritic cells is a key event in the cellular immune response against the influenza virus (4). Influenza-infected cells produce several chemotactic, pro-inflammatory and antiviral cytokines to facilitate the cellular immune response against the virus (review(5)).

Pattern recognition receptors (PRRs) are receptors which recognize pathogen-associated molecular patterns (review (6)). Toll-like receptors (TLRs) and CD14 are PRRs that recognize viral proteins and nucleic acid of several viruses. After recognition, cells become activated and produce antiviral cytokines like interferons (IFN) (7). CD14 is a glycosyl phosphatidylinositol (GPI) surface anchored molecule particularly expressed on monocytes and macrophages and to a lesser extent neutrophils (8-10). Infection of macrophages, neutrophils, dendritic cells and epithelial cells with influenza A is known to affect expression of TLRs and TLR-adaptor molecules like TRIF (Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing IFN-beta) and MyD88 (myeloid differentiation primary response gene 88) (11-15). Membrane bound CD14 lacks an intracellular domain and requires interaction with other receptors, like TLR2 and TLR4, for signal transduction (16). Pauligk et al. showed that CD14 is required for influenza-induced cytokine production during infection of murine macrophages; this CD14 function was not dependent on TLR2 and TLR4 (17). Interestingly, a recent study showed that CD14 can bind both ssRNA and double stranded (ds)RNA and mediates uptake of poly I:C (pIpC), a synthetic mimic of viral dsRNA (18). This may implicate that CD14 acts as a transporter of viral products or viruses (19). In addition, CD14 inhibits T cell proliferation and cytokine production (20, 21) and was recently shown to be expressed in a subpopulation of CD8+ lymphocytes (22) which are important effector cells involved in the clearance of influenza (23, 24). Together, these data indicate that CD14 may play a role in host defense against respiratory tract infection by influenza

A. However, thus far, such a potential role has not been directly addressed. Therefore, we here infected CD14 KO mice and WT mice intranasally with influenza A virus and determined the viral load and inflammatory response in the lungs during the course of infection.

Material and methods

Animals: Specific pathogen free 8-10 weeks old C57BL/6 mice (WT) were purchased from Charles River (Maastricht, The Netherlands). CD14 KO mice, backcrossed to a C57BL/6 genetic background, were obtained from Jackson Laboratory (Bar Harbor, Maine) and bred in the animal facility of the Academic Medical Center in Amsterdam. Age and sex matched mice were used in all experiments. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, the Netherlands).

Viral infection: The model of influenza pneumonia has been described earlier (25, 26). Briefly, mice were anesthesized by inhalation of isoflurane (Abbott Laboratories, Kent, UK) and inoculated intranasally with 50 µl phosphate buffered saline containing 1400 viral copies of influenza A/PR/8/34 (ATCC VR-95, Rockville, MD).

Measurement of viral load: Mice were anesthetized with Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Meidrecht, the Netherlands). Lungs were harvested and homogenized at 4°C. in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, UK). Hundred µl of lung homogenate was dissolved in TRIzol (Invitrogen, Breda, the Netherlands) and RNA was prepared according to manufacturer's protocol. Viral loads in lungs obtained 2, 4, 8 and 14 days after infection were determined using real-time quantitative polymerase chain reaction (PCR) (27).

Assays: Lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, and Pepstatin A, Leupeptin and Aprotinin (all 20 ng/ml; pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g at 4°C for 15 minutes, and supernatants were stored at -20°C until assays were performed. Tumor necrosis factor (TNF)-α, Interleukin (IL)-6, IL-10, IL-12p70, monocyte chemoattractant protein (MCP)-1 and

Interferon (IFN)-γ were measured by cytometric beads array (CBA) multiplex assay (BD Biosciences, San Jose, CA). Detection limits were 2.5 pg/ml. Myeloperoxidase (MPO) was measured by ELISA (HyCult, Uden, the Netherlands).

Histopathological analysis: Lungs were fixed in 10% formalin and embedded in paraffin. Four μm lung sections were stained with hemotoxylin and eosin (HE) and analyzed by a pathologist who was blinded for groups. To score lung inflammation and damage, a semi-quantitative scoring system was used; for this, the entire lung surface was analyzed with respect to the following parameters: pleuritis, bronchitis, edema, interstitial inflammation, intra-alveolar inflammation, and endothelialitis. Each parameter was graded on a scale of 0 to 4 with 0 as 'absent', 1 as 'slight', 2 as 'mild', 3 as 'moderate' and 4 as 'severe'. The total 'lung inflammation score' was expressed as the sum of the scores for each parameter, the maximum being 24.

Flow cytometry: Pulmonary and draining lymph node cell suspensions were obtained by dispersing tissue through nylon sieves and collected in FACS staining buffer (PBS with 0,5% (w/v) bovine serum albumine). Cells ($1x10^6$) were stained for 15 minutes at 4ºC. with anti-CD3-PE (clone KT3), anti-CD4-APC (clone RM4-5), anti-CD8-PerCP (clone 53-6.7) or CD69-FITC (clone H1.2F3). All antibodies were obtained from BD Pharming (San Diego, CA). FACS analysis was performed on a FACS calibur with Cell Quest software (Becton Dickinson, San Jose, CA).

Statistical analysis: Data are expressed as means ± SEM. Differences were analyzed by Mann Whitney U test. A value of $P < 0.05$ was considered statistically significant. For viral loads that were below the limit of detection of the assay (50 viral copies per lung) a value equivalent to half the detection limit was used for statistical analysis.

Results:

Body weights and viral loads

WT and CD14 KO mice were inoculated with influenza A and weight was measured 0, 2, 4, 8 and 14 days after viral infection. Bodyweights of both mouse strains declined equally, reaching a nadir at day 8, and both strains had recovered similarly at day 14 (Figure 1A). Next, we determined the viral loads in whole lung homogenates from CD14 KO and WT mice on day 2, 4, 8, and 14 using real-time quantitative PCR (Figure 1B). The viral loads in lungs from CD14 KO mice were significantly

decreased compared to WT mice 2 and 14 days after inoculation of influenza A (both $P<0.05$). Of note, at day 14, influenza virus was detectable in 6 out of 8 lung samples from WT mice (detection limit 50 viral copies) versus in 2 out of 8 lung samples from CD14 KO mice.

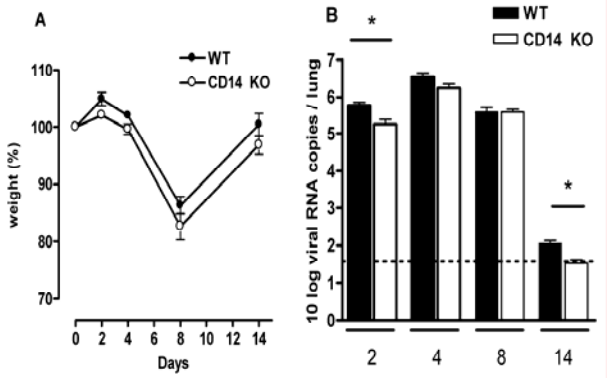


Figure 1: Body weights and viral loads. Body weight (1A) and viral load (1B) in WT (black symbols or bars) and CD14 KO mice (white symbols or bars) 2, 4, 8 and 14 days after infection with influenza. Figure 1A: body weight is expressed relatively to day 0. Figure 1B: dashed line indicates detection limit. Data are mean ± SEM (N=7-8 per group). * $P<0.05$ versus WT.

Lung histology and leukocyte recruitment

To further investigate the host response to influenza, we performed histopathological analysis of lung tissue slides of mice 2, 4 and 8 days after infection. Total lung pathology scores, determined as outlined in the Materials and Methods section, were similar in WT and CD14 KO mice at day 2 (5.0 ± 1.1 vs. 5.2 ± 0.4), day 4 (9.8 ± 1.0 vs. 9.1 ± 0.7) and day 8 (16.3 ± 1.9 vs. 16.7 ± 0.6). Figure 2 shows representative lung tissue slides of WT and CD14 KO mice from these time-points.

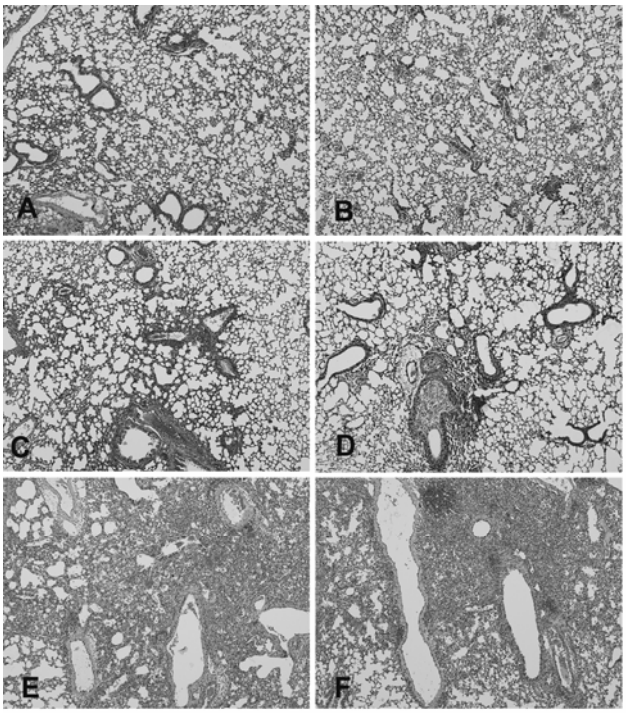
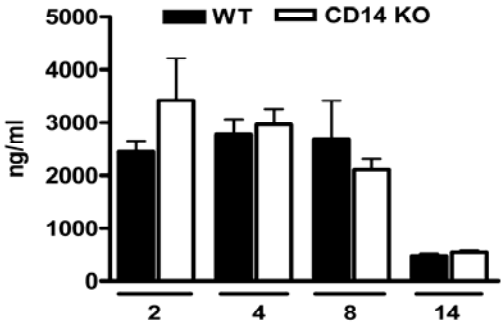


Figure 2: Histopathology.
Representative lung tissue slides of WT (panel A, C and E) and CD14 KO (B, D and F) obtained 2 (panel A and B), 4 (panel C and D) and 8 (panel E and F) days after infection with influenza. H&E staining. Magnification 4x.

To obtain insight into the role of CD14 in lymphocyte trafficking during influenza infection, we analyzed lymphocyte subsets in lungs and draining lymph nodes (DLN) 8 days after infection i.e. a time point frequently used to determine lymphocyte composition in these organs (25, 26). No differences in the percentage of CD4+ or CD8+ T cells were found in lungs or DLN of CD14 KO and WT mice (data not shown). In addition, the activation status of these T cells (measured as CD69 positivity) did not differ between mouse strains (data not shown). To further investigate lung inflammation we determined pulmonary MPO levels, reflecting the whole organ neutrophil content, in CD14 KO mice and WT mice. MPO levels were similar 2, 4, 8 and 14 days after infection between CD14 KO and WT mice (Figure 3).

Figure 3: Similar lung inflammation in CD14 KO mice. MPO levels in lungs of WT (black bars) and CD14 KO mice (white bars) 2, 4, 8 and 14 days after infection with influenza. Data are mean ± SEM (N=7-8 per group).



Cytokines and chemokines

To establish the contribution of CD14 to the pulmonary cytokine and chemokine response to influenza, we determined the lung concentrations of TNF-α, IL-6, IL-10, MCP-1, IFN-γ and IL-12p70 in whole lung homogenates obtained from CD14 KO mice and WT mice at day 2, 4, 8 and 14 after infection (Figure 4). Four days after infection CD14 KO mice displayed lower concentrations of TNF-α, IL-10 (both $P<0.05$) and IL-12p70 ($P<0.01$) in their lungs when compared to WT mice. Eight days after infection CD14 KO mice displayed higher concentrations of MCP-1 and IFN-γ (both $P<0.05$) and lower concentration of IL-10 ($P=0.05$) in their lungs compared to WT mice.

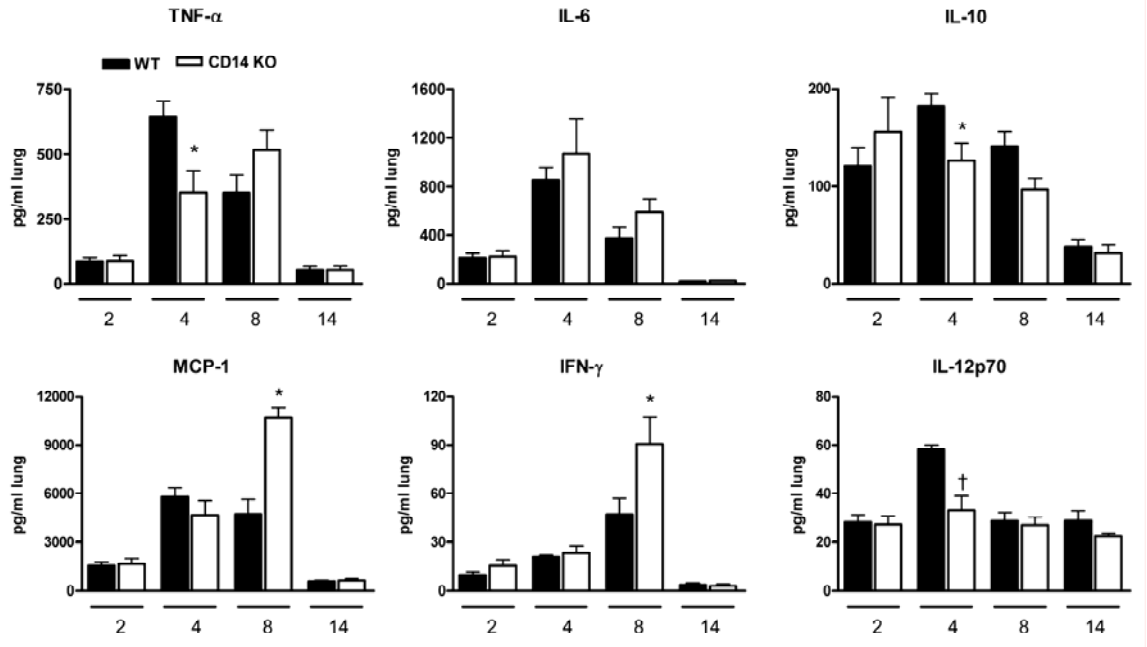


Figure 4: Pulmonary cytokine and chemokine concentrations. Pulmonary cytokine and chemokine levels from WT (black bars) and CD14 KO mice (white bars) 2, 4, 8 and 14 days after infection with influenza. Data are mean ± SEM (N=7-8 per group). * $P<0.05$ versus WT, † $P<0.01$ versus WT.

Discussion

Earlier studies have shown that CD14 is required for influenza-induced cytokine production by macrophages (17). Moreover, CD14 can bind ssRNA (18) and may be a transporter of viral products or viruses (19), suggesting an important role for CD14 during influenza infection. To investigate the role of CD14 in influenza pneumonia we inoculated WT and CD14 KO mice with a mouse adapted strain of influenza A and determined the pulmonary viral load, lymphocyte influx and cytokine and chemokine production. CD14 KO mice displayed a reduced viral load in the lungs, 2 and 14 days after infection and, in particular, an altered inflammatory mediator response, 4 and 8 days after infection. CD14 deficiency did not impact on lymphocyte migration or activation. Hence, although CD14 deficiency affects viral load and cytokine production during influenza infection, it does not critically impair clearance of the virus.

Although the role of CD14 in bacterial infections is widely documented (28-35), knowledge of its role in influenza infection is limited. CD14 is a 55 kDa GPI-linked protein present on the surface of several phagocytes like monocytes, macrophages and to a lesser extent neutrophils (8-10). CD14 is a known receptor for lipopolysaccharide (LPS) but can also bind lipoteichoic acid, pIpC, ssRNA and dsRNA (18, 36-38). CD14 has been associated with the cytokine response to respiratory syncytial virus and cytomegalovirus (39, 40). Recently, Pauligk et al. showed that influenza-induced cytokine production was impaired when human monocytes were treated with CD14 antibodies (17). Moreover, macrophages from CD14 KO mice were less responsive to influenza than WT macrophages (17). Since CD14 has no intracellular signaling domain it requires other receptors, like TLR2 or TLR4, for cell signaling (16). However, both in vitro and in vivo experiments have shown that TLR2 or TLR4 do not play a significant role in the immune response against influenza infection (17, 41, 42). These results show that CD14, together with a receptor other than TLR2 and TLR4, is a coreceptor for the recognition of influenza. Lee et al. showed that CD14 KO mice and macrophages derived from CD14 KO mice were less responsive to stimulation with pIpC than WT animals or cells (18). The authors showed that CD14 binds pIpC and internalizes thereafter. Once internalized, the pIpC-CD14 complex is recognized by TLR3 and induces an inflammatory response (18). Of note, influenza is

a ssRNA virus while pIpC mimics dsRNA. Stimulation of TLR3 deficient dendritic cells fully respond to ssRNA and influenza but not to dsRNA (12, 18, 43). TLR7 and TLR8 were identified as crucial receptors for the recognition of influenza and ssRNA respectively by dendritic cells (12, 43) but so far, an interaction between CD14 and TLR7 or TLR8 has not been reported. This indicates that CD14 may serve as a chaperone that facilitates binding and internalization of bacterial and viral products (19) and might be an entry-receptor for influenza. However, which receptor interacts with CD14 for the recognition of influenza in vivo is yet unknown. In our model, pulmonary viral load was decreased in CD14 KO mice early after infection, which might indicate that CD14 also plays a role in the internalization of influenza in vivo. Epithelial cells are the primary target cells of influenza A although macrophages can also be infected. CD14 expression is lacking on alveolar macrophages and epithelial cells in naïve mice but expression of CD14 is enhanced after inhalation of LPS (44). Whether CD14 contributes to the internalization of influenza in pre-stimulated epithelial cells (or macrophages) remains to be elucidated.

T-cell mediated immune response is important in protective immunity against influenza infection (45). Monocytes play a critical role in the activation of T cells through interaction with the T cell receptor (TCR)/CD3 complex with antigen bound to the MHCII class. In addition, monocytes provide the costimulatory signal required for the induction of IL-2 (46). CD8+ T cells are considered to be the primary effector cells in the clearance of influenza (23, 24). Recently, CD14 was shown to be expressed intracellularly by a subpopulation of CD8+ lymphocytes (22). CD14 either as a recombinant protein or as a native molecule secreted by monocytes can bind to the surface of in vitro activated human T cells (47). Importantly, (soluble) CD14 was shown to reduce monocyte-dependent T cell proliferation and release of cytokines like IL-2, IL-4 and IFN-γ (20, 21). In our influenza-model, lymphocyte influx is mainly present 8 days after inoculation of influenza (25, 26, 41). If CD14 would diminish T cell mediated IFN-γ production (21), this could explain the elevated pulmonary IFN-γ levels observed in CD14 KO mice, relative to WT mice, 8 days after infection. IFN-γ is an important antiviral mediator during influenza infection and induces several antiviral mechanisms, including inhibition of viral replication in virus infected cells by cytotoxic CD8+ T cells (48). The enhanced IFN-γ production in CD14 KO mice could facilitate clearance of the virus which was observed 14 days after infection.

Besides the enhanced IFN-γ production, the reduced IL-10 production in lungs from CD14 KO mice would favor a T helper (Th)-1 immune response, important for clearance of the virus (45).

In conclusion, we here show that CD14 plays a modest role in the clearance of influenza virus from the respiratory tract. Although CD14 deficiency impacts on pulmonary cytokine levels during influenza, its role in host defense seems redundant.

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Chapter 9

Gene-expression profiles in murine influenza pneumonia

Submitted

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Abstract

Infection of epithelial cells and leukocytes by influenza A is known to induce cytokine and chemokine expression and alter Toll-like receptor- (TLR) and tissue factor- expression. Although many individual *in vitro* studies have focused on gene expression in leukocytes, lung tissue or cell lines, knowledge on gene expression in these compartments *in vivo* is limited. To obtain insight in gene expression profiles during influenza infection, we determined multiple-gene expression by using a newly developed mouse specific Multiplex Ligation-dependent Probe Amplification (MLPA) assay. Genes involved in inflammation, TLR signaling, coagulation, fibrinolysis, cell adhesion, tissue repair and homeostasis were measured in lung tissue, leukocytes in bronchoalveolar lavage fluid and tracheal epithelial cells in mice before and after intranasal infection with influenza A. Most of the genes investigated were differentially expressed during the course of infection and returned to basal levels when mice had recovered from the infection. However, expression of several genes remained altered even though mice had completely cleared the virus. These data provide the first information on compartmentalized gene expression profiles in the respiratory tract during influenza.

Introduction

Respiratory influenza A infection is associated with symptoms like fever, sore throat, sneezing and nausea. These symptoms usually start two to four days after infection and may last one to two weeks (1, 2). Although most people infected with influenza recover, in rare cases infection may lead to life-threatening complications such as pneumonia. An average of about 36,000 people per year in the United States die from influenza, and 114,000 per year are admitted to a hospital as a result of this viral infection. Worldwide between 250,000 and 500,000 people die from influenza infection each year according to the World Health Organization (www.who.int/en/).

Influenza A virus primarily infects airway epithelial cells, but other cells like macrophages and leukocytes can also be infected (3). Influenza infected epithelial cells and leukocytes produce a diversity of cytokines and chemokines (4)(review (5)) and infection affects expression of Toll-like receptors (TLR) and TLR-adaptor molecules like TRIF (Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing IFN-beta) and MyD88 (myeloid differentiation primary response gene 88) (4, 6-10). Besides inflammatory pathways, influenza virus can trigger the coagulation system: it increases the expression of tissue factor (TF; the main initiator of coagulation) on endothelial cells and monocytes *in vitro* (11, 12) and has recently been shown to induce a prothrombotic state in mice by stimulation of coagulation and concurrent inhibition of fibrinolysis (13).

Several studies have examined the expression of a broad range of genes in mice and humans during infection with influenza (4, 14-20). However, individual studies have focused on one specific compartment, i.e. either blood, lung tissue or epithelial cells. Knowledge of the dynamics and extent of gene expression in several areas in the lung during influenza infection *in vivo* is relatively limited. Such knowledge may be important not only to obtain insight into the immune response to primary influenza infection but also for understanding the enhanced susceptibility for secondary bacterial pneumonia. Therefore, in the present study we sought to determine the relative expression of a set of 39 genes encoding cytokines, chemokines, proteins involved in coagulation and fibrinolysis, TLRs and associated proteins, and various other mediators implicated in the immune response to infection, in whole lung tissue,

leukocytes harvested from bronchoalveolar lavage fluid (BALF) and respiratory epithelial cells obtained from mice at various time points after intranasal infection with influenza A.

Material and methods

Animals: Specific pathogen free male 8-10 weeks old C57BL/6 mice (WT) were purchased from Charles River (Maastricht, The Netherlands). All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, the Netherlands).

Virus infection: The model of influenza pneumonia has been described in detail (21, 22). Briefly, mice were anesthetized by inhalation of isoflurane (Abbott Laboratories, Kent, UK) and inoculated intranasally with 50 µl phosphate buffered saline containing 1400 viral copies of influenza A/PR/8/34 (ATCC VR-95, Rockville, MD). Mice were sacrificed before and 2, 8 and 14 days after infection for the measurements described below.

Preparation of whole lung homogenates: Mice were anesthetized with Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Meidrecht, the Netherlands). Lungs were harvested and homogenized at 4°C in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, UK). Hundred µl of lung homogenate was immediately dissolved in TRIzol (Invitrogen, Breda, the Netherlands) and RNA was prepared according to manufacturers protocol. Determination of the viral load was done by using real-time quantitative polymerase chain reaction (PCR) (23).

Bronchoalveolar lavage: Bronchoalveolar lavage (BAL) was performed in separate mice as described earlier (21, 22). Briefly, the trachea was exposed through a midline incision, cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland) and two 0.5 ml aliquots of sterile PBS were instilled and retrieved thereafter. For cell count and differentiation: BAL was spun at 1500 RPM for 10 minutes and cells were resuspended in 100 µl PBS and counted by a Z2 Coulter particle count and size analyzer (Beckman-Coulter Inc, Miami, FL); differential cell counts were determined on cytospin preparations stained with Giemsa stain (Diff-Quick, Baxter, UK). For RNA preparations: BALF was spun at 1500 RPM for 5 minutes and cell

pellet was immediately dissolved in TRIzol and RNA was prepared according to manufacturers' protocol.

Brush: To obtain RNA from lung epithelial cells, the trachea was excised at the bifurcation of the bronchi and opened via longitudinal incision. Epithelial cells were scraped from the interior of the trachea using a sterile cotton stick, which was placed in TRIzol immediately thereafter. Several minutes later the cotton stick was removed and RNA was prepared according to manufacturer's protocol.

Multiplex ligation-dependent probe amplification: RNA was analyzed by multiplex ligation-dependent probe amplification (MLPA) as described for human samples earlier (24-28). In collaboration with MRC-Holland (Amsterdam, the Netherlands) we developed a mouse-specific kit for the simultaneous detection of 39 mRNA molecules (Table E1 -page 153- + E2 -online data supplement-). This set was designed to obtain a global insight into the induction of several inflammatory pathways implicated in the host response to infection. All samples were tested with the same batch of reagents. The levels of mRNA for each gene were expressed as a normalized ratio of the peak area divided by the peak area of the housekeeping gene transferrin receptor (TFRC; P90, CD71) (29), resulting in the relative abundance of mRNAs of genes of interest (24-28). Results on relative gene expression were similar when expressed to another housekeeping gene, i.e. TATA box binding protein (TBP) (data not shown).

Statistical analysis: Values are expressed as mean ± SEM. Differences between two groups were analyzed by Mann-Whitney test. Differences between more than two groups were analyzed by one-way analysis of variance. $P < 0.05$ was considered statistically significant.

Results:

Induction of influenza pneumonia

In accordance with previous reports from our laboratory (21, 22, 30-33), intranasal infection with 1400 viral copies of influenza A/PR/8/34 resulted in a transient weight loss reaching a nadir 8 days postinfection (Figure 1A; $P<0.05$ compared to day 0); body weight had recovered to baseline at day 14. Viral loads were determined 2, 8 and 14 days after infection (Figure 1B). Two and 8 days after infection, viral load had increased considerably and at day 14, influenza virus was cleared from the lungs. The cellular composition of BALF did not change during the first 2 days of the infection and mainly consisted of macrophages and very little amount of neutrophils (Figure 1C). At day 8, total leukocyte count had increased approximately 8-fold in BALF compared to day 0, which was caused by an influx of macrophages, neutrophils, monocytes and lymphocytes. The number of macrophages tended to be higher at day 8 ($P=0.06$) and remained significantly increased at day 14 compared to day 0 ($P<0.05$). Total neutrophil count was significantly increased at day 8 ($P<0.01$) and modestly increased at day 14 ($P=0.06$) compared to day 0. At day 14, no lymphocytes or monocytes could be detected in BALF.

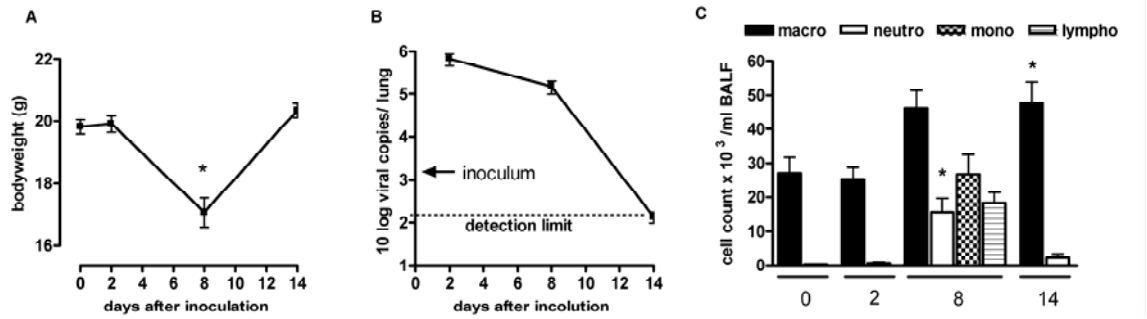

Figure 1: Body weights, viral loads and cell influx during influenza infection. Weight (Figure 1A), pulmonary viral load in lung homogenates (Figure 1B) and cell composition of BALF (Figure 1C) during influenza infection in WT mice. Data are mean ± SEM (N=8 per group). * $P<0.05$ versus uninfected mice (t=0 d).

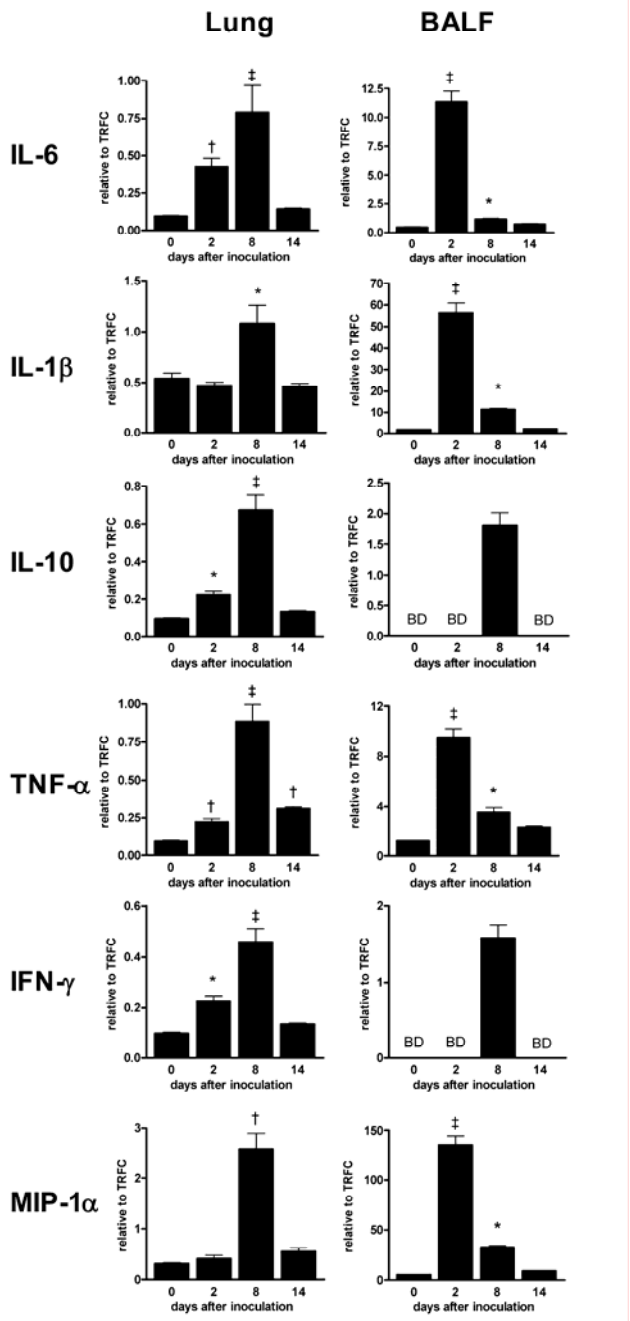

Figure 2: mRNA expression of several cytokines and chemokines. Naïve or influenza infected mice were sacrificed at specific time-points and mRNA was extracted from either lung tissue or cells from BALF. mRNA was measured by MLPA as described in the Methods section. Data are mean ± SEM (lung: N=8 per group, BALF: N=5 per group). * P<0.05, † P<0.01, ‡ P<0.001 versus uninfected mice. BD = below detection limit.

Cytokine and chemokine mRNA gene-expression

To determine mRNA expression of several cytokines and chemokines we analyzed mRNA profiles in lung tissue, leukocytes present in BALF and brushed epithelial cells (hereafter referred to as BRUSH). mRNA expression of IL-6, IL-1β, IL-10, TNF-α, IFN-γ and MIP-1α were detectable in lung tissue and BALF cells (Figure 2); of these only MIP-1α mRNA was detectable in BRUSH but did not alter during the course of infection (data not shown). mRNA expression of IL-6, IL-1β, IL-10, TNF-α, IFN-γ and MIP-1α increased significantly during the course of infection and, except for TNF-α expression in lung tissue, returned to basal level at day 14, i.e. when the mice had recovered from the infection. mRNA expression of cytokines and chemokines in general peaked earlier in BALF cells (2 days post infection) than in whole lung homogenates (8 days post infection), with the exception of IL-10 and IFN-γ mRNA's.

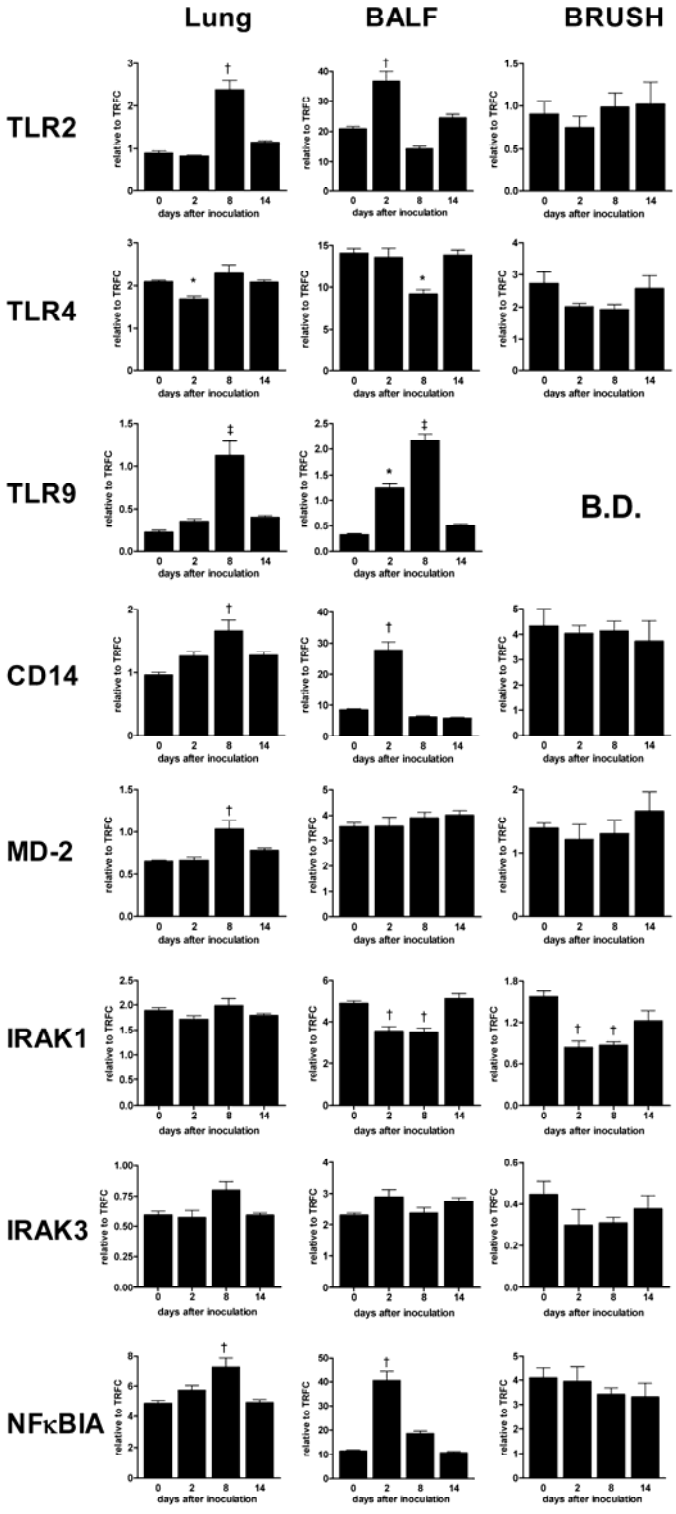

Figure 3: mRNA expression of TLRs and related molecules. Naïve or influenza infected mice were sacrificed at specific time-points and mRNA was extracted from lung tissue, BRUSH or cells from BALF. mRNA was measured by MLPA as described in the Methods section. Data are mean ± SEM (lung/BRUSH: N=8 per group, BALF: N=5 per group). * P<0.05, † P<0.01, ‡ P<0.001 versus uninfected mice. BD = below detection limit.

Toll-like receptor and associated proteins

Figure 3 shows mRNA expression of TLRs, CD14, MD-2 and proteins involved in TLR signaling in lung tissue, BALF cells and BRUSH. mRNA expression of TLR2, TLR4, CD14, MD-2, IRAK1, IRAK3 and NFκBIA were detectable in all three compartments, whereas TLR9 mRNA was only detectable in lung tissue and BALF cells. mRNA expression profiles during the course of the infection were largely similar in whole lung homogenates and BALF cells with in particular increases in mRNA levels for TLR2, TLR9, CD14, MD-2 and NF-κBIA and decreases in mRNA levels for TLR4. IRAK1 mRNA remained stable in lung homogenates but decreased in BALF cells and BRUSH. All mRNA's had returned to baseline levels 14 days after infection.

Coagulation and fibrinolysis

Figure 4 shows mRNA expression of several mediators involved in coagulation and fibrinolysis in lung tissue, BALF cells and BRUSH. mRNA levels of TF, TFPI and uPAR were detectable in all three compartments. mRNA levels of PAI-1 were detectable in lung tissue and BALF cells, mRNA levels of PAR-1 and PAR-2 were detectable in lung tissue and BRUSH and tPA mRNA was only detectable in lung tissue. Changes in mRNA levels were especially clear at 8 days after infection. At this time point TF and uPAR mRNA levels were increased in lung tissue but decreased in BALF cells and BRUSH. Similarly, mRNA was elevated in lung and decreased in BALF cells. TFPI mRNA levels declined in both lung tissue and BALF cells, whereas tPA mRNA levels increased in lung tissue. PAR mRNA's displayed few alterations during influenza with the exception of PAR-1 mRNA which was lower 8 days after infection. mRNA expression of most coagulation and fibrinolysis mediators had returned to basal levels at day 14, the expression of uPAR in BRUSH and tPA in lung tissue remained altered at this time point.

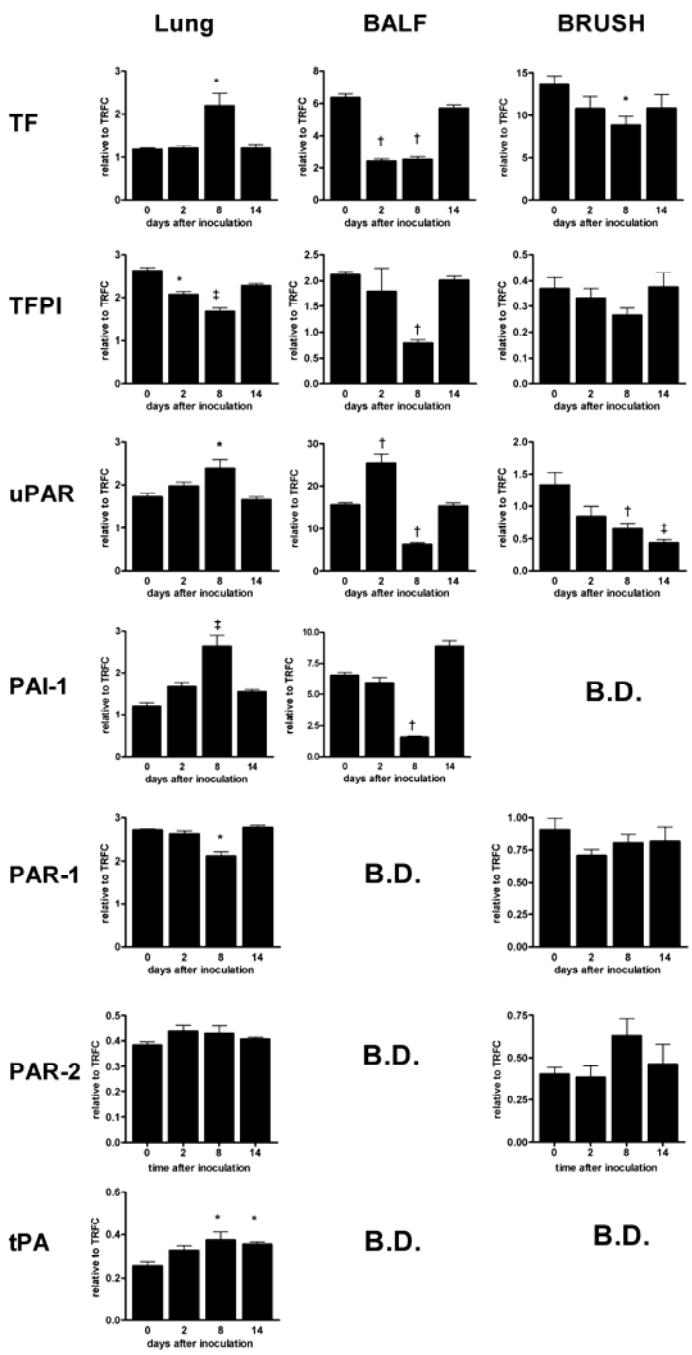

Figure 4: mRNA expression of mediators involved in coagulation and fibrinolysis. Naïve or influenza infected mice were sacrificed at specific time-points and mRNA was extracted from lung tissue, BRUSH or cells from BALF. mRNA was measured by MLPA as described in the Methods section. Data are mean ± SEM (lung/BRUSH: N=8 per group, BALF: N=5 per group). * P<0.05, † P<0.01, ‡ P<0.001 versus uninfected mice. BD = below detection limit.

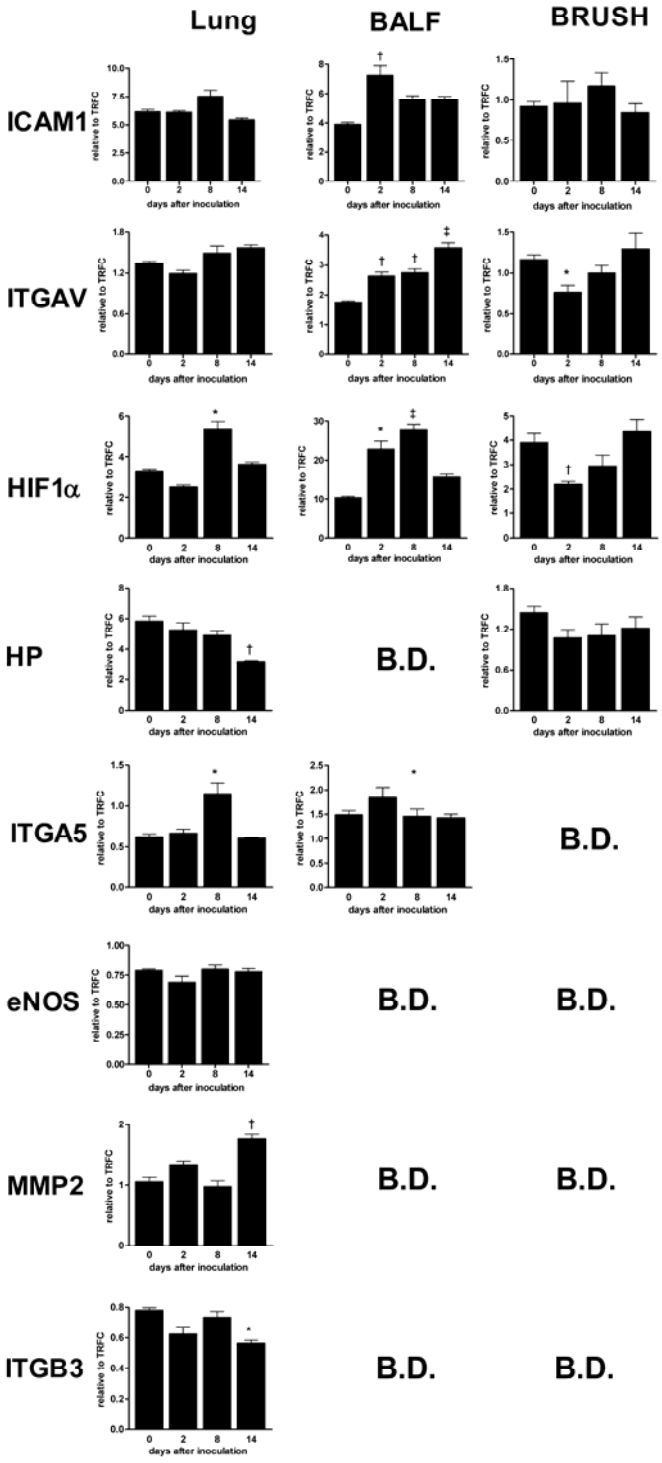

Figure 5: mRNA expression of integrins, tissue repair molecules and others. Naïve or influenza infected mice were sacrificed at specific time-points and mRNA was extracted from lung tissue, BRUSH or cells from BALF. mRNA was measured by MLPA as described in the Methods section. Data are mean ± SEM (lung/BRUSH: N=8 per group, BALF: N=5 per group). * P<0.05, † P<0.01, ‡ P<0.001 versus uninfected mice. BD = below detection limit.

Cell adhesion, tissue repair and homeostasis molecules

Figure 5 shows mRNA levels of integrins, metallopeptidases and related molecules in lung tissue, BALF cells and BRUSH. Detectable levels of ICAM-1, ITGAV and HIF-1α mRNA's were measured in all three compartments. HP mRNA expression was detectable in lung tissue and BRUSH, ITGA5 mRNA expression was detectable in lung tissue and BALF cells, whereas eNOS, MMP2 and ITGB3 mRNA's were only detectable in lung tissue. mRNA's of these mediators were differentially expressed during the course of infection in the three compartments studied. The most consistent changes were found in BALF cells: here increases in the mRNA's for ICAM-1, ITGAV and HIF-1α were detected. Fourteen days after infection, most mRNA's had returned to basal levels except for ITGAV mRNA in BALF cells and HP, MMP2 and ITGB3 mRNA's in lung tissue.

Discussion

Influenza is a common cause of upper respiratory tract infection and pneumonia. Although several studies have examined the host inflammatory response to influenza infection, our investigation represents the first attempt to evaluate the time-dependent expression of a set of inflammatory genes in three compartments within the lung (whole lung homogenates, BALF cells and tracheal epithelial cells) after inoculation of influenza A virus via the airways. For this we developed a mouse specific MLPA-kit that enables the simultaneous quantitative measurement of a range of genes involved in inflammation, TLR signaling and the coagulation- and fibrinolysis-pathways. Many of these genes were differentially expressed during the course of infection, returning to basal expression levels upon recovery 14 days after viral inoculation.

MLPA is a recently described technique that can detect one-copy number changes of chromosomal DNA sequences (24). Studies in humans have indicated the usefulness of this technique for relative quantification of up to 40 different mRNAs in a single reaction using very small amounts of sample RNA (equivalent to 10µl whole blood) (25). As such, MLPA represents a valuable tool to study gene expression profiles in

small animals. To our knowledge our article is the first to describe the use of MLPA in mice.

Many in vitro studies of influenza-induced cytokine production have been carried out in murine, rat or human monocytes/macrophages. The production of IFN-α, TNF-α, IL-6, and the chemokines MIP-1α, MIP-1β, MCP-1, MCP-3, IP-10 and RANTES in cell culture has been documented (34-37). Importantly, epithelial cells are the primary target cells of influenza and expression and production of IL-6, IL-8 and RANTES by influenza-infected epithelial cells have been reported (6, 38-41). In mice studies, infection with influenza enhanced mRNA expression in lung tissue of MIP-1α, MIP-1β, MIP-2, IP-10, RANTES and MCP-1 (42, 43). In addition, production of IL-1β, IL-6, TNF-α and IFN-γ have been shown to increase both in BALF and whole lung homogenates during infection with influenza (21, 33, 44, 45). In our study, mRNA expression of several cytokines and the chemokine MIP-1α were upregulated in lung tissue and BALF cells during the course of the infection, returning to baseline levels at day 14, i.e. when mice had recovered from the infection. mRNA expression of IL-6, IL-1β, TNF-α and MIP-1α in BALF cells were significantly up-regulated, 2 days after infection and was most likely to be expressed in resident alveolar macrophages, the main leukocyte-type present at that time and known to be capable of producing these mediators. IL-10 and IFN-γ mRNA expression was merely detectable 8 days after infection. IL-10 and especially IFN-γ are mainly produced by T lymphocytes. The expression profiles of these mediators corresponded with the cell-influx seen in BALF after influenza infection. Of note, we chose not to perform MLPA on purified cell populations from BALF or lung tissue in order to avoid artificial alterations in gene expression profiles due to the purification procedures. Together, these data demonstrate the immunokinetics of several cytokines and chemokines in mice during infection with influenza.

TLRs are pattern recognition receptors which recognize specific molecules expressed by bacteria and viruses (review (46)). TLRs are expressed by a wide range of leukocytes including cells from the innate immune system like macrophages/monocytes, neutrophils, dendritic cells, and also epithelial cells. In general, binding of a pathogen-related molecule to a TLR induces the recruitment of intracellular adaptor proteins to the TLR and the activation of several kinases, which

ultimately leads to the translocation of nuclear transcription factors and the transcription of several genes encoding pro- and anti-inflammatory cytokines, chemokines and interferons (review (46-48)). Several studies have shown that infection of human epithelial cells, macrophages and neutrophils altered TLR expression (4, 9, 10). A study performed by Seki et al. showed that mice infected with influenza had increased mRNA expression of TLR2, TLR4, TLR5 and TLR9 in lung tissue compared to uninfected mice (49). In this study, we showed that during infection with influenza, mRNA expression of TLR2 and TLR9 but also CD14, MD-2 and NFκBIA was upregulated in lung tissue which, except for MD-2, was also observed in leukocytes from BALF. Of note, 2 days after infection, macrophages are the foremost present leukocytes in BALF and alteration in mRNA expression of TLR2, TLR9, CD14, NFκBIA and IRAK1 most likely originated from these resident alveolar macrophages. Reduced mRNA expression of TLR4 in leukocytes in BALF 8 days after infection, could be explained by a relatively reduced expression of TLR4 of all leukocytes or influx of leukocytes with a lower TLR4 expression. We would have expected an increase in TLR4 mRNA expression during infection with influenza (49). However, altered TLR4 mRNA expression was not observed in lung tissue in our model. Little difference in TLR mRNA expression was observed in brush-obtained tracheal epithelial cells except for down-regulation of IRAK-1 mRNA during the course of infection, which returned to basal level 14 days after infection. Infection or stimulation of epithelial cells with influenza, IFN or poly(I:C), a synthetic compound known to mimic double-stranded RNA of viral origin, is known to alter mRNA expression of TLRs and related molecules (4, 6, 9, 50). Notably, cells obtained by the brush-procedure of the trachea do not include pulmonary bronchial or alveolar epithelial cells which are frequently used in vitro experiments. Therefore we do not exclude the possibility of altered TLR mRNA expression in mouse bronchial or alveolar epithelial cells during infection with influenza. These data show that during infection with influenza, mRNA expression of numerous TLRs and related molecules are differentially affected. The reduced expression of IRAK1 and the enhanced expression of the NFκB inhibitor NFκBIA at 2 days post infection suggest an oppression of the TLR pathway in BALF cells early after the entrance of influenza A virus in the respiratory tract.

The coagulation system is a narrowly controlled system with several proteins and receptors that control fibrin formation and degradation. Proteins involved in the coagulation system are TF, TFPI, PAR-1 and PAR-2, whereas uPAR, tPA and PAI-1 are members of the fibrinolysis pathway (review (51, 52)). Influenza has been shown to be capable of modulating inflammation and activating coagulation both *in vitro* and *in vivo*. Infection of human endothelial cells or monocytes with influenza induced procoagulant activity which was associated with an increase in TF expression (11, 12). In line, mice infected with influenza had elevated plasma levels of thrombin-antithrombin complexes, PAI-1 and D-dimer indicative of a prothrombotic state (13). Our present findings suggest that influenza associated coagulation activation at least in part is caused by a misbalance between TF and TFPI expression: whereas TF mRNA expression was upregulated in lung tissue 8 days after infection, TFPI was downregulated. Remarkably, TF mRNA expression was downregulated in BALF cells and BRUSH during the course of infection, suggesting that interstitial macrophages and/or endothelial cells were the source for TF. In addition, key players in the fibrinolytic system, uPAR, PAI-1 and tPA mRNA were also upregulated in lung tissue. The data on PAI-1 mRNA expression in lungs extend previous reports from our laboratory that documented enhanced pulmonary PAI-1 mRNA during bacterial pneumonia (53, 54).

In the last group of genes, several components involved in cell adhesion, tissue repair and cell homeostasis are joint together. Of these factors, ICAM-1, HIF-1α and integrin α5 were transiently increased in BALF cells. Integrin αv (ITGAV) expression was increased on day 2 and day 8 and even further increased on day 14. Integrin αv and subunit β3 (ITGB3) form a dimer which is a receptor for vitronectin (55). Integrin αvβ3 is expressed on endothelial cells, epithelial cells and leukocytes and plays a role in cell signaling and migration (55). However, expression of the β3 subunit could not be detected in BALF cells. In lung tissue, HIF-1α and integrin α5 were transiently upregulated during influenza virus infection, while MMP2 appeared to be upregulated on day 14 after infection. Pulmonary epithelial cells, macrophages and neutrophils are considered to be sources of MMPs (50). Infection of epithelial cells with influenza induced MMP-2 mRNA expression (56), while Poly(I:C)-stimulated epithelial cells increased secretion of MMP-1, MMP8-10 and MMP-13 (50). MMP2 and MMP-9 are proteinases which are capable of degrading type IV

collagen; thus they are considered to play an important role in the degeneration of epithelial cell layer (57). MMP2 expression may impair host defense against secondary bacterial infections by disruption of the airway epithelial barrier, thereby enhancing the colonization of invading pathogens. Alternatively, MMP2 expression may also impair host defense as a consequence of ongoing resolution of inflammatory cells. Both MMP2 and MMP9 have recently been implicated in the resolution of inflammatory cells *in vivo* (58). Finally, we observed decreased mRNA expression of HP in lung tissue, 14 days after infection. HP is considered to be an acute phase protein and plays numerous roles during immunological stress including antioxidant, antiinflammatory, antibacterial activities, and modulation of immune response (review (59)) Downregulation of HP in the lungs may therefore be considered as a potential mechanism by which influenza virus infection alters host defense against secondary infections.

We developed a mouse specific MLPA-kit to determine a high-throughput mRNA gene expression to profile a broad range of inflammation-related genes. We determined expression of several genes involved in inflammation, TLR signaling and coagulation/fibrinolyis pathway in several pulmonary compartments during infection with influenza in mice. Our data show that throughout the course of infection a broad range of genes were differentially expressed. Although expression of most of the genes returned to basal level, gene expression of uPAR, ITGAV, ITGB3, HP and MMP-2 remained altered when the virus was cleared from the lungs and mice had recovered from the infection.

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Table E1: Gene bank entry, symbol, name and function

Gene symbol	Gene name	Gene Ontology function
Cytokines and chemokines		
IL-6	Interleukin 6	Immune response / Apoptosis / Chemotaxis/ Acute phase response
IL-4	Interleukin 4	Immune response / Apoptosis
IL-1b	Interleukin 1beta	Immune response / Inflammatory response / Apoptosis
IL-10	Interleukin 10	Immune response / Inflammatory response / Apoptosis
Cxcl1 / KC	Chemokine (C-X-C motif) ligand 1	Immune response / Inflammatory response / Chemotaxis
TNF-α	Tumor necrosis factor	Immune response / Apoptosis / Defense response to bacterium
IFN-γ	Interferon gamma	Immune response / Apoptosis / Defense response to bacterium
Ccl3 / Mip1a	Chemokine (C-C motif) ligand 3	Immune response / Inflammatory response / Chemotaxis
Toll-like receptor		
TLR2	Toll-like receptor 2	Innate immunity / Inflammatory response / Defense response to bacterium
TLR4	Toll-like receptor 4	Innate immunity / Inflammatory response / Defense response to bacterium
TLR9	Toll-like receptor 9	Immune response / Inflammatory response / Defense response to bacterium
CD14	CD14 antigen	Immune response / Inflammatory response / Apoptosis
Ly96 / MD2	Lymphocyte antigen 96	Immune response / Inflammatory response / Defense response to bacterium
IRAK1	Interleukin-1 receptor-associated kinase 1	Cytokine and chemokine mediated signaling pathway / Activation of NF-kappaB-inducing kinase / Positive regulation of transcription
IRAK3	Interleukin-1 receptor-associated kinase 3	Cytokine and chemokine mediated signaling pathway
Nfkbia / IkBa	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	Regulation of NF-kappaB import into nucleus / Apoptosis
Coagulation and fibrinolysis		
TFPI	Tissue factor pathway inhibitor	Blood coagulation
F3 / TF	Coagulation factor III	Blood coagulation
Procr	Protein C receptor, endothelial / EPCR	Blood coagulation / Inflammatory response
Serpine1 / PAI-1	Serine (or cysteine) peptidase inhibitor, clade E, member 1	Blood coagulation / Fibrinolysis / Regulation of angiogenesis
Plat / tPA	Plasminogen activator, tissue	Blood coagulation / platelet-derived growth factor receptor signaling pathway
Plaur / uPAR	Plasminogen activator, urokinase receptor	Blood coagulation / cell surface receptor linked signal transduction / chemotaxis
F2r / PAR1	Coagulation factor II (thrombin) receptor	Blood coagulation / G-protein coupled receptor protein signaling pathway
F2rl1 / PAR2	Coagulation factor II (thrombin) receptor-like 1	Blood coagulation / G-protein coupled receptor protein signaling pathway / positive regulation of I-kappaB kinase/NF-kappaB cascade
other		
Nos3 / eNOS	Nitric oxide synthase 3, endothelial cell	Lipopolysaccharide-mediated signaling pathway / Cell motility
Icam1	Intercellular adhesion molecule	Regulation of cell adhesion
Sele/E-selectin	Selectin, endothelial cell	Regulation of cell adhesion / Inflammatory response
Itga5	Integrin alpha 5 (fibronectin receptor alpha)	Regulation of cell adhesion / Cell-substrate junction assembly
Itgav	Integrin alpha V	Regulation of cell adhesion / Cell-matrix adhesion
Itgb3	Integrin beta 3	Regulation of cell adhesion / Cell-matrix adhesion
Vcam1	Vascular cell adhesion molecule 1	Regulation of cell adhesion
Hif1a	Hypoxia inducible factor 1, alpha subunit	Response to hypoxia / Angiogenesis / Apoptosis
Mmp2	Matrix metallopeptidase 2	Proteolysis / Blood vessel maturation
Mmp9	Matrix metallopeptidase 9	Apoptosis / Macrophage differentiation / Proteolysis
Ela2	Elastase 2, neutrophil	Inflammatory response / Leukocyte migration / Phagocytosis
Hp	Haptoglobin	Defense response / Proteolysis
Housekeeping gene		
B2M	Beta-2 microglobulin	Immune response / Antigen processing and presentation of peptide, antigen via MHC class I
TBP	TATA box binding protein	Regulation of transcription
Tfrc	Transferrin receptor	Endocytosis / Proteolysis

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Part III

Postinfluenza pneumococcal pneumonia

Chapter 10

Toll-like receptor 2 does not contribute to host response during postinfluenza pneumococcal pneumonia

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Abstract

Influenza A can be complicated by secondary bacterial pneumonia, which is most frequently caused by *Streptococcus (S.) pneumoniae* and associated with uncontrolled pulmonary inflammation. Evidence points to Toll-like receptor (TLR) 2 as a possible mediator of this exaggerated lung inflammation: (1) TLR2 is the most important "sensor" for gram-positive stimuli, (2) TLR2 contributes to *S. pneumoniae* – induced inflammation, and (3) influenza A enhances TLR2 expression in various cell types. Therefore, the objective of this study was to determine the role of TLR2 in the host response to postinfluenza pneumococcal pneumonia. TLR2 knockout (KO) and wild-type (WT) mice were infected intranasally with influenza A virus. Fourteen days later they were administered with *S. pneumoniae* intranasally. Influenza was associated with a similar transient weight loss in TLR2 KO and WT mice. Both mouse strains were fully recovered and had completely cleared the virus at day 14. Importantly, no differences between TLR2 KO and WT mice were detected during postinfluenza pneumococcal pneumonia with respect to bacterial growth, lung inflammation and cytokine/chemokine concentrations, with the exception of lower pulmonary levels of cytokine-induced neutrophil chemoattractant in TLR2 KO mice. Toll-like receptor 2 does not contribute to host defense during murine postinfluenza pneumococcal pneumonia.

Introduction

Secondary bacterial pneumonia is a feared complication of respiratory tract infection by influenza A, responsible for at least 20,000 deaths annually in the United States alone (1). The most important pathogens causing postinfluenza pneumonia are *Staphylococcus aureus, Haemophilus influenzae* and in particular *Streptococcus (S.) pneumoniae* (2). Although *S. pneumoniae* is the most common pathogen isolated from previously healthy patients with community-acquired pneumonia (3), such primary pulmonary infections with the pneumococcus are usually less severe than secondary infections following influenza A (4). Thus far, knowledge about the precise mechanism by which influenza modulates the innate immune response to facilitate secondary bacterial infection in the lung is limited.

Our laboratory recently developed a model of postinfluenza pneumococcal pneumonia to obtain more insight into the pathogenetic mechanisms contributing the adverse outcome of secondary bacterial pneumonia (5-7). In this model mice are intranasally infected with a mouse adapted strain of influenza A, causing a mild illness characterized by transient weight loss and a complete recovery together with viral clearance by day 14. At this time point mice are infected with *S. pneumoniae*, which, in comparison with mice with primary pneumococcal pneumonia, results in an exaggerated pulmonary inflammatory response, a strongly enhanced bacterial outgrowth and a reduced survival (5-7).

S. pneumoniae can activate the innate immune system by an interaction with so-called pattern recognition receptors, among which Toll-like receptors (TLRs) prominently feature. Previous investigations have pointed to TLR2 as the key pattern recognition receptor in the immune response against gram-positive bacteria (8-10). In line, both *in vitro* and *in vivo* studies have indicated that *S. pneumoniae* activates the immune system at least in part via TLR2, although other TLRs, in particular TLR4, may also be involved (10-14). Moreover, our laboratory recently demonstrated that TLR2 contributes to the inflammatory response after primary pneumococcal pneumonia (15). We hypothesized that signaling of *S. pneumoniae* via TLR2 is an important mechanism by which this pathogen causes exaggerated lung inflammation during

infection following influenza A. This hypothesis, which was also put forward in a recent review on the induction of immune responses by *S. pneumoniae* (16), was supported by the fact that the expression of TLR2 has been found enhanced in mouse macrophages, human neutrophils and in human epithelial cells infected with influenza A (17) (18, 19). Thus, in the present study we sought to determine the role of TLR2 during postinfluenza pneumococcal pneumonia.

Material and methods

Animals: Specific pathogen free 8-10 weeks old female C57BL/6 mice (WT) were purchased from Charles River (Maastricht, The Netherlands). TLR2 knockout (KO) mice were generated as described previously (8) and backcrossed to C57BL/6 background 6 times; these mice were bred in the animal facility of the Academic Medical Center in Amsterdam. Age and sex matched mice were used in all experiments. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, the Netherlands).

Postinfluenza pneumonia: The model of postinfluenza pneumococcal pneumonia has been described in detail (5-7). In brief, influenza A/PR/8/34 (ATCC VR-95, Rockville, MD) was grown in LLC-MK2 cells. Mice were anesthesized by inhalation of isoflurane (Abbott Laboratories, Kent, UK) and inoculated intranasally with 50 µl phosphate buffered saline containing 1400 viral copies of influenza. Two, 8 and 14 days later the viral load was determined in lung homogenates using real-time quantitative polymerase chain reaction (PCR) (20). Pneumococcal pneumonia was induced 14 days after inoculation of influenza A by intranasal inoculation of 50 µl normal saline containing approximately 2 x 10^4 colony forming units (CFUs) of *S. pneumoniae* serotype 3 (ATCC 6303, Rockville, MD). In one experiment *S. pneumoniae* was administered 8 days after inoculation with influenza. For this *S. pneumoniae* was grown for 16 hours at 37^{o}C in 5% CO_2 in Todd Hewith broth; this suspension was diluted 100 times in fresh medium, grown for approximately 5 hours to logarithmic phase, washed twice in sterile normal saline and subsequently diluted to a final concentration of 2 x 10^4 CFUs/50 µl. Mice were killed 6 or 48 hours after inoculation of *S. pneumoniae*, whole lungs were harvested and homogenized at 4^0C in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, OK). Serial 10-fold dilutions in sterile isotonic saline were made from

whole lung homogenate and 50 µl volumes were plated onto sheep-blood agar plates. Blood was plated undiluted to check for bacteremia. Blood agar plates were incubated at 37^0C and 5% CO_2 and CFUs were counted after 16 hours.

Histopathological analysis*:* Lungs were fixed in 10% formalin and embedded in paraffin. Four µm lung sections were stained with hemotoxylin and eosin (HE) and analyzed by a pathologist who was blinded for the groups. To score lung inflammation and damage, a semi-quantitative scoring system was used (15, 21). For this the entire lung surface was analyzed with respect to the following parameters: pleuritis, bronchitis, edema, interstitial inflammation, percentage of pneumonia, and endothelialitis. Each parameter was graded on a scale of 0 to 4 with 0 as 'absent' and 4 as 'severe'. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 24.

Cytokine and chemokine measurement: For cytokine measurements, lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, and Pepstatin A, Leupeptin and Aprotinin (all 20 ng/ml; pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g at 4°C for 15 minutes, and supernatants were stored at -20°C until assays were performed. Tumor necrosis factor (TNF)-α, Interleukin (IL) -1β, IL-10, macrophage inflammatory protein (MIP)-2, cytokine-induced neutrophil chemoattractant (KC) and interferon (IFN)-γ were measured using specific ELISA's (R & D systems, Abingdon, UK) in accordance with the manufacturer's recommendations.

Statistical analysis: Data are expressed as means ± SEM. Differences were analyzed by Mann Whitney U test. A value of $P < 0.05$ was considered statistically significant.

Results:

Body weight and viral clearance during primary influenza infection

The primary goal of our study was to determine the possible contribution of TLR2 signaling in the exaggerated inflammatory response during *S. pneumoniae* pneumonia following influenza A infection. In order to adequately address this issue, we first established whether influenza has a different course in TLR2 KO mice than in WT mice, *i.e.* in case TLR2 KO mice would handle influenza infection in a different way, the "base-line condition" upon which pneumococcal pneumonia is superimposed would differ between the two mouse strains, hampering an adequate comparison between TLR2 KO and WT mice during postinfluenza pneumonia. Thus, TLR2 KO and WT mice were intranasally infected with influenza virus and followed for 14 days. As reported earlier by our and other laboratories (5-7, 22), influenza virus infection resulted in a transient loss of bodyweight in WT mice. This decrease in body weight, which reached a nadir at 8 days after infection and had completely recovered at 14 days, was similar in TLR2 KO mice (Fig. 1A). Next, we determined viral loads in whole lung homogenates prepared on day 2, 8 and 14 after influenza infection using real-time quantitative PCR. No differences in viral load were found in the lungs of WT and TLR2 KO mice at any time point. At 14 days after inoculation of the virus, influenza could not be detected anymore in lungs of either group, indicating that the virus had been cleared from the lungs of both TLR2 KO and WT mice (Fig. 1B).

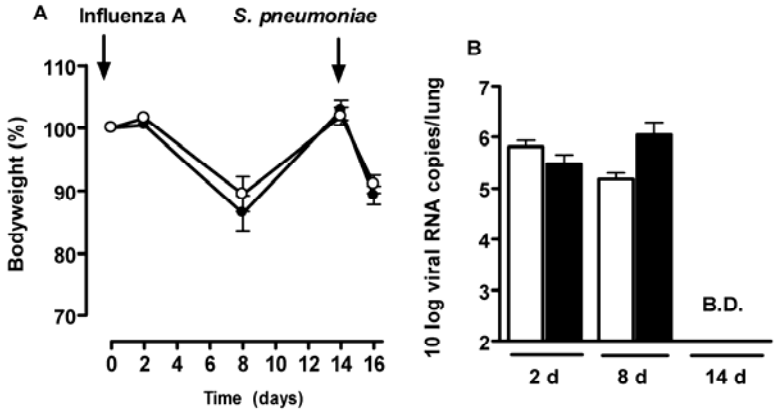

Fig. 1: Bodyweight and viral load of WT and TLR2 KO mice during (post)influenza pneumonia. WT (closed circles/bars) and TLR2 KO mice (open circles/bars) were given influenza A intranasally followed by *S. pneumoniae* 14 days later. **A**: Bodyweight relative to day 0. Data are mean ± SEM of 7-8 mice per group. **B**: Viral RNA copies per lung. Data are mean ± SEM of 4 mice per group. B.D.= below detection level.

Lung inflammation during primary influenza infection

To determine whether TLR2 deficiency influences the pulmonary cytokine and chemokine response during influenza, we measured the concentrations of TNF-α, IL-1β, IL-10, KC, MIP-2 and IFN-γ in lung homogenates obtained from TLR2 KO and WT mice at day 2, 8, 14 days after infection with influenza (Fig. 2). Although overall the levels of these mediators were relatively low, especially when compared to the levels measured after bacterial infection (see further; for reasons of clarity these latter data are also presented in Fig. 2), some differences were found between TLR2 KO and WT mice. In particular, the pulmonary levels of the anti-inflammatory cytokine IL-10 were higher in TLR2 KO mice at 8 and 14 days after infection (both $P < 0.05$ versus WT mice), whereas lung KC concentrations were lower in TLR2 KO mice 2 and 8 days after infection (both $P < 0.05$ versus WT mice). IFN-γ production tended to be higher in TLR2 KO mice 8 and 14 days after influenza inoculation although this was not significant ($P=0.05$ resp. $P=0.12$). Lung TNF-α, IL-1β, or MIP-2 levels did not differ between TLR2 KO and WT mice.

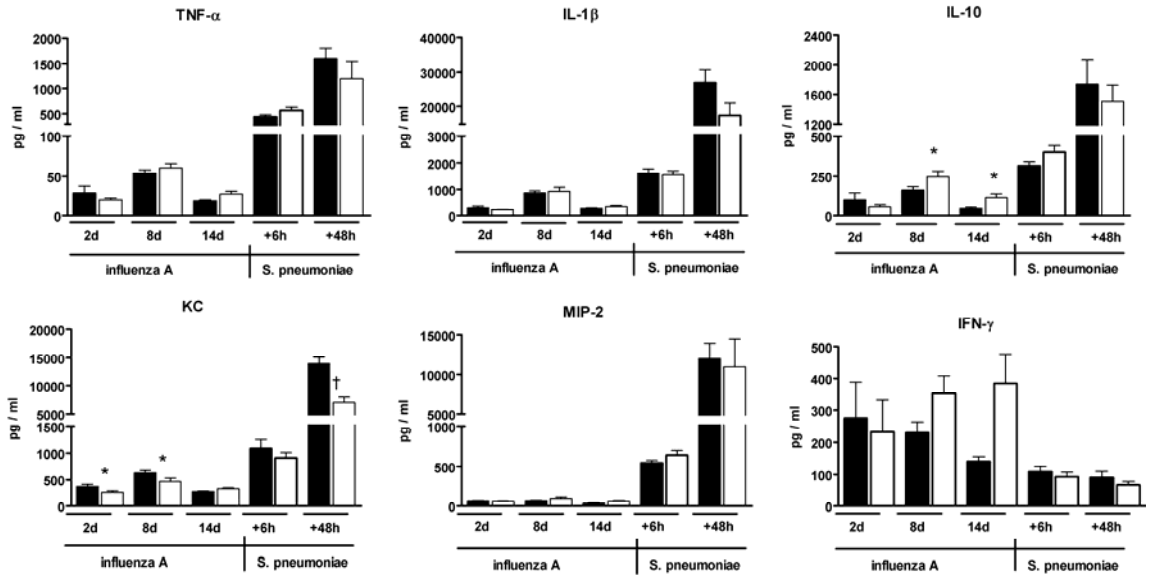

Fig. 2: Cytokine and chemokine concentrations in lungs of WT and TLR2 KO mice during (post)influenza pneumonia. Pulmonary levels of TNF-α, IL-1β, IL-10, KC, MIP-2 and IFN-γ from WT (closed bars) and TLR2 KO mice (open bars) during (post)influenza pneumonia. Data are mean ± SEM of 7-8 per group at each time point. * $P<0.05$ versus WT. † $P<0.001$ versus WT.

Body weight and bacterial outgrowth during postinfluenza pneumonia

At 14 days after infection with influenza, when all mice had completely recovered and the virus was no longer detectable in lungs, TLR2 KO and WT mice were intranasally infected with *S. pneumoniae*. Bacterial pneumonia resulted in a marked body weight loss 48 hours after infection; however, no differences were observed between TLR2 KO and WT mice (Fig. 1A). To determine whether TLR2 deficiency influences bacterial outgrowth during postinfluenza pneumonia we measured the number of *S. pneumoniae* CFU in the lungs of TLR2 KO and WT mice 6 and 48 hours after the bacterial inoculation. The 6 hour time point was chosen since TLR2 plays a role in early inflammatory response in murine pneumococcal pneumonia (15). The 48 hour time point was chosen because it is suitable to compare bacterial growth in this pneumonia model (23-25). At neither time point the pulmonary bacterial loads differed between the two mouse strains (Fig. 3). In addition, bacteremia occurred similarly in WT and TLR2 KO mice: whereas 6 hours after inoculation of *S. pneumoniae* neither WT nor TLR2 KO mice had positive blood cultures, 48 hours after bacterial infection all mice were bacteremic.

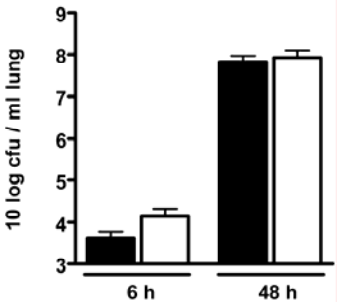

Fig. 3: Bacterial loads in lungs of WT and TLR2 KO mice during postinfluenza pneumonia. WT (closed bars) and TLR2 KO mice (open bars) were infected with $2x10^4$ CFU's of *S. pneumoniae* on day 14, *i.e.* after recovery of influenza infection and sacrificed 6 and 48 hours after secondary infection. Data are mean ± SEM of 7-8 per group at each time point.

Lung inflammation during postinfluenza pneumonia

Our laboratory previously showed that the lung inflammatory response to secondary *S. pneumoniae* infection of mice that have just recovered from influenza infection is strongly enhanced when compared to the inflammatory reaction in lungs of mice with primary *S. pneumoniae* pneumonia (5-7). Having established that TLR2 does not contribute to an effective antibacterial defense during postinfluenza pneumococcal pneumonia, we next wished to determine the possible role of TLR2 in the induction of lung inflammation after secondary bacterial respiratory tract infection. For this we semi-quantitatively scored lung tissue slides obtained from TLR2 KO and WT mice 6 and 48 hours after infection. No difference in pulmonary inflammation between TLR2

KO and WT mice were observed at both 6 and 48 hours after inoculation of *S. pneumoniae* (Fig. 4). To determine whether TLR2 KO mice had an altered cytokine/chemokine response to postinfluenza pneumonia, we measured several cytokines and chemokines in lung homogenates 6 and 48 hours after inoculation with *S. pneumoniae* (Fig. 2). Lung concentrations of TNF-α, IL-1β, IL-10, KC, MIP-2 and IFN-γ did not differ between TLR2 KO and WT mice at either time point with the exception of KC levels 48 hours after bacterial infection, which were lower in the former mouse strain.

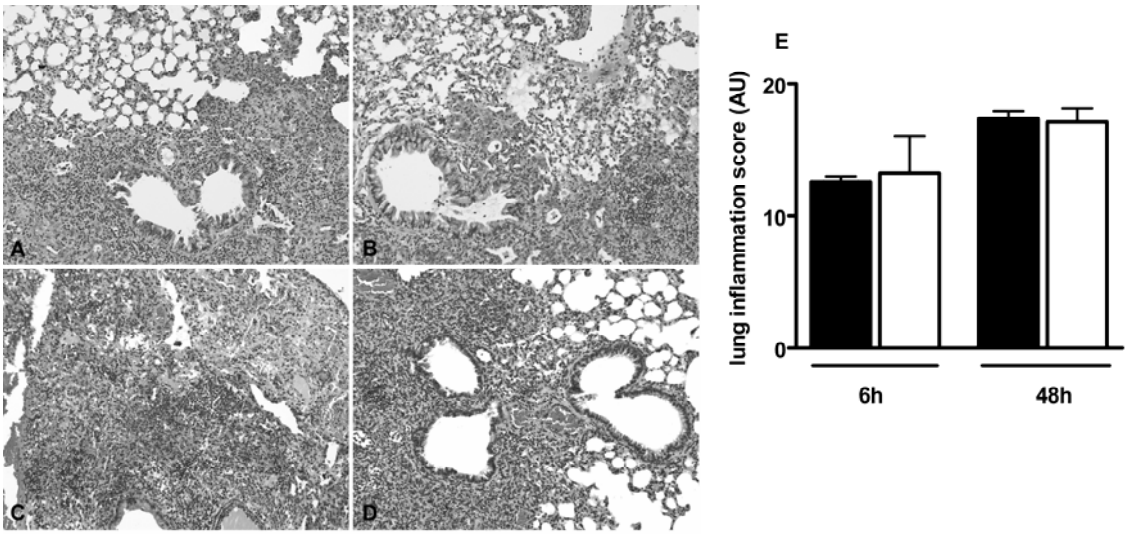

Fig. 4: Histopathology of lungs from WT and TLR2 KO mice during postinfluenza pneumonia. Representative lung slides of WT (A,C) and TLR2 KO mice (B,D) 6 hours (A,B) and 48 hours (C,D) after secondary infection with *S. pneumoniae*. H&E staining: magnification x 10 (Fig. 4A-D). Semi-quantitative histology scores, as determined by the scoring system described in the Methods section, from WT (open bars) and TLR2 KO mice (closed bars) 6 hours and 48 hours after secondary infection with *S. pneumoniae*. Data are mean ± SEM of 6-8 mice per group at each time point (Fig. 4E).

Induction of *S. pneumoniae* pneumonia 8 days after inoculation with influenza

To determine whether TLR2 plays a role in the inflammatory response to pneumococcal pneumonia superimposed on influenza induced 8 days earlier, we compared bacterial loads and cytokine/chemokine levels in lung homogenates prepared 6 hours after intranasal inoculation with *S. pneumoniae* in TLR2 KO and WT mice infected with influenza 8 days earlier. Of note, at 8 days after inoculation with influenza pulmonary viral loads were high and infected mice were severely ill as illustrated by their loss of weight (see figure 1). During the first 6 hours after

superinfection with *S. pneumoniae*, 3 of 7 WT mice and 3 of 7 TLR2 KO mice died. In the remaining mice, no differences were detected between TLR2 KO and WT mice with respect to bacterial loads or cytokine/chemokine levels in lungs (Table I).

Table I: Pulmonary bacterial load, cytokine and chemokine production in WT and TLR2 KO mice superinfected with *S. pneumoniae* during influenza infection.

	WT	TLR2 KO
Bacterial load	$3.1 \pm 1.1 \times 10^4$	$5.1 \pm 1.4 \times 10^4$
TNF-α	597 ± 52	413 ± 95
IL-1β	92 ± 16	51 ± 5
IL-10	402 ± 65	317 ± 22
KC	711 ± 421	412 ± 226
MIP-2	374 ± 129	273 ± 30
IFN-γ	332 ± 19	422 ± 102

Eight days after infection with influenza A, mice were superinfected with *S. pneumoniae* and lung homogenates were prepared 6 hours later. Bacterial load in CFU/ml, cytokine and chemokine production in pg/ml. Data are mean ± SEM (N=4 per group).

Discussion:

Postinfluenza pneumococcal pneumonia is associated with a much stronger inflammatory response in the lungs than primary pneumonia caused by *S. pneumoniae*. We here tested the hypothesis that TLR2 signaling contributes to this exaggerated pulmonary inflammation during *S. pneumoniae* pneumonia following influenza A infection. This hypothesis was based on the following lines of evidence: (1) TLR2 has been implicated as the most important TLR for sensing gram-positive bacteria (26), (2) TLR2 has been found important for the induction of inflammation upon infection with *S. pneumoniae in vivo* (11, 12, 15), and (3) TLR2 expression increased in macrophages, neutrophils and epithelial cells upon infection with influenza A (17-19). However, the main finding of this study is that, in contrast to our expectation, TLR2 does not play a role of importance in postinfluenza pneumococcal pneumonia.

In a first series of experiments we established that TLR2 is not involved in the host response to influenza A infection to a significant extent. Indeed, the transient body weight loss and viral clearance were unaltered in TLR2 KO mice when compared

with normal WT mice. Importantly, both mouse strains had completely cleared influenza virus at the time infection with *S. pneumoniae* was accomplished that is two weeks after intranasal inoculation of the virus. We used this time interval in this and our previous studies on postinfluenza pneumococcal pneumonia (5-7) since we wished to exclude a direct interaction between influenza virus and *S. pneumoniae* in the lungs and since clinical data indicate that two weeks is a common interval between influenza infection and the occurrence of secondary bacterial complications (2, 27). Notably, modest differences in pulmonary cytokine and chemokine levels were detected in TLR2 KO and WT mice infected with influenza A. In particular, TLR2 KO displayed higher pulmonary IL-10 concentrations during influenza, contrasting with findings in infections caused by other pathogens (*Yersinia enterocolitica* and *Candida albicans*) which have suggested that TLR2 stimulation results in a type 2 biased immune response characterized by increased IL-10 release (28). It is unlikely that the modestly elevated IL-10 levels in TLR2 KO mice at the time *S. pneumoniae* was administered biased our results: higher IL-10 concentrations in theory would have reduced lung inflammation during postinfluenza pneumonia (29) and thus would have made the expected diminished lung inflammation in TLR2 KO mice more profound; clearly this was not what we found in the current investigation. The same holds true for the slightly lower KC levels in TLR2 KO mice during the initial phase of influenza. We do not have a clear explanation for these small differences between the two mouse strains, especially since there is no evidence that TLR2 contributes to cellular responsiveness to influenza virus (30, 31). TLR2 does contribute to immune responses triggered by cytomegalovirus, varicella-zoster virus and herpes simplex (32-35). Within the TLR family in particular TLR3 is important for the innate recognition of double stranded viral RNA (31). Influenza A virus is a negative sense single stranded RNA virus with double stranded replication intermediates which are likely to be TLR3 ligands. Recently Le Goffic *et al*. showed a significant contribution of TLR3 during pulmonary infection with influenza (36). They reported that TLR3 is upregulated during viral infection and mice deficient of this receptor displayed significantly reduced inflammatory mediators and a lower number of CD8+ T lymphocytes in the bronchoalveolar airspace. Surprisingly, TLR3 KO mice had a survival advantage, despite a higher viral load in the lungs (36).

In light of the minor differences between TLR2 KO and WT mice during influenza, we considered it feasible to use TLR2 KO mice to establish the role of this receptor in the host response to postinfluenza pneumonia. The impact of TLR2 deficiency on lung inflammation during postinfluenza pneumococcal pneumonia was evaluated at 6 and 48 hours after bacterial infection. These time points were chosen in light of our previous investigation on the role of TLR2 in primary *S. pneumoniae* pneumonia (15). In that study, TLR2 KO mice were found to have lower pulmonary cytokine concentrations early after infection (6 hours), whereas at 48 hours post infection TLR2 KO mice displayed reduced lung inflammation upon semi-quantitative histological analysis (15). Such differences were not observed in the current study, although TLR2 KO mice did show reduced lung KC concentrations 48 hours after inoculation with *S. pneumoniae*. This latter finding presumably reflects the relatively strong TLR2 dependence of KC release induced by gram-positive stimuli, including *S. pneumoniae*, as indicated by profoundly diminished KC production by TLR2 KO alveolar macrophages *in vitro* and whole lungs from TLR2 KO mice *in vivo* upon exposure to *S. pneumoniae* (15). In line with our earlier study (15), TLR2 KO mice displayed similar bacterial loads in their lungs as WT mice and the occurrence of bacteremia was identical in both mouse strains. Together, these data indicate that the role of TLR2 in the host response to respiratory tract infection caused by *S. pneumoniae* is modest during primary infection and insignificant during postinfluenza pneumonia. Moreover, in additional experiments no difference in bacterial outgrowth and immune response were observed when WT and TLR2 KO mice were infected for 6 hours with *S. pneumoniae*, 8 days after infection with influenza. Apparently, other TLRs are capable to compensate for the absence of the "gram-positive sensor" TLR2 during pneumococcal infection. Indeed, mice with a functional loss of TLR4 and in particular mice with a targeted deletion of the gene encoding the TLR9 or common TLR adaptor MyD88 demonstrated an increased susceptibility to primary pneumococcal pneumonia (13, 14, 37, 38). Further studies are warranted to establish the role of these molecules in postinfluenza pneumonia.

It has been well established that influenza renders the host more susceptible to secondary infection with *S. pneumoniae*, which is associated with an uncontrolled inflammatory reaction in the lungs. We here investigated the potential role of TLR2 in the deregulated host response to pneumococcal pneumonia following influenza. In

contrast to our expectation, TLR2 deficiency had no impact on lung inflammation or bacterial growth, suggesting that other pattern recognition receptors can compensate for the loss of TLR2 in the innate recognition of *S. pneumoniae* during respiratory tract infection superimposed on influenza.

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Chapter 11

Summary and general discussion

Samenvatting en algemene discussie

Dankwoord

List of Publications

Curriculum vitae

Summary

Infectious diseases are major threats causing more morbidity and mortality than any other human disease. The lungs are prone to develop infections due to frequent exposure of pathogens and due to the large surface area. Cells of the innate immune system in the lungs, like macrophages, are the first to recognize these pathogens and induce an inflammatory response through receptors like Toll-like receptors (TLRs). After recognition of the pathogen, these immune cells produce several cytokines and chemokines which facilitate recruitment of other leukocytes and prevent dissemination of the pathogen. TLRs are evolutionary conserved receptors and important in sensing the presence of pathogens by recognition of "pathogen associated molecular patterns" (PAMPs). So far, 11 TLRs are known in mice of which each recognizes specific PAMPs. To determine the role of TLRs (or related molecules) during infection *in vivo*, we used mice with a targeted gene deletion (knock out -KO- mice). **Chapter 1** provides an introduction in the innate immune system in the lungs, the recognition of *Streptococcus (S.) pneumoniae* and influenza A and the role of TLRs and the chemokine monocyte chemoattractant protein (MCP)-1 herein. The **first part** of this thesis is focused on infections with *S. pneumoniae* and recognition of pneumococcal ligands like lipoteichoic acid (LTA) and pneumolysin (PLN). LTA is a compound found in the cell wall of *S. pneumoniae* that is released when bacteria are killed. LTA has profound inflammatory properties and so far, *in vitro* studies have shown that pneumococcal LTA is recognized by TLR2. In **Chapter 2** we showed that inoculation of pneumococcal LTA induces a dose-dependent inflammatory response and activation of coagulation *in vivo* which was TLR2 dependent. PLN which is an important virulence factor of *S. pneumoniae*. PLN has inflammatory, and in high doses, also lytic properties and was recently shown to be recognized by TLR4. In **Chapter 3** we showed that PLN dose dependently induces cytokine production and cell lysis *in vitro*. Using a low, non-lytic dose, no profound differences were observed between normal and TLR4 KO mice with respect to inflammatory responses. Using a higher, lytic dose, TLR4 KO mice responded less to PLN as revealed by a reduced inflammatory response and neutrophil recruitment in these mice. However, a similar phenotype was observed in TLR2 KO mice. Similarities between these two mutant strains could be caused by the PLN–induced release of endogenous mediators which

also signal through TLRs, thereby skewing clear PLN-TLR4 signaling. The role of PLN during infection with live bacteria is further discussed in **Chapter 4**. Earlier studies in our laboratory have shown a minor role for TLR2 in pneumococcal pneumonia. We hypothesized that lack of TLR2 signaling can be compensated by ligand-dependent signaling through another TLR. To investigate this hypothesis we determined whether in the absence of TLR2, *S. pneumoniae* can still be sensed by the immune system through an interaction between PLN and TLR4. Indeed, *S. pneumoniae* deficient of its TLR4-ligand PLN was able to grow in TLR2 KO mice which was not observed using normal, PLN-producing, *S. pneumoniae*. This shows that TLR4-PLN signaling can compensate for TLR2 deficiency in TLR2 KO mice. CD14 is a scavenger receptor which recognizes several PAMPs. CD14 has no intracellular signaling domain and requires TLRs for cell signaling. The role of CD14 has been investigated in several gram-negative bacterial infections but knowledge of its role in gram-positive bacterial infections was limited. The unexpected, significant role of CD14 in pneumococcal pneumonia is described in **Chapter 5**. We showed that (soluble) CD14 has a detrimental role in the pathogenesis of pneumococcal pneumonia. CD14 KO mice displayed a reduced migration of pneumococci from the bronchoalveolar compartment into the lung tissue and systemic compartment resulting in an improved survival. The reduced bacterial outgrowth was in line with the reduced pulmonary and systemic inflammatory response. In wild type (WT) mice, soluble (s) CD14 increased during the course of infection and instillation of sCD14 in CD14 KO mice changed these mice into the WT phenotype. This implicates that CD14 might be a transporter receptor for pneumococci which facilitates invasive respiratory tract infection.

MCP-1 is known to primarily attract monocytes and T lymphocytes and may contribute to neutrophil recruitment during severe bacterial infections. In addition, MCP-1 is highly expressed during pneumococcal pneumonia. In **Chapter 6** we showed that MCP-1 production is correlated to the bacterial load during pneumococcal pneumonia. However, mice deficient of MCP-1 showed a similar antibacterial defense and inflammatory response compared to WT mice.

The **second part** of this thesis is focused on pulmonary infection with influenza A virus. Whereas MCP-1 deficiency has no significant role during pneumococcal

pneumonia (chapter 6), MCP-1 deficiency resulted in an impaired immune response against viral pneumonia. **Chapter 7** presents data from experiments with MCP-1 KO mice showing that MCP-1 contributes to an adequate immune response during pulmonary infection with influenza A virus. These mice displayed reduced leukocyte recruitment into the infected lungs resulting in an enhanced viral burden, inflammatory response and weight loss. Although MCP-1 KO mice showed a reduced impaired antiviral mechanism, eventually viral clearance was similar to WT mice. This shows that MCP-1 is not the primary key player in resolving the infection and may be compensated for by other chemokines/cytokines. A recent study showed that CD14 was required for influenza A-induced cytokine and chemokine production in macrophages. In addition, CD14 is known to inhibit proliferation and activation of lymphocytes, which are important in the clearance of viruses. **Chapter 8** describes the role of CD14 in influenza pneumonia. Mice deficient of CD14 displayed a reduced viral load at the relative early and late phase of infection and an altered inflammatory response. However, this had no impact on the lymphocyte recruitment or weight loss. Together, this shows that CD14 deficiency mildly affects viral pneumonia in contrast to its pivotal role in pneumococcal pneumonia (chapter 5). **Chapter 9** describes the development and usage of a technique (Multiplex Ligation-dependent Probe Amplification -MLPA-) to determine a wide gene-expression profile of genes involved in the inflammatory response, induction of coagulation, TLR signaling and cell repair mechanisms in mice. We investigated gene-expression in different compartments of the respiratory tract during infection of mice with influenza A. Most of the genes investigated were differentially expressed during the course of infection and returned to basal level when the virus was cleared from the lungs. However, expression of a few genes remained altered when mice had cleared the virus. These genes could potentially be of interest in the mechanism behind postinfluenza pneumococcal pneumonia (see below).

The **third part** of the thesis is focused on secondary bacterial infection. Postinfluenza pneumonia is a common cause of severe bacterial infection. Secondary bacterial infections are more severe than primary bacterial infections. *S. pneumoniae* is a commonly isolated pathogen during secondary bacterial infection. Influenza is known to affect TLR2 expression in several cells from the innate immune system. Since TLR2 is the most important sensor for gram-positive stimuli, alteration in TLR2 expression, due to the preceeding viral infection, may contribute to the uncontrolled

pulmonary infection observed in postinfluenza pneumonia. A commonly used model for secondary bacterial infection is inoculation of the bacteria after recovery from a primary, viral infection. In **Chapter 10** we describe experiments showing that TLR2 deficiency does not/minimally impair(s) the host immune response to primary viral infection, secondary bacterial infection and super-infection, a model in which we inoculate bacteria when the viral infection has reached its 'zenith'.

General discussion

The recognition of invading pathogens by the innate immune system is a crucial first line defense which is controlled by several receptors; TLRs are new key players herein. Research on these receptors in the last decade has shown that, like the adaptive immune system, the innate immune system also has specificity. However, direct interaction of TLRs and pathogens, or components of pathogens, has only recently become more elucidated. To develop new tools for treatment of infections it is necessary to fully understand pathogen-host interactions. In the experiments described in this thesis, we intended to gain more insight in host defense mechanisms against pulmonary tract infection caused by either *S. pneumoniae* or influenza A. In the first part of this thesis we focused on pulmonary tract infections caused by *S. pneumoniae* or inflammation caused by components derived from this pathogen. We clearly show that recognition of pneumococcal LTA *in vivo* depends on TLR2. Interestingly, studies have shown a minimal contribution of TLR2 in the antibacterial defense during pulmonary infection with *S. pneumoniae*. Although the experiments with purified components or live bacteria differ significantly from each other, the strong differences in host response in TLR2 KO mice during the recognition of pneumococci or LTA are remarkable. One hypothesis is the recognition of pneumococci by several different TLRs. Indeed, we showed that signaling of TLR4 via PLN can compensate for TLR2 deficiency. In addition, others have shown that mice deficient for the TLR adaptor molecule MyD88 also displayed an impaired antibacterial defense. Taken together, this shows the redundancy in the recognition of a pathogen by the host immune system through a variety of TLRs. The contribution of (s)CD14 herein is believed to be TLR independent. sCD14 could be in important candidate to block during pneumococcal pneumonia and may prevent the occurrence of bacteremia after pulmonary pneumococcal infections.

The study on gene expression during influenza infection in mice has shown that many genes are differentially expressed in different 'compartments' in the lungs during infection. This showed us that although TLR2 and CD14 were highly upregulated during viral infection, deficiency of either of these receptors had minimal and respectively some impact on the clearance of influenza. Surprisingly, whereas MCP-1 contributed to an adequate immune response against viral infection, no role was found during pneumococcal infection even though in both respiratory tract infections MCP-1 is highly expressed.

In our experiments we have used mice deficient for a specific receptor or chemokine. Although this is a very elegant method to determine the role of a specific protein during infections *in vivo*, the possibility exists that these genetically modified mice developed compensation in the immune system for the genetic deletion. In addition, different laboratories investigating pneumococcal pneumonia frequently use different serotypes of *S. pneumoniae* and diversity exists in inoculation; i.e. some laboratories introduce lower-, while others introduce upper respiratory tract infections. More importantly, when interpreting our data, we need to be careful in extrapolating results obtained from mice experiments to the human situation. One could speculate about the intervention of TLRs or (s)CD14 during severe pneumococcal infection in humans. If anything, we could hypothesize that intervention of (s)CD14 and TLR2 may hamper the translocation of pneumococci to the circulation and respectively reduce excessive inflammatory response to released LTA which is involved in post infectious sequelae like septic shock. However, the possibility exists that other, opportunistic pathogens arise when patients are treated with TLR or (s)CD14 antibodies.

We gradually come to understand the complexity of the interaction between innate immunity and pathogens like *S. pneumoniae* and influenza A. Insightful research over the next decades may lead to the development of non-conventional, alternative therapies for infectious diseases.

Samenvatting

Infectieziekten vormen een grote bedreiging en zorgen voor meer ziekte en sterfte dan andere ziekten. Vooral de longen zijn gevat voor infecties door het veelvuldig in aanraking komen met micro-organismen en het grote oppervlakte van de longen. Cellen in de longen van het 'innate immune' systeem, zoals macrofagen, zijn de eerste die de micro-organismen herkennen via receptoren zoals 'Toll-like receptoren' (TLRs) en een ontsteking induceren. Na de herkenning van de micro-organismen produceren deze cellen diverse cytokinen en chemokinen die het aantrekken van andere immune cellen bevorderd om zo verspreiding van de micro-organismen te voorkomen. TLRs zijn receptoren die een belangrijke rol spelen in het herkennen van structuren van deze micro-organismen. Tot dus ver zijn er 11 TLRs bekend in de muis die elke een specifieke structuur herkennen van verschillende soorten micro-organismen. Om de rol van TLRs te bestuderen tijdens infecties in het levende organisme (*in vivo)* hebben we gebruik gemaakt van muizen met een deletie in een specifiek gen (knock out -KO- muizen). **Hoofdstuk 1** geeft een introductie over het 'innate immune' systeem in de longen, de herkenning van *Streptococcus (S.) pneumoniae* en het influenza virus en de rol van TLRs en chemokine MCP-1 hierin. Het eerste deel van dit proefschrift is gericht op infecties met *S. pneumoniae* en de herkenning van onderdelen van de pneumococ zoals lipoteichoic acid (LTA) en pneumolysine (PLN). LTA is een onderdeel van de celwand van *S. pneumoniae* welke vrijkomt als de bacterie gedood word. LTA heeft sterke inflammatoire eigenschappen en tot nu toe hebben celkweek studies (*in vitro)* aangetoond dat LTA herkend wordt door TLR2. In **Hoofdstuk 2** laten we zien dan LTA, afkomstig van *S. pneumoniae*, een dosis afhankelijke ontsteking en activatie van de stollingscascade induceert *in vivo* welke TLR2 afhankelijk was. PLN is een belangrijke virulente factor van *S. pneumoniae* en induceert ontsteking en bij hogere dosis ook cel dood (lytisch). Recentelijk is aangetoond dat PLN herkend wordt door TLR4. In **Hoofdstuk 3** laten we zien dat PLN een dosis afhankelijke cytokine productie en cel dood induceert *in vitro*. We vonden geen grote verschillen mbt ontsteking tussen normale muizen en TLR4 KO muizen bij gebruik van een lage, niet lytische dosis. Bij gebruik van een hogere, lytische dosis, reageerde TLR4 KO muizen minder op PLN mbt het induceren

van een ontsteking en het aantrekken van neutrophilen. Echter, een vergelijkend beeld was ook terug te zien in TLR2 KO muizen. Overeenkomsten tussen TLR2 KO en TLR4 KO muizen zouden veroorzaakt kunnen worden doordat PLN het vrijkomen van endogene eiwitten bij longschade induceert, welke ook herkend worden door TLRs, waardoor een duidelijk PLN-TLR4 interactie vertroebeld wordt. In **Hoofdstuk 4** wordt de rol van PLN tijdens infecties met levende bacteriën beschreven. Eerdere studies in ons laboratorium hebben laten zien dat TLR2 geen grote rol speelt tijdens longontsteking geïnduceerd door *S. pneumoniae*. Wij veronderstelden dat de afwezigheid van TLR2 gecompenseerd werd door signalering via andere TLRs. Om dit te onderzoeken hebben we gekeken of in de afwezigheid van TLR2, *S. pneumoniae* nog steeds herkend wordt door het immune systeem via PLN en TLR4. *S. pneumoniae* zonder PLN productie groeide uit in TLR2 KO muizen wat niet gebeurde tijdens infecties met PLN producerende *S. pneumoniae*. Dit laat zien dat TLR4-PLN signalering kan compenseren voor de afwezigheid van TLR2. CD14 is een receptor welke verschillende producten van micro-organismen kan herkennen. CD14 heeft geen intracellulaire signaleringsstructuren en heeft TLRs nodig om na binding met ligand, cellen te kunnen activeren. De rol van CD14 is onderzocht in verscheiden studies over infecties met gram negatieve bacteriën maar de rol tijdens infecties met gram positieve bacteriën is beperkt. In **Hoofdstuk 5** beschrijven we de onverwachtse rol van CD14 tijdens infectie met *S. pneumoniae*. Wij laten zien dat (vrij)CD14 een negatieve rol speelt tijdens deze infectie. CD14 KO muizen hebben een verminderde translocatie van de bacterie vanuit het long weefsel naar de circulatie. De verminderde hoeveelheid bacteriën in longen en bloed ging samen met een verminderde ontsteking. In normale muizen neemt vrij CD14 toe tijdens de infectie en het toedienen van exogeen vrij CD14 in CD14 KO muizen veranderde het fenotype van deze CD14 KO muizen in normale muizen tijdens infectie. Dit geeft aan dat CD14 een receptor kan zijn voor het verplaatsen van de bacterie en zorgt voor een invasieve longontsteking.
MCP-1 trekt monocyten en T-cellen aan en kan ook bijdragen aan het aantrekken van neutrofielen tijdens ernstige bacteriële infecties. Daarnaast komt MCP-1 in hoge mate voor tijdens infectie met *S. pneumoniae*. In **Hoofdstuk 6** laten we zien dat MCP-1 productie gecorreleerd is aan de hoeveelheid bacteriën in de longen tijdens infectie met *S. pneumoniae*. Maar tijdens infectie geven muizen zonder MCP-1 productie,

MCP-1 KO muizen, een gelijke bacteriële uitgroei en inductie van ontstekings mediatoren in vergelijking met normale muizen.

Het tweede deel van dit proefschrift richt zich op infecties van de longen (respiratoir) met het influenza virus. Daar waar MCP-1 deficiëntie geen effect had bij respiratoire infecties met *S. pneumoniae* (hoofdstuk 6), had MCP-1 deficiëntie wel effect bij infectie met influenza; MCP-1 KO muizen hadden een verslechterd immune response tegen respiratoire infectie met influenza A virus. **Hoofdstuk 7** laat zien dat MCP-1 bijdraagt aan een efficiënt immune response tijdens deze infectie. MCP-1 KO muizen hebben een verminderde influx van verschillende leukocyten in de geïnfecteerde longen wat resulteert in een verhoogde virale load, ontsteking en gewichtsverlies. Ondanks dat deze MCP-1 KO muizen een verslechterd immune response vertonen, is het virus, na herstel van de infectie, evengoed geklaard als bij de normale muis. Dit geeft aan dat MCP-1 niet de hoofdfactor is bij virale infectie en dat deficiëntie van dit gen opgevangen wordt door andere ontstekings-mediatoren. Een recente studie gaf aan dat CD14 nodig is voor het induceren van een immune response bij macrofagen tijdens infectie met influenza virus. Daarnaast is bekend dat CD14 de proliferatie en activatie van lymfocyten voorkomt welke belangrijk zijn voor het klaren van influenza. **Hoofdstuk 8** beschrijft de rol van CD14 tijdens respiratoire infectie met influenza. CD14 KO muizen hadden een verminderde virale load tijdens de vroege en late fase van de infectie en een veranderd immune response. Echter, dit had geen effect op de influx van leukocyten in de geïnfecteerde longen of gewichtsverlies. Dit alles geeft aan dat CD14 minimaal bijdraagt tijdens virale infectie, dit in tegenstelling tot infectie met *S. pneumoniae* (hoofdstuk 5). **Hoofdstuk 9** beschrijft een nieuwe techniek (Multiplex Ligation-dependent Probe Amplification -MLPA-) voor het bepalen van een breed scala aan genexpressie van genen die betrokken zijn bij ontsteking, inductie van de stollingscascade, TLR signalering en genen betrokken bij herstelmechanisme in muizen. Wij hebben de genexpressie bestudeerd van de muis in verschillende compartimenten van de longen tijdens infectie met influenza. De meeste genen die we onderzocht hadden, hadden een verschillend expressie patroon tijdens de infectie en herstelden naar basaal waarde als het virus geklaard was. Echter, expressie van sommige genen bleef veranderd na herstel van het virus en deze genen

zouden bij kunnen dragen aan het mechanisme verantwoordelijk voor de excessieve ontsteking tijdens postinfluenza pneumonie.

Het derde deel van dit proefschrift is gericht op secundaire bacteriële infectie. Een secundaire bacteriële infectie is vaak ernstiger dan een primaire bacteriële infectie. Postinfluenza pneumonie is een veel voorkomende oorzaak van ernstige, secundaire bacteriële infectie van de longen. *S. pneumoniae* is een veel voorkomende bacterie tijdens postinfluenza pneumonie. Studies hebben aangetoond dat influenza TLR2 expressie kan beïnvloeden in verschillende cellen van het 'innate immune' systeem. Omdat TLR2 de belangrijkste receptor is voor de herkenning van producten afkomstig van gram-positieve micro-organismen, is verandering in TLR2 expressie door de virale infectie, mogelijk betrokken bij de excessieve infectie bij postinfluenza pneumonie. Een gebruikelijk model voor secundaire bacteriële infectie is het inoculeren van bacteriën na herstel van een primaire virale infectie. In **Hoofdstuk 10** laten we zien dat TLR2 deficiëntie niet/minimaal bijdraagt aan het immune response tijdens primaire infectie met influenza, secondaire bacteriële infectie of super-infectie, een model waarbij bacteriën worden geinoculeerd als de virale infectie op zijn 'top' is.

Algemene discussie

De herkenning van binnendringende micro-organismes door het 'innate immune' systeem wordt veroorzaakt door verschillende receptoren zoals TLRs en is van cruciaal belang voor het afweermechanisme. Onderzoek naar deze TLRs heeft laten zien dat net als het 'adaptive immune' systeem, het 'innate immune' systeem ook specificiteit heeft. De directe interactie tussen TLRs en micro-organismen of onderdelen van deze micro-organismen wordt de laatste jaren pas echt duidelijk. Om nieuwe behandelingen te ontwikkelen tegen infecties is het van belang om de interactie tussen micro-organsime en de gastheer goed te begrijpen. In de experimenten beschreven in dit proefschrift, proberen we meer inzicht te krijgen in het immune systeem van de gastheer tijdens respiratoire infecties met *S. pneumoniae* of influenza A. In het eerste deel van dit proefschrift richten we ons op infecties met *S. pneumoniae* of ontsteking veroorzaakt door fragmenten van dit micro-organisme. We laten zien dat LTA van *S. pneumoniae* door TLR2 herkend wordt *in vivo*. Opvallend is dat eerdere studies hebben laten zien dat TLR2 geen/minimale rol speelt

bij het antibacteriële mechanisme tijdens respiratoire infecties met *S. pneumoniae*. Ondanks dat de muis modellen waarbij componenten van *S. pneumoniae* of met levende bacteriën geinoculeerd wordt, significant van elkaar verschillen is het verschil in de resultaten met TLR2 KO muizen opvallend. Een verklaring zou kunnen zijn dat *S. pneumoniae* door meerdere TLRs herkend wordt. We laten zien dat signalering van TLR4 via PLN kan compenseren voor een deficiëntie in TLR2. Daarnaast hebben andere laten zien dat muizen deficiënt in het TLR adaptor molecuul MyD88 ook een verminderd antibacterieel mechanisme vertonen tijdens infectie met *S. pneumoniae*. Dit alles laat zien dat er een grote mate van overvloed is in het systeem voor het herkennen van de bacterie door het immune systeem. De bijdrage van (vrij) CD14 hierin is TLR onafhankelijk. Vrij CD14 zou een mogelijke kandidaat kunnen zijn om te blokkeren tijdens respiratoire infectie met *S. pneumoniae* om zo translocatie van de bacterie naar de bloedbaan te voorkomen.
De studie mbt genexpressie bij influenza in muizen laat zien dat vele genen verschillend tot expressie komen in diverse onderdelen van de long. De studie liet o.a. zien dat TLR2 en CD14 sterk werden up-gereguleerd tijdens infectie met influenza. Echter deficiëntie van deze receptoren had een minimale en respectievelijk afwezige rol voor het klaren van influenza. Opvallend was dat daar waar MCP-1 een bijdrage leverde tijdens virale infectie, MCP-1 geen rol speelde tijdens infectie met *S. pneumoniae* terwijl MCP-1 in beide modellen sterk toenam in hoeveelheid.

In onze experimenten hebben we gebruik gemaakt van muizen welke deficiënt zijn voor een specifieke receptor of chemokine. Ondanks dat dit een mooie methode is om de rol van een specifiek eiwit te bepalen tijdens infecties *in vivo*, bestaat de mogelijkheid dat deze genetisch gemodificeerd muizen een compensatie hebben ontwikkeld in hun immune systeem. Daarnaast is het zo dat verschillende laboratoria soms andere serotypen *S. pneumoniae* gebruiken en er is variabiliteit in het inoculeren: sommige laboratoria induceren een lagere luchtweg infectie terwijl andere een hogere luchtweg infectie induceren. Ook is het van belang behoedzaam te zijn met het extrapoleren van de resultaten uit muizen studies naar de menselijke situatie. Je zou kunnen speculeren dat de interventie van (vrij) CD14 en TLR2 de translocatie van *S. pneumoniae* vanuit de longen naar de circulatie zou kunnen belemmeren en respectievelijk een excessieve ontsteking door circulerend LTA zou kunnen

voorkomen. Echter de mogelijkheid bestaat dat opportunistische micro-organismen de kop op steken als patiënten worden behandeld met CD14 of TLR antilichamen.

Langzamerhand komen we tot het begrijpen van de complexiteit van de interactie tussen het ‘innate immune’ systeem en micro-organismen zoals *S. pneumoniae* en influenza virus. Intensief vervolg onderzoek in het volgende decennium zou kunnen leiden tot de ontwikkeling van nog niet bekende, alternatieve therapieën voor infectieziekten.

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Mark

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Curriculum vitae

Mark Christianus Dessing werd geboren op 1 april 1976 te Gouda. Hij behaalde zijn diploma als proefdierkundige in 1998 aan het Hogere Laboratorium Opleiding te Utrecht. Zijn eerste wetenschappelijke stage heeft hij uitgevoerd op de afdeling Farmacologie en Pathofysiologie aan de Universiteit Utrecht ob.v. Prof. dr. F.P. Nijkamp. Tijdens deze stage werd de rol van 'Nerve Growth Factor' bij luchtweg hyperreactiviteit bestudeerd. Vervolgens heeft hij de opleiding Biologie aan de Universiteit Utrecht afgerond in 2001. Zijn tweede stage volgde hij op het Rudolf Magnus Institute for neuroscience aan de Universiteit Urecht o.b.v. Prof. dr. W.H. Gispen. Daar heeft hij het effect van diabetis mellitus en insuline op de zenuwimpuls activiteit in de hippocampus bestudeerd. Na een verfrissende wereldreis van een half jaar heeft hij bij het Institute for Risk Assessment Sciences & AM-Pharma Holding te Utrecht een half jaar meegewerkt aan het opzetten van een nieuwe behandeling tegen sepsis. Vervolgens begon hij als assistent in opleiding op de afdeling Laboratorium Experimentele Inwendige Geneeskunde van het Academisch medisch centrum te Amsterdam o.b.v. Prof. dr. Tom van der Poll. Doel van het onderzoek was om meer inzicht te krijgen in voornamelijk de rol van Toll-like receptors tijdens infectie van de luchtwegen met *Streptococcus pneumoniae* of influenza A.