Inflammatory Response to Severe Bacterial Infections

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INFLAMMATORY RESPONSE TO SEVERE BACTERIAL INFECTIONS

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The studies presented in this thesis have been conducted in the Laboratory of Experimental Internal Medicine, Academic Medical Center, University of Amsterdam, the Netherlands.

In memory of my father, Walter Knapp.

Contents

CHAPTER 1

General Introduction and Outline of the Thesis

Introduction

Described herein are studies pertaining to innate immune responses to bacterial infections. This thesis illustrates and focuses on three distinct models— respiratory tract infection, peritonitis, and endotoxemia.

The first part of this thesis (Chapters 2-7) investigates and elucidates the *in vivo* role of specific inflammatory mediators and immune cells during pneumonia. The second part of this thesis (Chapters 8-18) is dedicated to studies focusing on pattern recognition receptors and their impact on the innate immune response during pneumonia, peritonitis and endotoxemia *in vivo*.

The general aim of this thesis is to refine our current knowledge about the complex world of distinct mediators and receptors during bacterial infections. The more specific objectives of each individual study are delineated in the respective chapter.

The general introduction to this thesis is kept concise since published reviews precede each section. However, in the following paragraphs I briefly describe why we chose these specific models by explaining their clinical relevance.

Respiratory tract infections

Infections of the respiratory tract are the 7th leading cause of mortality in the United States and bacterial pneumonia is the most frequent source of sepsis (1-4). According to the acquisition of pneumonia and the pathogens involved, community-acquired pneumonia (CAP) can be distinguished from hospital-acquired pneumonia (HAP) (also called nosocomial pneumonia).

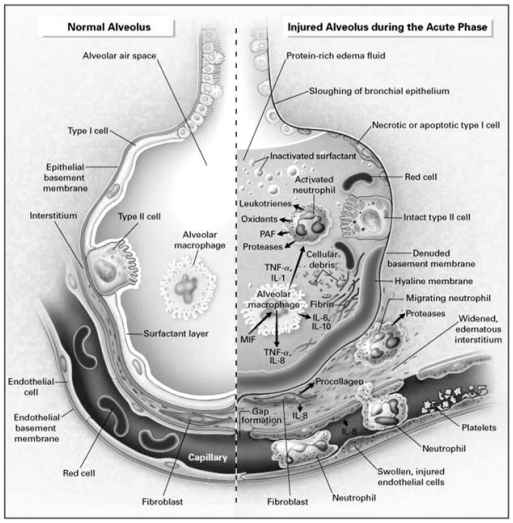

Fig. 1
Depicted is a normal (left side) and inflamed (right side) alveolus as it is observed during respiratory tract infections (adapted from (9)).

While *Streptococcus pneumoniae* is the single most frequent pathogen causing CAP, counting for up to 60% of cases, *Haemophilus influenzae*, *Staphylococcus aureus* and *Klebsiella*

pneumoniae are encountered in about 10% each (5-7). Pneumococcal pneumonia causes more than 150.000 hospitalizations in the United States annually and *Streptococcus pneumoniae* is the most frequent cause of lethal CAP (5, 8). Due to this high clinical importance and worldwide significance, several studies described in this thesis investigated host defense pathways in this particular disease.

Community Acquired Pneumonia		
Pathogen	**%**	**Ref.**
S. pneumoniae	20-75	(7)
H. influenzae	3-10	(7)
S. aureus	3-5	(7)
Gram negative	3-10	(7)
Legionella	2-8	(7)
Mycoplasma	1-18	(7)
Chlamydia	4-6	(7)

Table 1: Causative pathogens of CAP

Nosocomial pneumonia usually occurs in patients with preexisting conditions and can be induced by various pathogens including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacteriaceae* or *Acinetobacter baumannii* (10-13). As a result of improved medical treatments, the wide-spread use of antibiotics and increasing numbers of patients admitted to intensive care units, nosocomial pneumonia is more frequently encountered these days (14). In this thesis, we studied specific immune response pathways using models of nosocomial pneumonia induced by *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. In addition, we took the emergence of bacteria into account that have been associated with nosocomial pneumonia only recently, and set up a new model of HAP, induced by *Acinetobacter baumannii*. *Acinetobacter baumannii* is well-known for its broad resistance to common antibiotics and ability to cause pneumonia outbreaks in intensive care and burn units (14-16). 15-25% of ventilator-associated pneumonias in intensive care units have been ascribed to *Acinteobacter baumannii* and mortality rates up to 75% have been reported (10, 12, 17-20). Considering these high mortality rates and lack of appropriate models, we deemed it necessary to investigate host defense mechanisms against this pathogen.

Hospital Acquired Pneumonia		
Pathogen	**%**	**Ref.**
P. aeruginosa	23-31	(12, 13, 17)
S. aureus	10-33	(12, 17)
A. baumannii	15-34	(12, 13)
K. pneumoniae	12	(12)
E. coli	5	(13)

Table 2: Most frequent causative bacterial pathogens of HAP

Bacterial peritonitis

The second entity of bacterial infections that was investigated and is described in this thesis is peritonitis. Peritonitis is caused by the presence of bacteria in the otherwise germ-free peritoneal cavity and is invariably caused by the perforation of intestines. Bacterial peritonitis is a serious and potentially life-threatening infection that requires immediate surgical intervention, fluid resuscitation and proper antibiotic therapy (21-23). Despite proper medical treatment, mortality rates remain very high (23, 24). Due to the site of infection, causative bacteria have the tendency to quickly spread via the circulation, resulting in systemic inflammation and sepsis (25, 26). The magnitude of systemic inflammation and consecutive organ failure determines survival (27). Mortality rates of 50-80% have been described in patients with more than two failing organs (27). Therefore, the outcome of peritonitis critically depends on the adequacy of the immediate local and systemic host defense intended to eliminate causative pathogens and to control the inflammatory response (24). *Escherichia coli* counts for 60% of bacteria recovered from peritonitis cases, which led us to use a model of *Escherichia coli* peritonitis in mice (Table 3) (28, 29).

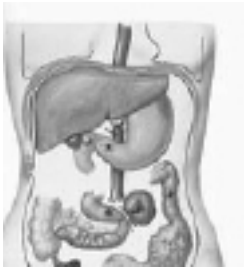

Fig. 2
Peritoneal cavity.

Peritonitis	
Pathogen	**%**
E. coli	60
Enterobacter/Klebsiella	26
Proteus	22
Pseudomonas	8

Table 3: Most frequent Gram-negative bacteria isolated from intra-abdominal infections (from (27)).

Endotoxemia

Systemic inflammation and sepsis is a leading cause of mortality worldwide. The incidence of sepsis is constantly increasing, reaching the 10th most frequent cause of death in the U.S. in 2002 (1, 30). Depending on the severity of inflammation and resulting organ failure, mortality rates as high as 65% have been reported (31, 32). While any bacterial infections can theoretically progress and cause systemic inflammation, respiratory tract as well as abdominal infections are the two most frequent sources for sepsis (Fig. 3) (2, 33).

The systemic spread of endotoxin (lipopolysaccharide, LPS) derived from Gram-negative bacteria is considered one of the most important correlates of sepsis. Intravenous injection of LPS results in activation of several inflammatory pathways, thereby mimicking – in a qualitative way – the systemic host response seen in sepsis. As such, this model allows for a

close examination of the dynamic changes in mediators, receptors and cells during systemic inflammation in humans.

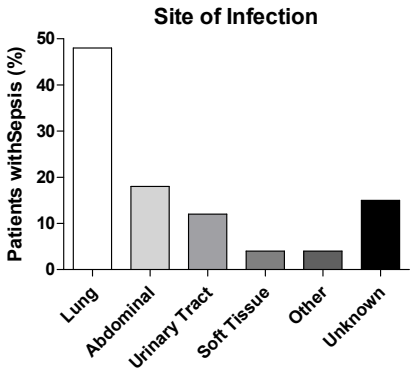

Fig. 3
Site of infection in patients with sepsis (adapted from (33)).

Outline of the Thesis

Part I: Innate Immune Response Mechanisms during Pneumonia
In order to investigate host defense mechanisms and the biological *in vivo* significance herein, the use of animal models proved to be very helpful. **Chapter 2** gives an introduction and overview about commonly used pneumonia-models and summarizes some key findings considering the innate immune response in these models.
Alveolar macrophages are resident phagocytes within the alveolar compartment and considered the first cells that encounter the presence of bacteria within the lungs. In turn, alveolar macrophages are thought to initiate the inflammatory response and orchestrate the activation and performance of attracted neutrophils and resident epithelial cells in order to effectively eliminate bacteria. Our study, in which we investigated the precise role of alveolar macrophages during *Streptococcus pneumoniae* pneumonia is presented in **Chapter 3**.
Host defense against respiratory pathogens involves the coordinated interaction of cells such as macrophages, neutrophils, epithelial and endothelial cells. To allow for a coordinated immune response, these cells secrete mediators that in turn instruct and assist other cells to perform their task. Granulocyte colony-stimulating-factor (G-CSF) is one of these mediators that are released by macrophages upon activation by bacteria. G-CSF in turn acts locally as an immuno-modulatory mediator, with both pro- and anti-inflammatory properties. This attractive profile together with the commercial availability and low incidence of side-effects of G-CSF led to the initiation of clinical studies where G-CSF was administered to patients with pneumococcal pneumonia. However, most data regarding G-CSF came from *in vitro* studies or from unrelated diseases whereas the use and potential benefit of G-CSF during pneumococcal pneumonia has not been studied before. **Chapter 4** describes a study where we

investigated the biological role of endogenous G-CSF during *Streptococcus pneumoniae* pneumonia *in vivo*.

Interleukin (IL)-18 is a pro-inflammatory cytokine, produced by monocytes, macrophages and dendritic cells in response to various stimuli. Just like IL-1, IL-18 is produced as a pro-from that needs further cleavage by interleukin-1 converting enzyme (ICE) to become functionally active. In models of *Streptococcus pneumoniae* pneumonia, both IL-1 and IL-18 have been proven to importantly contribute to survival and bacterial clearance (34, 35). However, we knew from earlier studies that the requirement for distinct pro-inflammatory mediators differs in models of *Streptococcus pneumoniae* versus nosocomial pneumonia (36-40). In an attempt to understand the role of IL-18, IL-1 and ICE during nosocomial pneumonia we studied these mediators in models of *Pseudomonas aeruginosa* (**Chapter 5**) and *Acinetobacter baumannii* (**Chapter 6**) infection in mice. Nosocomial pneumonia is almost invariably preceded by critical illness. One major insult that typically occurs in intensive care units is the aspiration of gastric contents. The impact of acid aspiration on the development of nosocomial pneumonia is poorly understood. We therefore developed a "two-hit" model of acid aspiration followed by infection with Klebsiella pneumoniae; the results are described in **Chapter 7**.

Part II: The role of pattern recognition receptors in the innate immune response:

The second part of this thesis focuses on pattern recognition receptors, such as Toll-like receptors (TLR), CD14 and LPS-binding protein (LBP). **Chapter 8** provides an introduction and general overview about the importance of TLRs, CD14 and LBP during acute infections.

LBP is an acute phase protein and lipid-transfer protein that facilitates and augments the inflammatory response to LPS. As a consequence, $LBP^{-/-}$ mice were protected from lethality in a model of endotoxemia (41). However, these specific mice did not show a clear phentotype when infected with Gram-negative bacteria and we therefore investigated the role of LBP during Gram-negative sepsis, induced by *Escherichia coli*, in mice (**Chapter 9**). Lethality during Gram-negative peritonitis is related to overwhelming inflammation leading to sepsis and multiple organ failure. Oxidized phospholipids are endogenous side products of inflammation that have been shown to inhibit the interaction of LPS with LBP and CD14, thereby preventing hyperinflammation and lethality during endotoxemia in mice (42). Assuming that these lipids might avoid overwhelming inflammation during Gram-negative peritonitis, we studied the role of these lipids during *Escherichia coli* peritonitis; the respective study is presented in **Chapter 10**. Beside LBP's role as an acute phase protein, synthesized by hepatocytes, LBP has been shown to be released by respiratory epithelial cells, suggesting a role during pulmonary inflammation (43). We consequently were interested in the function of pulmonary LBP and investigated its role during LPS-induced lung inflammation using $LBP^{-/-}$ animals. The results of this study are described in **Chapter 11**.

While the innate immune response to Gram-negative pathogens largely depends on the immunogenic properties of LPS, lipoteichoic acid (LTA) is considered the counterpart derived from Gram-positive bacteria. LTA signals its presence via TLR2 and reports exist that indicate a role for LBP and CD14 herein (44-47). Since most of these data were derived from *in vitro* experiments, we decided to elucidate the role of LBP, CD14, TLR2 and 4 during

LTA-induced lung injury *in vivo* (**Chapter 12**). CD14 serves as a co-receptor for TLR4 that can be found membrane-bound on myeloid cells and as a soluble form circulating in plasma (48, 49). Although it is known that CD14 plays a crucial role during LPS-induced inflammation, its function during Gram-positive infections is less well understood. Adding the fact that CD14 can be detected within the pulmonary compartment, we decided to study the functional properties of CD14 during pneumococcal pneumonia using $CD14^{-/-}$ mice (**Chapter 13**). Gram-positive bacteria like *Streptococcus pneumoniae* express different molecules such as LTA, peptidoglycan and lipopeptides that all have been identified as TLR2 ligands (44, 45, 50-53). To investigate the biological and functional relevance of these molecules *in vivo*, we studied the role of TLR2 in this pneumonia model in mice (**Chapter 14**). Furthermore, new reports indicated that TLR4 ligands are released by *S. pneumoniae* (54), which gave rise to another study that elucidated the role of TLR4 in pneumococcal pneumonia as well as *Klebsiella* pneumonia (**Chapter 16**). Nosocomial pneumonias instigate distinct defense mechanisms as compared to pneumococcal pneumonia. Being interested in the emerging nosocomial pathogen *Acinetobacter baumannii*, we then moved on and set up a respective animal model and investigated the role of CD14, TLR2 and 4 using respective gene-deficient mice (**Chapter 16**). A similar approach was undertaken to clarify the role of pattern recognition receptors during lung inflammation induced by lipoarabinomannan, a major molecule expressed by mycobacteria (**Chapter 17**). Last but not least, we investigated a novel receptor, termed triggering receptor expressed on myeloid cells (TREM)-1 that has been shown to amplify the TLR-mediated inflammatory response in mice. Curious about its regulation during sepsis in humans we studied the course of TREM-1 expression and secretion of its soluble form in volunteers challenged with LPS. These results are given in **Chapter 18**.

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Part I

Innate Immune Response Mechanisms during Pneumonia

CHAPTER 2

Pneumonia models and innate immunity to respiratory bacterial pathogens

Sylvia Knapp, Marcus J. Schultz, Tom van der Poll

Shock. 2005; Suppl 1:12-18

Introduction

Preclinical sepsis-models have been used for decades to study the pathophysiologic processes during sepsis and shock. Although these studies revealed promising immunomodulating agents for the treatment of sepsis, clinical trials evaluating the efficacy of these new agents in septic patients were disappointing. The main reason for this unsatisfactory experience might be that - unlike the clinical situation - most of these preclinical models are devoid of a localized infectious source from which the infection disseminates. Studies on the effects of several immunomodulating strategies have demonstrated strikingly opposite results when sepsis models with a more natural route of infection, such as pneumonia, were used. In this review we will give insights into pneumonia models and discuss results and differences in the innate immune responses during distinct pulmonary infection models.

Pneumonia models

The airways are continuously exposed to respiratory pathogens, either by inhalation or (micro)-aspiration. Infectious agents that have passed structural defenses enter the terminal airways, where they may cause pneumonia, one of the most common infectious diseases and the leading cause of sepsis. The two major types of lung infections are community-acquired pneumonia (CAP) and nosocomial / hospital-acquired pneumonia. The most frequent causative pathogen in CAP worldwide is *Streptococcus pneumoniae* (*S. pneumoniae*) whereas *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Klebsiella pneumoniae* (*K. pneumoniae*) are prominent bacteria causing nosocomial pneumonia.
Experimental murine pneumonia with *S. pneumoniae*, *K. pneumoniae* and *P. aeruginosa* are the three best described and most commonly used bacterial pneumonia models. Pneumonia is induced by direct administration of bacteria into the trachea or into the nose of anaesthetized animals. The intra-tracheal infection is usually performed with the help of a small catheter that is inserted into the trachea – either surgically or via the mouth - followed by administration of a small volume of fluid containing the desired number of bacteria. One disadvantage of this method is the need of skilled personnel and the fact that this manipulation can be associated with tissue injury and trauma that might affect outcome. The intranasal route is easy to perform and when carried out in properly anesthetized animals, the pulmonary infection rate is close to 100% without additional injury. An alternative approach is the exposure of mice to aerosolized bacteria in a whole-animal chamber.

Host defense mechanisms and differences between pneumonia models

Pulmonary host defense includes both innate and specific immune responses. In the upper respiratory tract, physical mechanisms such as coughing and sneezing are utilized to remove potential pathogens (Fig. 1). The innate immune response is primarily responsible for the elimination of bacterial pathogens from the alveolar space whereas the specific immune response is involved in the eradication of encapsulated pathogens, and pathogens that survive after phagocytosis. In this review we will focus on the innate immune response. Innate

defenses consist of structural defenses, antimicrobial molecules, and phagocytosis by resident alveolar macrophages (AMs) and recruited polymorphonuclear cells (PMNs) (Fig. 1). AMs and PMNs play a prominent role in innate immunity in the lungs. These cells can phagocytose and kill microorganisms, and PMNs can release antibacterial contents of their granules. AMs and PMNs need to communicate in mounting an effective host defense against invading pathogens. Pro-inflammatory and anti-inflammatory cytokines, chemokines, and colony-stimulating factors play a critical role in this process.

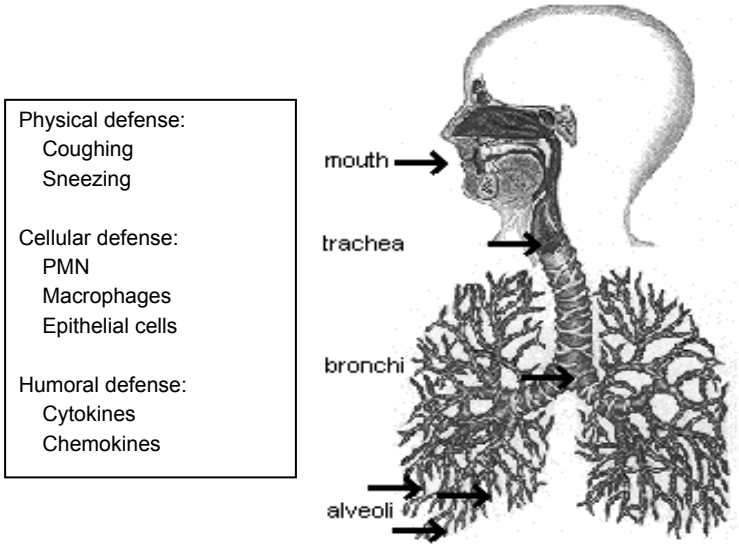

Fig. 1: Overview of pulmonary host defense mechanisms

Cellular component of pulmonary host response

Respiratory pathogens that have entered the terminal airways are first encountered by AMs - the resident pulmonary macrophages. AMs can bind, phagocytose, and kill pathogens, and thus can directly influence the bacterial load (1, 2). In addition, AMs can also secrete a large range of inflammatory mediators that act directly on the respiratory pathogens or exert indirect effects by recruiting and activating PMNs (1, 3, 4). The recruitment of large numbers of PMNs in the alveolar space from the marginated pool of leukocytes in the pulmonary circulation is initiated when the microbial challenge is too large or too virulent to be contained by the AMs alone. These attracted PMNs provide auxiliary phagocytic capacities that are critical for the effective eradication of pathogens. Last but not least, AMs can phagocytose apoptotic PMNs and thereby contribute to the resolution of pneumonia (5). From this it can be concluded that AMs play a regulating role in the pathogenesis of pneumonia, both in the initiation and resolution phase of the inflammatory response.

AMs and bacterial clearance/survival

A body of evidence illustrates the importance of AMs in host defense against different respiratory pathogens. In a murine *Mycoplasma* pneumonia model, AM-depletion resulted in

elevated outgrowth of *Mycoplasma* from the lungs (6). AM-depletion exacerbated the *Mycoplasma* infection in *Mycoplasma*-resistant mice by reducing killing of the pathogen to a level comparable to that in *Mycoplasma*-susceptible mice without AM-depletion, suggesting that defective AM-function is the likely explanation for the difference between susceptible and resistant mice. Interestingly, higher levels of PMNs in lavage fluids were recovered from the AM-depleted mice, indicating that the differences in killing of the invading pathogen could not be explained by an effect on the recruitment of PMNs. In a murine *K. pneumoniae* pneumonia model, AM-depletion influenced mortality dramatically, with 100% lethality after 3 days in AM-depleted mice while all control mice (i.e. non-depleted) survived (7). This was accompanied by an increased outgrowth of *K. pneumoniae* from lungs and blood in AM-depleted mice, and similar to the study on *Mycoplasma pneumoniae*, an increase in PMN-influx into the pulmonary compartment. Comparable data were reported in a murine pneumonia model with *P. aeruginosa*, in which AM-depletion resulted in a delayed bacterial clearance compared to control mice (8). However, it was also demonstrated that early after the initiation of pneumonia, compared with control mice, AM-depleted mice had lower concentrations of chemokines and fewer PMNs in BALF, and a significant improvement in lung edema. Two other studies underlined the role of AMs in the early attraction of PMNs to the lung: Compared with control rats, PMN-numbers were decreased in bronchoalveolar lavage fluids (BALF) of AM-depleted rats after instillation of a sublethal dose of *P. aeruginosa* into the airways, concordant with significantly decreased levels of tumor necrosis factor-α (TNF) and macrophage inflammatory protein (MIP)-2 mRNA expression in one study (9). In the second study, in which AMs from lipopolysaccharide (LPS)-exposed mice were transferred to the lungs of naive recipient mice, nearly a threefold increase in the number of PMNs in BALF was found compared to instillation of AMs from unexposed mice (10).

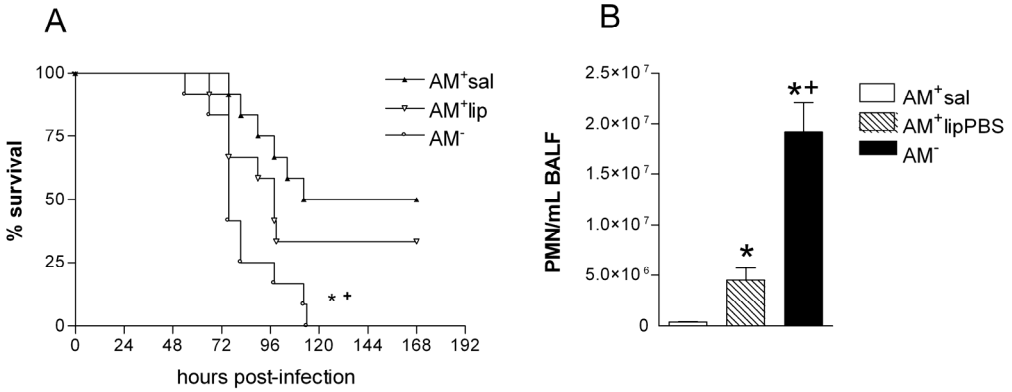

Fig. 2: Increased mortality and pulmonary PMN influx of AM$^-$ mice following *S. pneumoniae* infection. A: Survival of BALB/c mice (n=12 per group) that were depleted of AM with liposomal clodronate (AM-) or received control liposomes (AM$^+$lip) or saline (AM$^+$sal) 48 hours prior to bacterial challenge with 5x10^4 CFUs *S. pneumoniae*. $^+$ $p \leq 0.05$ AM$^-$mice vs AM$^+$lip, ***** $p \leq 0.05$ AM$^-$ vs AM$^+$sal. **B:** PMN counts obtained in broncho-alveolar lavage fluid (BALF) 44 hours post inoculation with *S. pneumoniae*. $^+$ $p \leq 0.05$ AM$^-$mice vs AM$^+$lip, ***** $p \leq 0.05$ AM$^-$ vs AM$^+$sal. Adapted and modified from reference (11).

Impaired survival was found in mice depleted of AMs in a murine *S. pneumoniae* pneumonia model (Fig. 2). In this study, PMN-recruitment was strongly increased in AM-depleted mice, but no difference in bacterial outgrowth could be observed (Fig. 2) (11). In a more recent study, Dockrell et al. made use of a model of resolving pneumococcal pneumonia and found an impaired bacterial clearance in AM-depleted animals (12). These data suggest that AMs are particularly important phagocytic cells during low dose pneumococcal infection whereas PMNs are required to combat infections with high bacterial counts.

AMs and resolution of inflammation

Whereas early on AMs readily phagocytose and thereby eliminate certain inhaled pathogens, they later represent important effector cells in the resolution process (13). The above mentioned observation of overzealous and persistent PMN-infiltration in the absence of AMs during pneumococcal pneumonia may be related to the equally important function of AMs during the resolution of inflammation (11). To restitute tissue homeostasis all processes involved in the initiation of inflammation must be reversed and one important prerequisite for resolution to occur is the removal of extravasated PMNs. Several lines of evidence support the hypothesis that PMNs undergo apoptosis followed by rapid clearance by AM (5, 13). Apoptosis thereby provides an injury-limiting mechanism since the membrane of PMNs remains intact, preventing potentially injurious granule contents from being released. The role of macrophages as regulators of inflammation has further been illustrated by studies demonstrating that macrophages engulf apoptotic PMNs and consecutively release anti-inflammatory mediators (14). These data indicate that the role of AMs in host defense is not limited to the generation of the initial inflammatory response, but extends to regulation of inflammation, including the elimination of aged PMNs.

Together, these studies in different pneumonia models illustrate the importance of AMs in the orchestration of the inflammatory response in pneumonia. The early initiation of pulmonary inflammation and elimination of low bacterial doses depends on the presence of AM. At later timepoints, AMs contribute to the modulation and subsequent resolution of the inflammatory response.

Role of PMNs in pneumonia

Depending on the causative pathogen during pneumonia, PMNs migrate from the pulmonary circulation in a $\beta_2$integrin (CD11b/CD18) dependent or independent way (15). Once they reach the lungs, PMNs are potent phagocytes that help eliminating bacteria and secrete an array of pro-inflammatory mediators. A deficient influx of PMNs into the lungs, either by antibody mediated PMN depletion or by blocking CXC chemokine receptors expressed by PMNs, is associated with a strongly reduced resistance against bacterial pneumonia (16). Generally spoken, Gram-negative bacterial lung infections rely on a CD11b/CD18 dependent and Gram-positive infection on a CD11b/CD18 independent PMN recruitment (17, 18). Studies in *P. aeruginosa* pneumonia revealed that factors interacting with the CD11b/CD18 pathway impair PMN recruitment and bacterial clearance (19). On the other hand, the absence

of the CD11b/CD18 ligands P-selectin or ICAM had no effect on PMN recruitment during *S. pneumoniae* pneumonia (15).

Role of respiratory epithelium

The respiratory epithelium lines the airways and immediately senses inhaled pathogens. The epithelial response consists of an increased release of antimicrobial peptides, chemokines and cytokines. Antimicrobial peptides are a growing number of mediators that display microbicidal activity or inhibit growth of bacteria (20). The principal families are named defensins and cathelicidins and are released during pneumonia. Delayed bacterial elimination has been demonstrated in mouse β-defensin-1 deficient mice infected with *Haemophilus influenzae* (21).

Humoral component of host defense

A large series of animal studies have demonstrated the importance of pro-inflammatory cytokines in host defense during pneumonia (Table 1). The cytokines TNF and IL-1β are increased early during bacterial pneumonia, both in humans (22) and in animals (23-26). TNF and IL-1 activate phagocytes, leading to augmented phagocytosis, oxidative burst, protein release and bacterial killing (27-29). Moreover, TNF contributes to the recruitment of PMNs by stimulating the expression of adhesion molecules (29), and inducing the production of chemokines (30).

Table 1: Cytokines influence host defense during respiratory tract infections

Cytokine	*S. pneumoniae*	*K. pneumoniae*	*P. aeruginosa*
TNF	+	+	+/-
IL-1β	+	?	-
IL-10	-	-	+/-
IL-12	0	+	?
IL-18	+	?	-
IFN-γ	+/-	?	+/-

+, indicates a beneficial role in respiratory infection with the specified pathogen; -, indicates a detrimental role in respiratory infection with the specified pathogen; +/-, indicates conflicting data; ?, indicates that there are no data on the effect of this cytokine: 0, indicates no effect. See text for details.

There is ample evidence that underlines the importance of TNF and IL-1β in host defense in bacterial pneumonia: In a murine *S. pneumoniae* pneumonia model, treatment with a neutralizing anti-TNF moAb strongly impaired antibacterial defense (23). Anti-TNF-treatment resulted in an enhanced outgrowth of *S. pneumoniae* from the lungs, and anti-TNF-treated mice died significantly earlier from pneumococcal pneumonia than control mice. These findings are in line with results from another study on pneumococcal pneumonia (24). In this study, treatment with anti-TNF moAb accelerated bacterial proliferation in blood and increased lethality. Similar data were obtained in a murine model of Gram-negative pneumonia: Passive immunization against TNF prior to *K. pneumoniae* inoculation resulted in

significantly higher numbers of *K. pneumoniae* in lung and plasma which was associated with markedly decreased short- and long-term survival (25). In addition, during *K. pneumoniae* pneumonia, both intratracheal administration of a TNF agonist peptide (31) and augmentation of TNF expression within the lung by means of a recombinant adenoviral vector (32) have been shown to enhance clearance of bacteria, and to significantly improve survival compared to that of animals receiving control therapy. Comparable results were obtained for IL-1. IL-1 receptor type I deficient mice infected with *S. pneumoniae* displayed an increased bacterial outgrowth together with a reduced capacity to form inflammatory infiltrates (33). Of considerable interest, treating IL-1 receptor deficient mice with a neutralizing anti-TNF antibody made them extremely susceptible to pneumococcal pneumonia - more so than IL-1 receptor knockout mice treated with a control antibody and wild type mice treated with anti-TNF (Fig. 3) (33). Thus, whereas excessive production of pro-inflammatory cytokines at the systemic level causes organ failure and death in animal models of fulminant sepsis, the local production of pro-inflammatory cytokines importantly contributes to host defense during pulmonary infection.

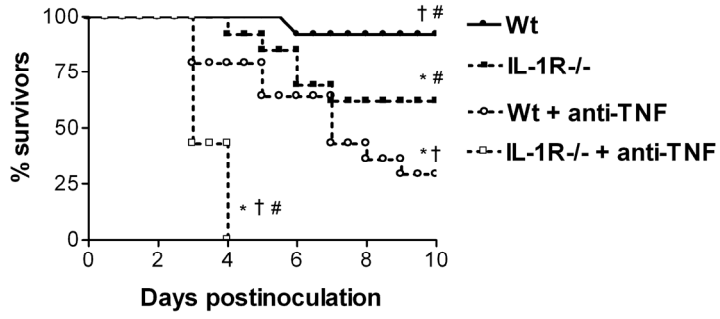

Fig. 3: Role of IL-1 and TNF in lethality induced by *S. pneumoniae*. Survival after intranasal inoculation with S. pneumoniae in mice pre-treated with a neutralizing anti-mouse TNF mAb (open circles for Wt and open squares for IL-1R-deficient mice) or control Ab (closed circles for Wt and closed squares for IL-1R deficient mice). N=12-14 per group; * $p<0.05$ vs Wt; $^{+}$ $p<0.05$ vs. IL-1R deficient; $^{\#}$ $p<0.05$ vs. Wt and anti-TNF. Adapted and modified from (33).

Interferon (IFN)- γ is a cytokine mainly produced by antigen activated T and natural killer (NK) cells and exerts several immune regulatory activities, including activation of phagocytes, stimulation of antigen presentation, orchestration of leukocyte-endothelium interactions and stimulation of the respiratory burst (34, 35). The secretion of IFN- γ is induced by TNF and IL-12 and the production of IFN- γ as well as IL-12 is enhanced during murine pneumonia (36-38). The role of IFN- γ in the setting of bacterial pneumonia is not quite clear. Whereas in one study IFN- γ knockout mice demonstrated higher mortality compared to wild type mice (39), and in other studies local overexpression of IFN- γ in rats resulted in increased bacterial clearance from the pulmonary compartment (40), other data suggest that endogenous IFN- γ may impair an effective pulmonary defense in pneumonia, since pulmonary clearance of *S. pneumoniae* was attenuated in IFN- γ -R knockout mice, as well as in IFN-γ knockout mice compared to wild type mice (37). During *P. aeruginosa* pneumonia endogenous IFN-γ impairs the bacterial clearance (36). Neutralization of IL-12 attenuated bacterial clearance and increased mortality of mice with *K. pneumoniae* pneumonia, while overexpression of IL-12 reduced mortality (38). In contrast, IL-12

knockout mice had a normal host defense against *S. pneumoniae*: bacterial outgrowth from lung tissue and survival were similar to bacterial outgrowth and survival in wild type mice in a pneumonia model with this pathogen (41).

Considering the opposite effects of pro-inflammatory cytokines and anti-inflammatory cytokines in sepsis, it was hypothesized that IL-10 might play a negative role during pneumonia. Indeed, immunizing mice with anti-IL-10 serum before infection with *K. pneumoniae* pneumonia resulted in an enhanced bacterial clearance, increased expression of pro-inflammatory cytokines and prolonged survival (42). In addition, treatment with recombinant IL-10 during *S. pneumoniae* pneumonia resulted in decreased TNF concentrations together with increased bacterial counts in lungs and blood, and early lethality (43). In accordance, pretreatment with an anti-IL-10 moAb led to reduced bacterial counts in lungs and plasma and prolonged survival in pneumococcal pneumonia (43). Thus, while the anti-inflammatory cytokine IL-10 is protective in models of overzealous systemic inflammation, it impairs host defense during local pulmonary infections induced by *K. pneumoniae* or *S. pneumoniae*.

However, in contrast to pneumonia models with *S. pneumoniae* or *K. pneumoniae*, pro-inflammatory cytokines attenuate host defense while anti-inflammatory pathways are beneficial during experimental murine pneumonia induced by *P. aeruginosa*. Both TNF receptor I and IL-1 receptor deficient mice demonstrated improved bacterial clearance after induction of *P. aeruginosa* pneumonia (44, 45). Likewise, the outcome in neutropenic rats suffering from *P. aeruginosa* sepsis significantly improved after treatment with either IL-1ra or TNF-binding protein (46). Of great interest, the combination of IL-1ra and TNF-binding protein not only worsened outcome of septic rats but was associated with diffuse bacterial microabscesses throughout the lungs (46). In addition, treatment with rIL-10 significantly decreased lung injury and mortality in a model of *P. aeruginosa* pneumonia (47). However, it must be emphasized that the murine *Pseudomonas* pneumonia model has some similarities with the intravenous administration of bacteria models for sepsis. First, a rather large amount of bacteria must be installed into the airways to induce pneumonia in mice; using lower inocula, bacteria are cleared from the airways and no pneumonia develops. Second, compared with the other experimental pneumonia models in which pneumonia develops in several days, in this model acute pneumonia develops within 6-24 hours. Both the inoculum size and the character of inflammation that develops upon the bacterial challenge may not adequately reflect the clinical situation.

Granulocyte colony-stimulating factor (G-CSF) is a cytokine produced by monocytes/macrophages that has gained much attention as potential adjunct therapy in patients with pneumonia. G-CSF is produced locally at the site of infection during pneumonia (48) and offers a couple of attractive properties: beside its role as hematopoietic growth factor, it has been shown to enhance the recruitment of PMNs to the lungs during pneumonia *in vivo* and to improve phagocytic and bactericidal activity of PMNs *in vitro* (49, 50). In addition, G-CSF exerts anti-inflammatory properties by stimulating the production of mediators such as IL-1 receptor antagonist (IL-1ra), soluble TNF receptor (sTNF-R) I and II

and IL-10, and by attenuating the release of pro-inflammatory mediators like IL-8, macrophage inflammatory protein-2 (MIP-2), TNF-α, IL-1β, IL-18 and interferon-γ (51). In pneumococcal pneumonia models, a survival benefit has been described in G-CSF treated rats and splenectomized mice (52). Another study only found beneficial effects of adjunct G-CSF when mice were infected with a low bacterial dose (53). We recently investigated the role of endogenous G-CSF in murine *S. pneumoniae* pneumonia and could demonstrate that G-CSF moderately contributed to the activation of PMNs and down-regulated TNF, IL-1 and KC production (48). However, blocking endogenous G-CSF with antiserum did not influence bacterial outgrowth or survival in mice. This finding is in line with studies in humans that so far failed to reveal beneficial effects of adjunct G-CSF therapy in CAP (54, 55). In experimental *K. pneumoniae* pneumonia, treating mice with G-CSF even showed detrimental effects (56). Due to a direct interaction of G-CSF with bacteria that led to increased capsular polysaccharide production, G-CSF treatment promoted bacterial replication in the liver and spleen (56). However, the possibility exists, that adjunct G-CSF might be a helpful immunomodulatory therapy in models of systemic inflammation and pulmonary infection. Indeed, G-CSF administered to rats with systemic inflammation induced by cecal ligation puncture (CLP) followed by *P. aeruginosa* pneumonia improved lung bacterial clearance (57).

The role of CD14 and Toll-like Receptors in pulmonary host response

The primary challenge to the innate immune system is the recognition and discrimination of potential pathogens from self. Toll-like receptors (TLRs) are key molecules that recognize pathogen associated molecular patterns (PAMPs) and induce an inflammatory response (58). Out of the eleven described TLRs, TLR2 and 4 are of great importance in bacterial infections and have been studied in pneumonia models. TLR4 recognizes lipopolysaccharide (LPS) of Gram-negative bacteria and subsequently induces an inflammatory response. Accordingly, mice lacking a functional TLR4 displayed an impaired anti-bacterial defense against the Gram-negative pathogen *K. pneumoniae* (59). As a consequence, mortality during *K. pneumoniae* pneumonia was significantly higher in TLR4-mutant mice than controls (59). Since the Gram-positive PAMPs lipoteichoic acid, lipoproteins and peptidoglycan are all ligands of TLR2 we recently investigated the role of TLR2 in pneumococcal pneumonia. To our surprise, TLR2 played a limited role during this Gram-positive infection. Mice lacking TLR2 had lower chemokine concentrations in lung tissue together with a reduced pulmonary PMN influx, but cytokine levels, bacterial outgrowth and survival did not differ when compared to wild-type animals (Fig. 3) (60). This finding can be explained by another report that revealed pneumolysin, one of the crucial virulence factors of *S. pneumoniae*, as a TLR4 ligand (61). The recognition of pneumolysin by TLR4 has been demonstrated to correlate with the invasiveness of pneumococcal disease *in vivo* (61). In addition, a moderately impaired survival and increased bacterial outgrowth was found in TLR4-mutant mice with invasive pneumococcal pneumonia (59).

Data on the role of single TLRs during *P. aeruginosa* pneumonia are currently not available. However, mice lacking the common TLR-adaptor protein myeloid differentiation factor (MyD) 88 rapidly died when infected with *P. aeruginosa* (62). In the absence of MyD88 these mice were incapable of mounting an inflammatory response or control bacterial replication (62). Beside its role in TLR signaling, MyD88 also links IL-1 and IL-18 receptor associated inflammatory responses. However, since both IL-1 and IL-18 deficiency were associated with improved bacterial clearance in *P. aeruginosa* infections (45, 63), the impaired host response found in MyD88 deficient mice most likely results from defects in TLR signaling. *P. aeruginosa* contains diverse TLR ligands such as LPS (TLR4), lipoproteins (TLR2), flagellin (TLR5) and CpG DNA (TLR9) that might – together – induce an appropriate immune response against this pathogen.

The role of CD14, the cellular LPS receptor that facilitates the interaction with TLR4, has also been investigated in pneumonia models. While neutralizing CD14 in systemic endotoxemia in humans proved beneficial, CD14 contributes substantially to the local host defense in Gram-negative pneumonia (64). Mice infected with a low number of virulent *K. pneumoniae* died rapidly due to overwhelming infection when pre-treated with a neutralizing Ab against CD14 (65). The same Ab had no impact on high dose infection with *K. pneumoniae* or *Escherichia coli* pneumonia (both high and low dose). Another study investigated the role of CD14 in rabbits that were infected intratracheally with *E. coli*. Although anti-CD14 improved the mean arterial pressure, these animals died of an increased bacterial burden (66). Together, although the blockade of CD14 improved systemic side effects of Gram-negative infection, the simultaneously impaired bacterial clearance proved to be harmful during pneumonia.

The influence of pre-existing inflammation

In animals and humans it has been demonstrated that during severe inflammation, such as sepsis, the immune response follows a biphasic pattern: the initial hyper-inflammatory phase is counterbalanced by a subsequent anti-inflammatory response. Sepsis is therefore characterized by the imbalance of the pro- and anti-inflammatory response. The overwhelming pro-inflammatory response leads to tissue injury while excessive activation of anti-inflammatory pathways results in impaired pulmonary host defense. Potential mechanisms of the impaired pulmonary host defense during systemic inflammation include dysfunctional mononuclear cells, apoptosis of immune cells, and the production of anti-inflammatory cytokines, such as IL-10 (67, 68)

Very few investigators attempted to combine systemic inflammation and pneumonia in one model: in a very interesting study, sepsis (induced by CLP) and *P. aeruginosa* pneumonia have been combined (69). The authors nicely demonstrate that a pre-existing systemic inflammation impairs the pulmonary host defense against *P. aeruginosa* (69). Mice that underwent sublethal CLP were more susceptible to *P. aeruginosa* pneumonia than sham-operated or normal animals. Preceding CLP was associated with increased pulmonary IL-10 expression and the administration of neutralizing anti-IL-10 antibodies improved bacterial

clearance and reduced mortality (69). Likewise, adenoviral mediated transgenic expression of TNF in the lungs could also reverse the sepsis-induced impairment in pulmonary host response (70). A very similar study published recently confirmed and extended these findings by demonstrating decreased interferon (IFN)-γ and IL-12 production in mice that underwent CLP before induction of *P. aeruginosa* pneumonia (71). The importance of IFN-γ in sepsis-induced immuno-suppression has also been investigated in a model of stroke (72). Preceding stroke made mice highly susceptible to bacterial infections, predominately within the lungs, which was related to impaired IFN-γ synthesis. Prass and coauthors could illustrate that the sympathetic nervous system mediates immunosuppressive pathways that lead to defects in the early lymphocyte activation after stroke. An earlier study showed that downregulated HLA-DR expression on monocytes can be regarded a valuable marker of the compensatory anti-inflammatory response syndrome (73). Importantly, administration of IFN- γ to septic patients not only restored the levels of HLA-DR expression but also re-established the capacity of monocytes to produce IL-6 and TNF (73).

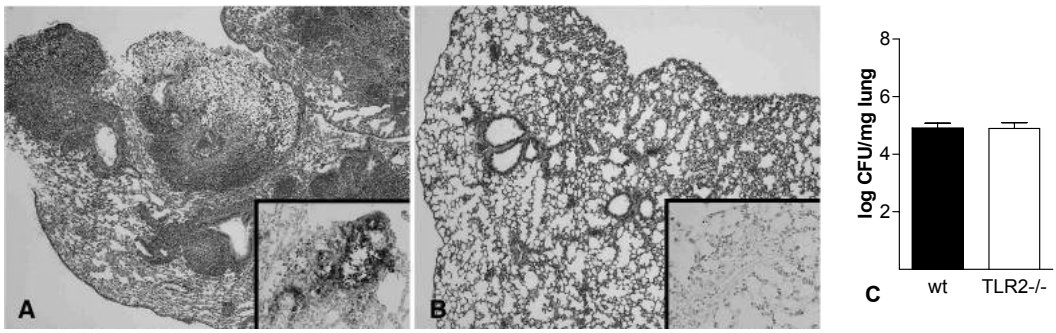

Fig. 4: Impaired PMN recruitment but unaltered bacterial outgrowth in TLR2 deficient mice during pneumococcal pneumonia. Representative lung histology of wt (**A**) and TLR2 deficient (**B**) mice 48 h after infection with 10^4 CFU *S. pneumoniae* showing significantly more inflammation in wt mice compared to TLR2 deficient animals. The insets are representative pictures of immunostaining for granulocytes, confirming the dense granulocytic infiltration in wt mice. The histological lung sections are representative for at least 5 mice per group. H&E staining: magnification x 4; insert (Ly-6 staining): magnification x 20. **C**: Lung bacterial outgrowth in mice infected with S. pneumoniae 48h after infection. Mean ± SEM of 7 mice per strain. Adapted and modified from reference (60).

Our laboratory performed a series of experiments in which *P. aeruginosa* pneumonia was preceded by an acute phase response (induced by subcutaneous turpentine injections) (Renckens et al., unpublished data). Turpentine injection into the limb causes local abscesses and an acute phase reaction, which peaks after two days. This model was used to mimic profound acute phase protein responses as seen in trauma and post-surgical patients. Pulmonary clearance of *P. aeruginosa* was impaired in turpentine-injected mice, compared with controls. Further, bacteremia was more frequent in the turpentine-injected animals. Thus, during an acute phase response mice have a significantly impaired host defense during *P. aeruginosa* pneumonia. These studies clearly demonstrate that local pulmonary host defense during respiratory tract infection is influenced by sepsis, or sepsis-like syndromes. Further

studies are necessary to see whether a strategy aimed at restoration of mononuclear functions in the anti-inflammatory state of sepsis is beneficial during secondary respiratory infections.

Conclusion

These data suggest that in more gradually developing pneumonias, such as caused by *S. pneumoniae* and *K. pneumoniae*, a certain pro-inflammatory cytokine response within the pulmonary compartment is required to combat the invading micro-organism, while in a more acute form of pneumonia, such as caused by *P. aeruginosa*, an excessive inflammatory response contributes to an adverse outcome. On the other hand, the activation of anti-inflammatory mechanisms in clinically relevant situations with a pre-existing systemic inflammation due to e.g. peritonitis, stroke or sterile acute phase response, likely is responsible for the impaired pulmonary host defense against *P. aeruginosa*.

Based on the results of these studies we conclude that inflammatory processes "at a distance" influence pulmonary host defense mechanisms. Indeed, the actual immune status may be of importance in the decision to stimulate or to dampen inflammatory responses during pneumonia.

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CHAPTER 3

Alveolar Macrophages have a Protective Anti-Inflammatory Role during Murine Pneumococcal Pneumonia

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Abstract

Alveolar macrophages (AM) are considered major effector cells in host defense against respiratory tract infections by virtue of their potent phagocytic properties. In addition, AM may regulate the host inflammatory response to infection by production of cytokines and by their capacity to phagocytose apoptotic polymorphonuclear cells (PMN). To elucidate the *in vivo* contribution of AM to host defense against pneumococcal pneumonia we depleted mice of AM via pulmonary application of liposomal dichloromethylene-bisphosphonate (AM^-mice) before inoculation with *Streptococcus pneumoniae*; controls received saline (AM^+sal) or liposomal PBS (AM^+lip) before bacterial inoculation. AM^-mice displayed a significantly higher mortality compared to AM^+controls whereas bacterial clearance did not differ. Poor outcome of AM^-mice was accompanied by a pronounced increase of local pro-inflammatory cytokine production as well as strongly elevated and prolonged pulmonary PMN accumulation. Closer examination of infiltrating PMN in AM^-mice disclosed high proportions of apoptotic and secondary necrotic cells reflecting the lack of efficient clearance mechanisms in the absence of AM. Furthermore, caspase-3 staining showed only slightly higher activity in AM^-mice, arguing against accelerated apoptosis per se. These data suggest that AM are indispensable in the host response to pneumococcal pneumonia by means of their capacity to modulate inflammation possibly via elimination of apoptotic PMN.

Introduction

Streptococcus pneumoniae is a leading causative pathogen in community-acquired pneumonia (1-3). Despite adequate antimicrobial therapy, pneumococcal pneumonia remains a major cause of morbidity and mortality. Furthermore, with an increasing incidence of antibiotic resistance in this pathogen, there is an urgent need to expand our knowledge of the pathogenic and host defense mechanisms that influence outcome in *S. pneumoniae* pneumonia (2, 4).

Alveolar macrophages (AM), located at the interphase between air and lung tissue, provide the first line of cellular defense against microbes (5, 6). Most *in vivo* data concerning the role of AM in pulmonary host defense come from studies in which AM were depleted by administration of liposome-encapsulated dichloromethylene bisphosphonate (Cl_2MBP) to the pulmonary tract. In this way the role of AM as phagocytes has been demonstrated in mice challenged with *Pseudomonas aeruginosa* or *Klebsiella pneumoniae*. Mice lacking AM showed a delayed and impaired bacterial clearance as compared to control mice (7, 8), although conflicting data exist about the phagocytic properties of AM in a mouse model with unopsonized *P. aeruginosa* (9). In sharp contrast to uncapsulated bacteria, *S. pneumoniae* are known to bind poorly to macrophages without prior opsonization (10-12). Moreover, mouse AM show minimal expression of receptors for C3b/iC3b (CR1/CR3/CR4) (13), indicating that AM use other, nonopsonic phagocytosis mechanisms. Alternatively, the contribution of AM to pulmonary host defense in *S. pneumoniae* pneumonia may rely on different, as yet unidentified mechanisms.

Until recently, most investigations dealing with pneumonia concentrated on the onset of inflammation. Within the past few years more effort has been made to study mechanisms responsible for the resolution of inflammation and restitution of tissue homeostasis. Through this it has become evident that persistent inflammation, leading to tissue injury and organ malfunction, may not only be due to prolonged proinflammatory events but may equally likely arise from inefficient resolution processes (14). AM have been implicated as major effector cells in this resolution process, mainly by phagocytosing apoptotic PMN (15). The rapid elimination of extravasated, apoptotic PMN by AM may provide an injury-limiting mechanism since the membrane of PMN remain intact, preventing potential injurious granule contents from being released. AM can therefore be regarded as major modulators of pulmonary host defense. They readily phagocytose and eliminate certain inhaled pathogens and act as effector cells in the equally important resolution process (14, 15).

To our knowledge the proposed dual function of AM, facilitating both the clearance of bacteria and the resolution of the ensuing inflammatory response in the pulmonary compartment, has never been investigated directly in a model of bacterial pneumonia. We therefore sought to obtain insight into the *in vivo* contribution of AM to the host response to pneumococcal pneumonia in a well-established animal model. For this purpose, mice were depleted of AM by administration of liposomal Cl_2MBP prior to intranasal (i.n.) infection with *S. pneumoniae*. Intranasal Cl_2MBP administration selectively depletes AM without

damaging other cells (16). We thereby investigated the traditional role of AM as phagocytes as well as their contribution to resolution processes.

Materials and Methods

Mice

Pathogen-free 6-8 wk-old female BALB/c mice were obtained from Harlan Sprague-Dawley (Horst, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam (The Netherlands) approved all experiments.

In vivo AM depletion

Cl_2MBP was a gift from Roche Diagnostics (Mannheim, Germany). Preparation of liposomes containing Cl_2MBP was performed as described previously (16). 100µl Cl_2MBP was administered intranasally (i.n.) (AM^- mice) 48 hours prior to bacterial inoculation according to methods described by our laboratory previously (17). Controls received liposomal PBS or saline, respectively.

Induction of pneumonia

Pneumonia was induced as described previously (18-20). *S. pneumoniae* serotype 3 was obtained from American Type Culture Collection (ATCC 6303; Rockville, MD) and 50 µl (approximately $5x10^4$ CFU) were inoculated intranasally.

Preparation of lung homogenates

At 20 and 44hours after infection, mice were anesthetized and sacrificed - whole lungs were homogenized for CFU determination and for cytokine measurements exactly as described previously (17-20). Cytokines and chemokines were measured using specific ELISAs (R&D Systems, Minneapolis, MN) according to the manufacturers` instructions. The detection limits were 31 pg/ml for tumor necrosis factor-α (TNF-α) and Interleukin (IL-) 10, 12 pg/ml for the mouse chemokine KC and 8 pg/ml for IL-1β.

FACS analysis of BALF and lung suspension

Bronchoalveolar Lavage (BAL) and leukocyte differentiation was done as described previously (17, 18, 21). Pulmonary cell suspensions were obtained using an automated disaggregation device (Medimachine-System; Dako, Glostrup, Denmark) exactly as described previously (17, 20). For FACS analysis of apoptotic PMN, BALF and lung cells were gated for PMN by forward and side scatter and stained with Annexin-V PE and 7-Amino-Actinomycin D (7-AAD). In these experiments, BALF and lung suspension cells were obtained from the same mice in order to simultaneously address both alveolar and interstitial lung compartments. Annexin V positive and 7-AAD negative cells were considered apoptotic, double positive PMN as necrotic (22). To correct for aspecific staining, an appropriate

isotype-control Ab was used. Staining was performed in the presence of 20% normal mouse serum to block nonspecific binding to FcγR. All reagents were purchased from Pharmingen (San Diego, CA) and used in concentrations recommended by the manufacturer. Samples were analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA).

Histology

Lungs for histology were fixed in 4% formaline, embedded in paraffin and 4μm sections were stained with hematoxylin and eosin (H&E). Granulocyte staining was done as described previously (19, 21). To detect apoptotic bodies, deparaffinized slides were boiled in citrate buffer (pH 6.0). After blocking of non-specific binding and endogenous peroxidase activity, slides were incubated with rabbit anti-human active caspase-3 polyclonal antibody (Cell Signaling, Beverly, MA) followed by biotinylated swine anti-rabbit antibody (Dako, Glostrup, Denmark) and further revealed as described for granulocyte staining. Mucosa associated lymphatic tissue of mice treated with Staphylococcal enterotoxin B for 8 hours (known to strongly induce apoptosis) served as positive control in these stainings. All antibodies were used in concentrations recommended by the manufacturers`.

Myeloperoxidase (MPO) assay

MPO-activity was determined by measuring the H_2O_2-dependent oxidation of 3,3`5,5`tetramethylbensidine (TMB) as described previously (23). MPO-activity is expressed as activity/gram lung-tissue/reaction-time. All reagents were purchased from Sigma (St.Louis, MO).

Statistical analysis

Differences between groups were calculated by Mann-Whitney *U* test. For survival analyses, Kaplan-Meier analysis followed by log rank test was performed. Values are expressed as mean ± SEM. A p-value ≤ 0.05 was considered statistically significant.

Results

Depletion of AM by Cl_2MBP

We and others have previously demonstrated that the intrapulmonary delivery of liposomal Cl_2MBP results in a selective depletion of AM in BALF (7, 8, 16, 17, 24, 25). To confirm this finding for the present series of experiments we administered saline (AM^+sal), liposomal PBS (AM^+lip) or liposomal Cl_2MBP (AM^-) intranasally (n=6 per group) and determined the number of AM in BALF 48 hours later (i.e. at the time point mice were designated to receive *S. pneumoniae*). Cl_2MBP led to a depletion of 74±1.4 % of AM relative to AM^+sal mice ($p<0.05$). The number of AM in AM^+lip mice did not differ from that in AM^+sal mice (data not shown).

AM$^-$ mice are more susceptible to *S. pneumoniae* pneumonia

To determine the role of AM in host defense against pneumonia *in vivo*, we first assessed the survival of mice depleted of AM (AM$^-$) and control mice (AM$^+$sal or AM$^+$lip) after i.n. inoculation with 5x10^4 *S. pneumoniae* CFU. All AM$^-$ mice died within 5 days after the bacterial challenge, whereas 33% (4/12) of AM$^+$lip (p=0.04 versus AM$^-$) and 50% (6/12) of AM$^+$sal mice (p=0.001 versus AM$^-$) survived (Fig.1). All mice surviving 5 days appeared to no longer be clinically ill.

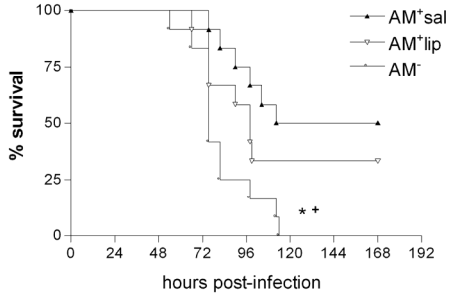

Fig. 1: Increased mortality of AM$^-$ mice following *S. pneumoniae* infection. BALB/c mice (n=12 per group) were i.n. administered with Cl_2MBP (AM$^-$), liposomes (AM$^+$lip) or saline (AM$^+$sal) 48 hours prior to bacterial challenge with 5x10^4 CFUs *S. pneumoniae*. $^+$ $p \leq 0.05$ AM$^-$mice vs AM$^+$lip, * $p \leq 0.05$ AM$^-$ vs AM$^+$sal.

Increased mortality of AM$^-$ mice is not related to an impaired bacterial clearance

Since AM are considered an important first line phagocytic defense against inhaled pathogens, we next sought to investigate whether the increased lethality in AM$^-$ mice was related to an impaired clearance of *S. pneumoniae*. Both at 20 and 44 hours post inoculation with *S. pneumoniae* the number of CFUs in lungs was similar in all three groups (Fig. 2A). Of interest, the lowest bacterial outgrowth at 44 hours was found in AM$^-$ mice, although the difference with the two control groups did not reach statistical significance

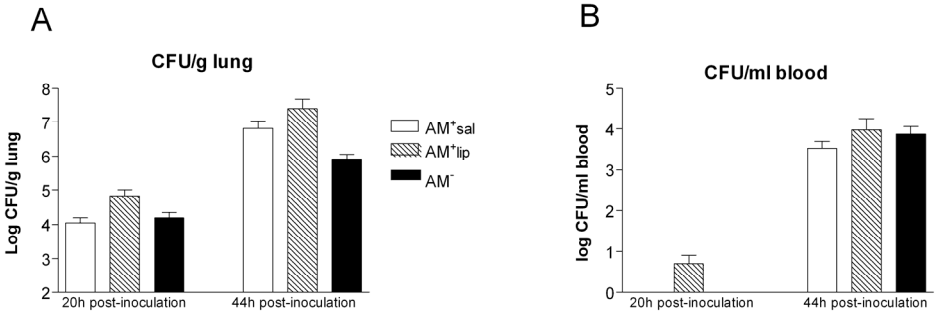

Fig. 2: AM depletion does not influence the outgrowth of pneumococci. Similar bacterial outgrowth in lungs (A) and blood (B) of AM$^-$ and AM$^+$ mice 20 and 44 hours after inoculation with *S. pneumoniae*. Data are mean ± SEM of eight mice per group at each time point.

In addition, blood from all 3 groups contained the same amount of bacteria at 20 and 44 hours postinoculation (Fig. 2B). Hence, bacterial clearance was not impaired in the absence of AM and therefore can not explain differences in survival.

AM$^-$ mice display an increased inflammatory response within the pulmonary compartment

Neutrophilic influx to the lungs as well as the production of proinflammatory cytokines and CXC-chemokines are regarded as major host defense mechanisms in bacterial pneumonia (26, 27). AM are thought to play a key role in the initial recruitment of PMN through their capacity to secrete many of these proinflammatory mediators. We therefore investigated these early host responses to obtain insight in the mechanism by which AM$^-$ mice are more susceptible to pneumococcal pneumonia. The highest level of pulmonary cell recruitment was found in AM$^-$ mice (Table 1). Differential counts of whole lung cell suspensions displayed an impressive predominance of PMN.

Lung	Cells/ml x10^4	PMN (%)	Macrophage (%)	Lymphocyte (%)
20 h				
AM+ sal	150 ± 29	39 ± 5	35 ± 2	27 ± 5
AM+ lip	378 ± 108 *	59 ± 6 *	31 ± 3	10 ± 4 *
AM-	454 ± 95 *	86 ± 2 *†	9 ± 1*†	5 ± 1 *
44 h				
AM+ sal	633 ± 151	27 ± 6	44 ± 3	29 ± 4
AM+ lip	1285 ± 187 *	39 ± 4	42 ± 3	19 ± 2
AM-	2985 ± 422 *†	65 ± 4 *†	26 ± 3 *†	10 ± 2 *†

Table 1: Cellular Composition of Lungs. Data are mean ± SEM of 8 mice per group for each timepoint. Mice received 5x10^4 CFU *S. pneumoniae* i.n. at t=0, preceeded (-48 hours) by i.n. saline (AM$^+$sal), liposomes (AM$^+$lip) or Cl_2MBP (AM$^-$). Cell counts and differentials were done on whole lung suspensions as described in the Methods. * $p < 0.05$ AM$^-$ vs AM$^+$sal, † $p < 0.05$ AM$^-$mice vs AM$^+$lip.

This observation was confirmed by elevated MPO-activity in lungs of AM$^-$ mice (p=0.0002 versus AM$^+$sal and p=0.04 versus AM$^+$lip) and a profoundly elevated PMN number in BALF of AM$^-$ mice (p=0.002 versus AM$^+$sal and p=0.002 versus AM$^+$lip) (Fig. 3).

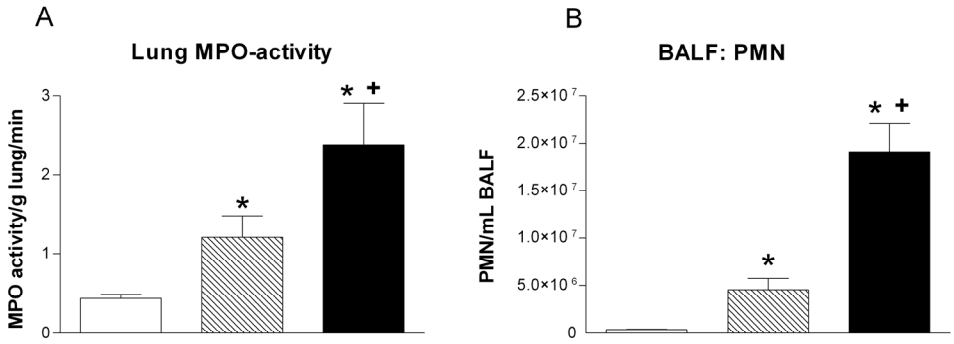

Fig. 3:. **Increased pulmonary PMN influx in AM$^-$ mice. A:** Increased MPO activity in lungs of AM$^-$ mice 44 hours after bacterial challenge. **B:** PMN counts obtained in BALF 44 hours post inoculation with *S. pneumoniae*. Data are from eight mice per group (mean ± SEM); $^+$ $p \leq 0.05$ AM$^-$mice vs. AM$^+$lip, * $p \leq 0.05$ AM$^-$ vs. AM$^+$sal.

Forty-four hours after *S. pneumoniae* inoculation both groups of AM^+ control mice had a predominantly interstitial pulmonary infiltrate (Fig. 4A and C), composed of monocytes, lymphocytes and only few granulocytes (Fig 4B and D) compatible with the clearance phase of the infection. In sharp contrast, the lungs of AM^- mice displayed dense and diffuse inflammatory infiltrates with focal destruction of the lung parenchyma (Fig 4E). These inflammatory infiltrates were mostly composed of PMN as demonstrated by immunostaining with the mouse granulocyte marker Ly-6G (Fig 4F).

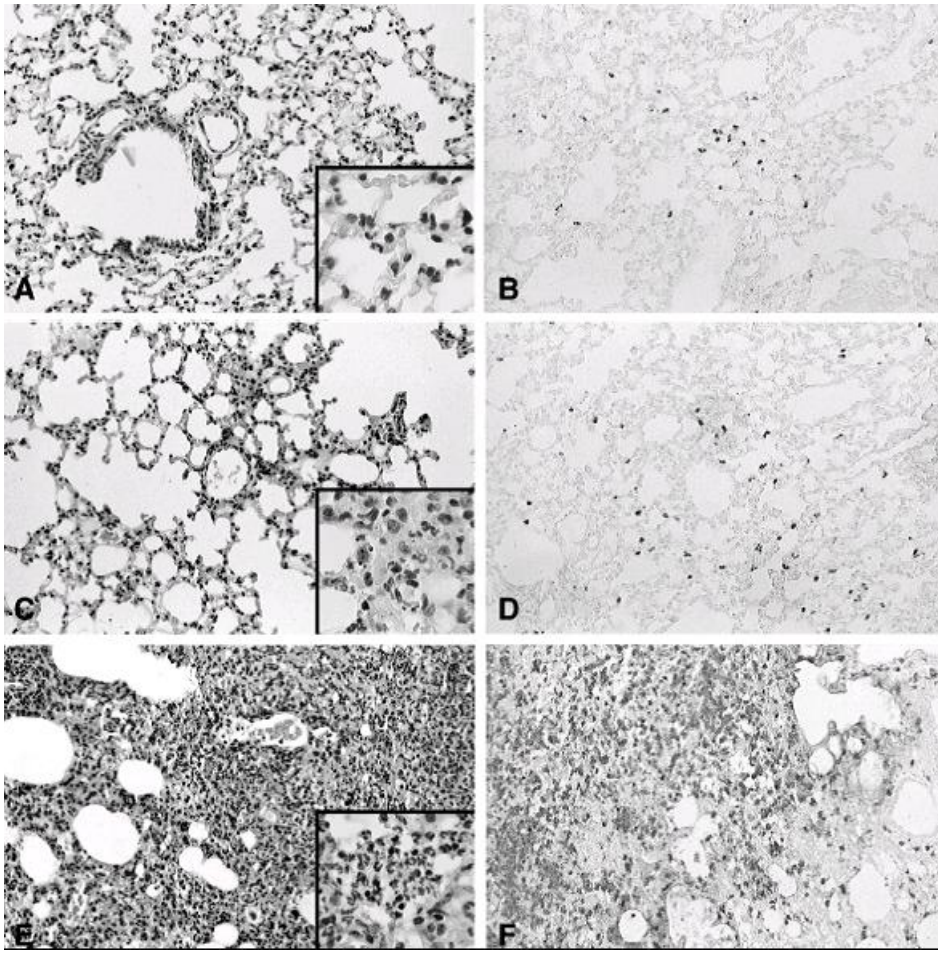

Fig. 4: Histopathology at 44 hours after infection. Both lungs of AM^+ mice showed a histological picture compatible with the clearance phase of the pneumonia characterized by a slight interstitial influx, mostly composed of monocytes (**A:** AM^+sal and **C:** AM^+lip, H&E staining, x 50) with sparse granulocytes as shown in the higher magnified inserts (A and C, x 400) and by immunostaining with Ly-6G (**B:** AM^+sal and **D:** AM^+lip, anti-Ly-6G immunostaining, x 50). In contrast, lungs of AM^- mice displayed diffuse and dense inflammatory infiltrates with effacement of the lung parenchyma (**E:** AM^-, H&E staining, x 50). As shown in the insert (E, x 400) and on panel **F**, granulocytes were the predominant cell type in this process (anti-Ly-6G immunostaining, x 50).

This increased PMN influx in AM^- mice was accompanied by an early and marked increase in pulmonary TNFα, IL-1β and KC levels, whereas in both AM^+ control groups a less impressive and less sustained elevation in these cytokines was found (Fig. 5).

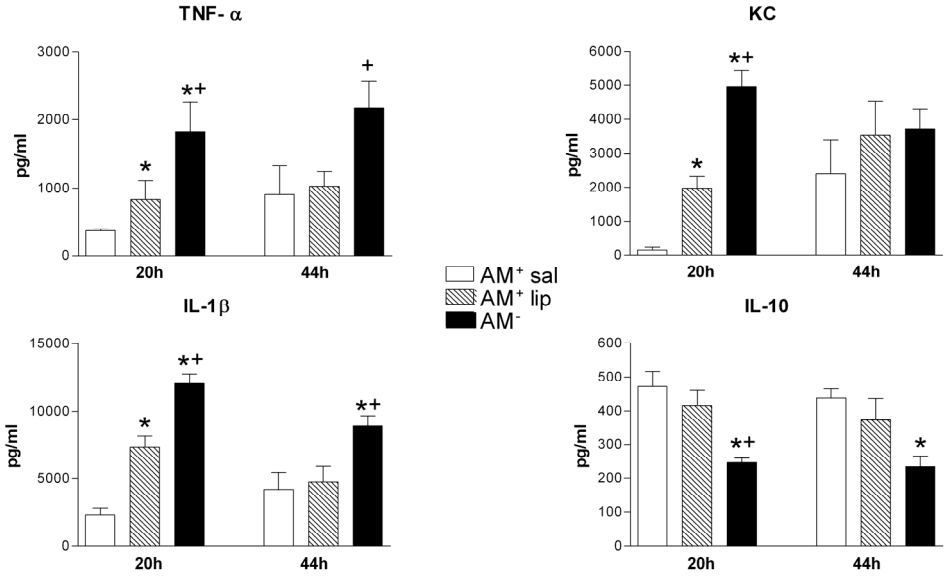

Fig. 5: Pro-inflammatory cytokines predominate in AM$^-$ mice. Elevated lung concentrations of the pro-inflammatory mediators TNF-α, IL-1β and KC and reduced pulmonary IL-10 levels in AM$^-$mice at 20 and 44 hours postinfection. Data represent mean ± SEM of eight mice per time point and group; $^+$ $p \leq 0.05$ AM$^-$mice vs AM$^+$lip, * $p \leq 0.05$ AM$^-$ compared to AM$^+$sal.

Interestingly, lung concentrations of the anti-inflammatory cytokine IL-10 were significantly lower in AM$^-$ mice than in AM$^+$ mice. Theoretically the administration of Cl_2MBP could have been responsible for the increased inflammatory response in AM$^-$ mice, rather than the infection with *S. pneumoniae*. Therefore, we treated mice (n=6 per group) with liposomal Cl_2MBP, liposomal PBS or saline at 48 hours before i.n. administration of sterile saline (instead of *S. pneumoniae*). At 44 hours after saline administration, lung MPO and TNF-α levels, and histopathology were similar in the three groups, and not different from normal control mice (data not shown). Together these data suggest that *S. pneumoniae* induces an exaggerated inflammatory response in AM$^-$ mice, which likely accounts for the increased mortality in these animals.

AM-deficiency impedes the clearance of apoptotic neutrophils

The deleterious effects of AM depletion in mice with pneumonia seemed to result from prolonged and exaggerated inflammation rather than from impaired bacterial clearance. Apart from participating in the first line of defense, AM have been implicated in the resolution of inflammation due to their ability to phagocytose and degrade PMN that are undergoing apoptosis (14, 15). Indeed, in BALF cytospins of AM$^+$ mice with moderate persistent pulmonary inflammation 44 hours post inoculation, we not only found apoptotic PMN but also AM with phagocytosed apoptotic bodies (Fig. 6).

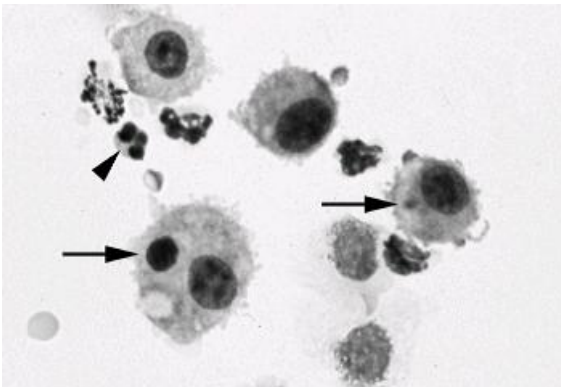

Fig. 6: AM phagocytose apoptotic PMN. Cytospin preparation (Giemsa staining) of a BALF specimen of AM^+ mouse showing PMN undergoing apoptosis (triangle) and phagocytosed apoptotic bodies within the cytoplasm of AM (arrows).

These data indicate that the depletion of AM may impair physiological repair mechanisms including elimination of apoptotic PMN, thereby promoting prolonged inflammation. If this is the case, an increase of apoptotic and/or secondary necrotic PMN should be detectable in lungs of these mice. We therefore investigated the proportion of apoptotic and necrotic PMN in BALF and whole lung suspensions. Among the elevated number of PMN, we indeed found higher proportions of apoptotic (Annexin V positive, 7-AAD negative) PMN in BALF and lung suspensions of AM^- mice (Fig. 7).

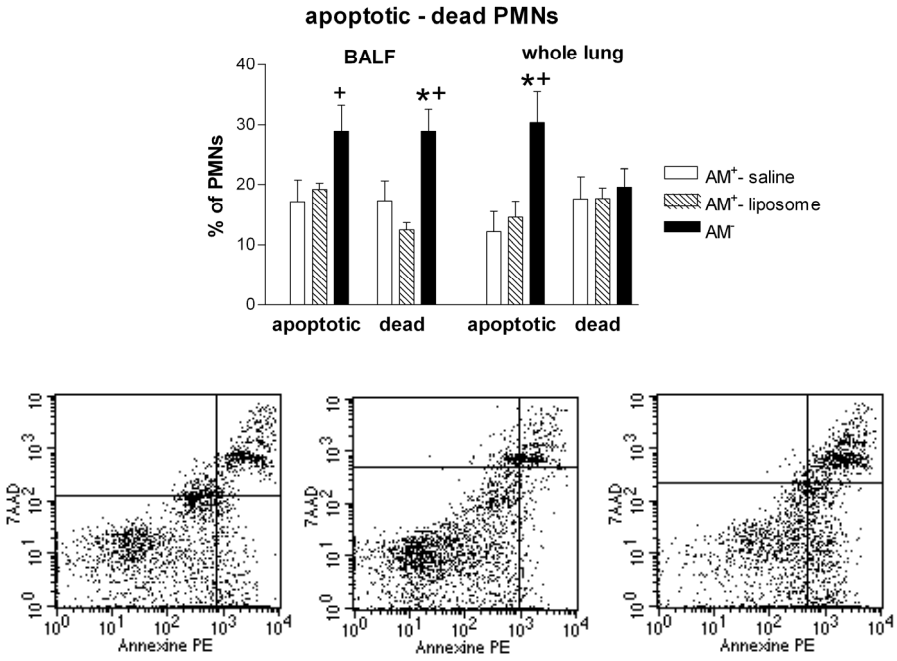

Fig. 7: Accumulation of apoptotic and dead PMN in AM^- mice. top: BALF and lung suspension obtained 44 hours post bacterial inoculation were analyzed for apoptotic and dead PMN by FACS as described in the Methods. Annexin V/7-AAD double positive PMN were considered "dead", single Annexin V positive PMN "apoptotic". Representative FACS plots of lung suspension samples are shown in the bottom part of the Fig. – quadrants were set according to respective isotype controls. Data are mean ± SEM of eight mice per group; $^+ p \leq 0.05$ AM^-mice vs AM^+lip, $* p \leq 0.05$ AM^- compared to AM^+sal.

Moreover, the percentage of dead (Annexin V and 7-AAD double-positive) PMN in BALF was significantly higher in AM^- mice as compared to AM^+lip and AM^+sal controls. This was confirmed by histological analysis of AM^- lungs, showing at higher magnification large numbers of shrunken cells, cellular debris and pyknotic nuclei characteristic of apoptotic bodies (Fig. 8A).

Caspase-3 gets activated during the early stages of apoptosis and is involved in the subsequent disassembly of the cell. Active caspase-3 is therefore regarded as an early marker of cells undergoing apoptosis (28). In sharp contrast to the tremendous amount of characteristic apoptotic bodies seen by conventional H&E staining in AM^- mice, anti-active caspase-3 immunostaining of lung specimens showed only few positive cells (Fig. 8B). Moreover, these mice displayed only slightly elevated active caspase-3 activity compared to both AM^+ controls (Fig. 8C and D). Therefore, the increase in apoptotic cells in AM^- mice is not due to an increase in apoptosis, further supporting the theory that it is due to an imbalance between apoptosis and rapid elimination.

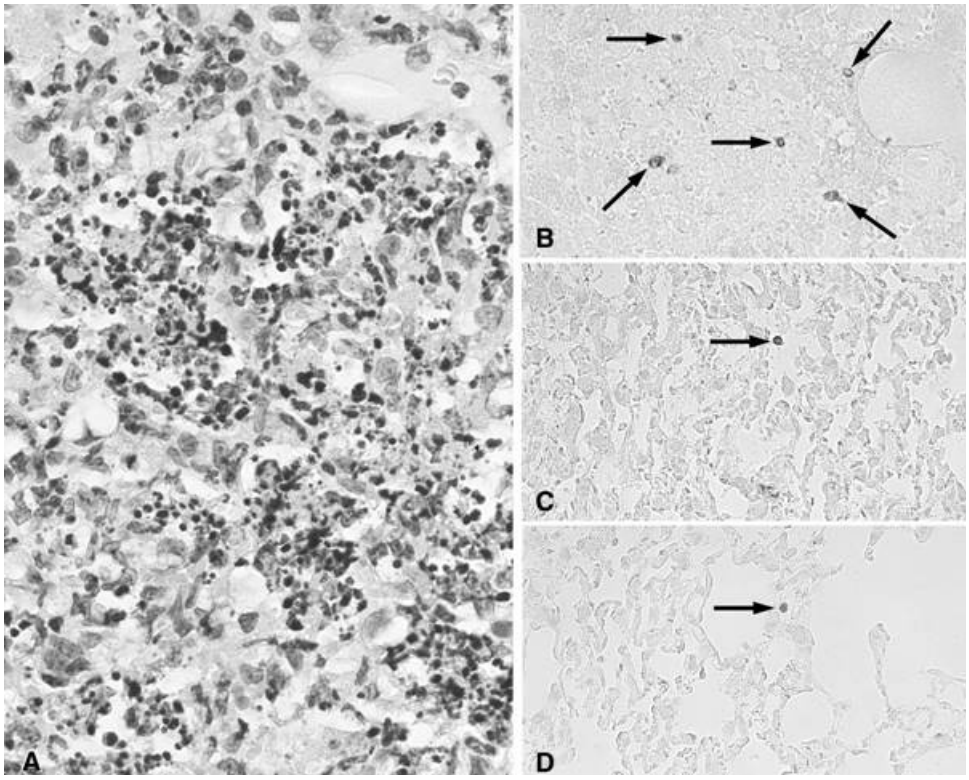

Fig. 8:. **Many apoptotic bodies but low caspase-3 activity.** Close-up on the inflammation observed in AM^- lungs 44 hours after infection showing large amounts of pyknotic nuclei, cellular debris and shrunken cells, compatible with apoptotic bodies (**A:** H&E staining, x 160). Immunostaining for active caspase-3 showed a discrepancy between the amount of apoptotic bodies and the active caspase-3 activity in the lungs of AM^- mice (**B:** x 60). Moreover, given the 10-fold higher number of PMN in lungs of AM^- mice, active caspase-3 activity seemed to be only marginally higher than in both control AM^+ groups (**C:** AM^+sal and **D:** AM^+lip, x 60). Arrows indicate caspase-3 positive cells.

Discussion

AM are considered major effector cells of innate immunity, capable of participating in both the initiation and resolution process of pulmonary inflammation (14). Previous publications have reported the importance of AM in the rapid elimination of Gram-negative pathogens like *P. aeruginosa* and *K. pneumoniae* from the respiratory tract (7, 8), whereas others have emphasized the role of AM in early PMN recruitment to the alveolar space (24). Together these reports concentrated on the role of AM in initiating and orchestrating the immediate pulmonary host defense against invading pathogens. So far, however, evidence for the equally important regulatory role of AM in the resolution process comes from *in vitro* experiments or *in vivo* studies with intact AM using oleic acid, ozone or LPS (15, 29, 30). Therefore, to examine the contribution of AM to resolution of pneumonia, we used a direct approach and depleted mice of AM before infection with *S. pneumoniae* serotype 3.

In the present study we demonstrate the importance of AM as major effector cells in the resolution process of pneumococcal pneumonia *in vivo*. The lack of AM led to overwhelming inflammation and an insufficient clearance of apoptotic PMN, which was associated with an increased lethality. The classical role of AM as phagocytes of invading *S. pneumoniae* seems to be of less importance, as illustrated by unaltered bacterial outgrowth in AM^- mice.

AM^- mice exhibited a pronounced and prolonged influx of PMN within the alveoli and interstitial space. This finding is consistent with previous reports on Gram-negative pneumonia. However, in contrast to our observations of an unaltered bacterial clearance, these earlier investigations found increased bacterial loads in AM^- animals and the authors apparently contributed the prolonged influx of PMN to the more extensive proinflammatory stimulus provided by the increased bacterial burden (7, 8). We further examined these pulmonary infiltrates and revealed a high proportion of apoptotic or dead PMN. Apoptosis, the process of programmed cell death, is believed to play a major regulatory role in the inflammatory response as invading PMN undergo apoptosis and are readily phagocytosed by surrounding AM. This process prevents the release of potentially toxic or immunogenic intracellular contents and thereby allows an injury-limiting elimination of potentially harmful PMN (14). Thus, the higher number of apoptotic and secondary necrotic PMN we found in AM^- mice is very likely the result of inefficiency of the normal resolution process in the absence of AM, thereby tipping the balance toward persistent inflammation and tissue injury.

It is interesting to note that apoptosis of PMN is known to be delayed in both systemic and local inflammation, including pneumonia and ARDS (31-33). This increased PMN longevity has been attributed to factors like GM-CSF, G-CSF, IFN-γ and, to some degree, to TNF-α (33-36). In accordance with this, despite the high number of apoptotic/necrotic PMN in lungs of AM^- mice, we could not find signs of accelerated PMN apoptosis itself. In apoptosis, caspase-3 is known as a key effector protease that, once activated by initiator proteases like caspase-8 or 9, irreversibly leads to cell disassembly (28, 37). Immunostaining for active caspase-3 revealed only slightly increased numbers of positive cells in AM^- lungs as

compared to AM^+ mice. This slight elevation of caspase-3 seems almost negligible compared to the enormous number of infiltrating PMN in these mice (Fig. 4).

In addition to the unquestionable importance of rapid elimination of aged PMN, it has been shown that the uptake of apoptotic PMN induces an anti-inflammatory phenotype in macrophages, as it actively inhibits the production of IL-1-β, IL-8 and TNF-α by human monocyte-derived macrophages (38-40). Local cytokine production in mice with intact AM, and therefore effective elimination of senescent PMN, was relatively diminished in parallel with a partial resolution of the inflammatory response. The substantial and prolonged elevation of pro-inflammatory cytokines like TNF-α and IL-1β in AM^- mice is likely to result from ongoing inflammation fuelled by intracellular contents released from necrotic PMN. The potential cellular source of these mediators after depletion of AM can only be speculated upon, but bronchial epithelial cells, alveolar epithelial cells type II as well as interstitial macrophages are well known for their secretory capacity upon stimulation with pro-inflammatory cytokines, LPS or reactive oxygen intermediates (41-44). One might argue that the prolonged and exaggerated inflammation in AM^- mice results from the lack of anti-inflammatory cytokines like IL-10 after AM depletion. We certainly can not rule out this possibility, but the low IL-10 levels found in AM^- mice can not be attributed to the lack of AM itself, since murine AM (including Balb/c) do not produce IL-10 (45). Potential sources of IL-10 in the lungs are interstitial macrophages and T-cells as well as infiltrating alveolar monocytes. We hypothesize that the impaired clearance of apoptotic cells in AM^- mice and the concomitant prolonged inflammation precludes the "switch" of monocytic cells to an anti-inflammatory cytokine production profile – as is known to occur in the resolution phase of inflammation (14, 38). This in turn could explain lower IL-10 levels in AM^- mice.

The exposure of AM to liposome encapsulated Cl_2MBP leads to selective apoptosis of AM. Both *in vivo* and *in vitro* experiments have demonstrated that PMN are morphologically and functionally unaffected by this compound (16). Thus, an effect of Cl_2MBP on PMNs can not explain our findings. In addition, the intra-alveolar inflammatory reaction in response to Cl_2MBP itself is minimal as documented here and in earlier reports from other laboratories (7, 17, 24, 25, 46). Liposomes were used to encapsulate Cl_2MBP to facilitate uptake by macrophages. As controls, we used mice pretreated with saline (AM^+sal) or liposomes (AM^+lip) only. Interestingly both outcome and inflammatory responses in AM^+lip mice ranged between those found in AM^+sal and AM^- mice, indicating that liposomes themselves somewhat impair or influence the functional properties of AM. Indeed, liposomes can reduce the phagocytic and migratory behavior of AM (47). Similar observations have been made in earlier studies (16, 17).

Whole lung suspensions revealed an increased proportion of monocytic cells in all 3 groups 48 hours after bacterial inoculation (Table 1). This observation can be explained by an influx of alveolar and interstitial monocytes, known to reach peak levels 48 hours after induction of inflammation (48). However, in sharp contrast to AM^+ control mice with intact AM, the number of PMN consistently outnumbered monocytic cells in AM^- mice. This persistent

imbalance of the PMN – monocyte/macrophage ratio in AM^- mice most likely accounts for the observed impaired PMN clearance.

Pneumonia remains a leading cause of morbidity and mortality, and *S. pneumoniae* is the most frequently isolated pathogen in community-acquired pneumonia. Using a well established model of murine pneumococcal pneumonia, we demonstrate that the selective depletion of AM results in an exaggerated inflammatory response and enhanced lethality together with an accumulation of apoptotic and necrotic PMN. The clearance of pneumococci from the lungs was not influenced by AM depletion. These data indicate that AM play an essential role in the regulation of the lung inflammatory response during pneumococcal pneumonia.

Acknowledgements/Support/Footnote

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CHAPTER 4

Activation of neutrophils and inhibition of the proinflammatory cytokine response by endogenous granulocyte colony-stimulating factor in murine pneumococcal pneumonia

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Abstract

Granulocyte colony-stimulating factor (G-CSF) is considered to improve host defense during infection via increased recruitment and performance of neutrophils and subsequent inhibition of potentially harmful pro-inflammatory mediators. The present study sought to determine the role of endogenous G-CSF in host defense against pneumococcal pneumonia. Patients with unilateral community acquired pneumonia demonstrated elevated G-CSF concentrations in bronchoalveolar lavage fluid obtained from the infected, but not from the contralateral site. Treatment with an anti-G-CSF antibody of mice with pneumococcal pneumonia tended to reduce neutrophil numbers in lung tissue, diminished CD11b expression on pulmonary neutrophils, but increased the lung concentrations of tumor necrosis factor-α, interleukin-1β and KC. Anti-G-CSF did not influence the outgrowth of pneumococci in lungs, the dissemination of the infection, or survival in murine pneumonia. G-CSF is produced locally at the site of the infection during pneumococcal pneumonia where it exerts both pro- and anti-inflammatory effects.

Introduction

Pneumonia is a leading cause of morbidity and mortality and the most frequent source of infection in severe sepsis [1, 2]. The Gram-positive bacterium *Streptococcus pneumoniae* is responsible for more than 50% of community-acquired pneumonias (CAP) [3-5]. Despite adequate antimicrobial therapy, mortality rates up to 25% have been reported in hospitalized patients with pneumococcal pneumonia [5, 6]. In addition, the growing resistance of *S. pneumoniae* to antibiotics is an issue of worldwide concern [4, 7] that warrants expansion of our knowledge of the pathogenesis and host defense mechanisms in order to identify adjunctive treatment options.

The recruitment of polymorphonuclear cells (PMNs) to the lungs is a crucial component of the host defense in pneumonia. Due to their potent phagocytic properties, PMNs are critical for the elimination of bacteria in the lower respiratory tract [8]. Among host mediators involved in the function and production of PMNs, granulocyte colony-stimulating factor (G-CSF) prominently features. G-CSF is generally known as a hematopoietic growth factor, responsible for proliferation and maturation of bone marrow stem cells to PMNs [9, 10]. In addition, elevated systemic and local G-CSF concentrations are found in patients with infections [11-13]. G-CSF is regarded as a cytokine that is produced by monocytes/macrophages at the site of infection [14]. Within the pulmonary compartment, alveolar macrophages produce G-CSF upon stimulation with lipopolysaccharide (LPS), interleukin (IL)-1 or tumor necrosis factor-α (TNF-α) [15]. Exogenously administered G-CSF has been demonstrated to enhance the recruitment of PMNs in response to pulmonary infection and inflammation in vivo [16, 17]. Beside this increase in chemotaxis [18], improved phagocytic activity, bactericidal function [19] and respiratory burst [20] have been attributed to G-CSF in in vitro systems with isolated human PMNs. Together, G-CSF is considered to act locally, contributing to the recruitment and performance of PMNs at the site of infection, and systemically, stimulating the formation of additional PMNs, thus reinforcing the host response until the infection is resolved [21].

Of note, uncontrolled stimulation of pro-inflammatory pathways and recruitment of PMNs is associated with tissue injury [22]. Prolonged attraction of PMNs, due to release of pulmonary chemokines, is a known risk factor for the development of acute lung injury [23, 24]. G-CSF has been shown to exert anti-inflammatory effects on the cytokine network, adding to the favorable properties of this cytokine [25]. G-CSF does so by stimulating the production of anti-inflammatory mediators such as IL-1 receptor antagonist (IL-1ra) [26-28], soluble TNF receptor (sTNF-R) I and II [26, 28] and IL-10 [29], and by attenuating the release of IL-8 [27], macrophage inflammatory protein-2 (MIP-2) [16], TNF-α, IL-1β, IL-18 and interferon-γ [26, 30, 31]. This attractive profile of G-CSF and the concomitant availability of recombinant G-CSF led to numerous studies investigating the effect of recombinant G-CSF on the course of infections. Indeed, G-CSF administration improved survival rates in endotoxemia and various sepsis models in animals (reviewed in [32]), which encouraged several groups to

investigate the usefulness of G-CSF in pneumonia. In pneumococcal pneumonia, a survival benefit has been described in G-CSF treated rats and splenectomized mice [33-35]. Another study found an improved survival only in mice infected with a low inoculum of *S. pneumoniae* [36]. However, two recent studies conducted in patients with multilobar pneumonia or pneumonia complicated by severe sepsis did not reveal beneficial effects of adjunct G-CSF treatment [37, 38].
Despite the fact that many studies investigated the utility of adjunct G-CSF treatment in pneumonia, the role of endogenous G-CSF in the pulmonary host defense in vivo has not been examined thus far. Therefore, in the present study, we investigated local G-CSF concentrations at the site of the infection in patients with unilateral CAP and in mice with experimentally induced pneumococcal pneumonia, and studied the effect of passive immunization against G-CSF in murine pneumonia.

Materials and Methods

Patient study

Patients

Four patients, 3 men and 1 woman (mean age: 41 ± 5 years; mean ± SE), with a unilateral CAP were enrolled in the study. Samples obtained from these patients were used previously to investigate local alterations in fibrinolysis during CAP [39]. All patients fulfilled the following criteria: fever (>37.7ºC), new unilateral infiltrate on chest X-ray within 2 days after admission, no antibiotic pre-treatment and paO_2 > 7.5 kPa while breathing room air. Exclusion criteria were hospitalization within two weeks prior to admission and any immunosuppressive therapy. Ten healthy volunteers (mean age: 32 ± 8 years; mean ± SE) not taking any medication, served as controls. The protocol was reviewed and approved by the Medical Ethics Committee of the University of Amsterdam and written informed consent was obtained from all subjects.

Broncho-alveolar lavage (BAL)

Within 12 h after admission, BAL was performed in a standardized fashion according to the guidelines of the American Thoracic Society, using a flexible fiberoptic video-bronchoscope. Seven successive 20ml aliquots of prewarmed isotonic saline were instilled in a subsegment of the lung and aspirated immediately with low suction. BAL was first performed at the uninfected side of the middle lobe or lingula, followed by lavaging a subsegment of the infected lobe. Generally, 10-15ml of the instilled 20ml was recovered. There was no difference between the recovered volumes from the infected or uninfected side.
Specimen processing: BAL fluid (BALF) was kept at 4ºC until processing, which was performed within 30 minutes. The samples were centrifuged at 3000 rpm for 15 minutes at

4ºC. The first three recoveries were sent to the microbiology department for culture and the remaining supernatant was stored at -80ºC until G-CSF concentrations were assessed. G-CSF levels were measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN) according to the instructions of the manufacturer. The detection limit of the assay was 31 pg/ml.

Mouse studies

Animals

Pathogen-free 6-8 wk-old female BALB/c mice were obtained from Harlan Sprague-Dawley (Horst, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments.
Anti-G-CSF Antibody: Sheep polyclonal IgG Ab to mouse G-CSF was generated as described previously [40]. Administration of this Ab to mice in a dose also used in the present study resulted in a complete neutralization of endogenous G-CSF for 2-3 days, which was associated with an approximate 50% reduction of PMN counts in peripheral blood 2 days post-injection [40]. The biological activity of the anti-G-CSF batch used in the current investigation was confirmed by demonstrating a similar 50% decrease in circulating PMNs 24h after injection of healthy mice with anti-G-CSF, relative to mice that received sheep Ig (Sigma, St. Louis, MO)(data not shown).

Induction of pneumonia and design

Pneumonia was induced as described previously [41, 42]. Briefly, *S. pneumoniae* serotype 3 was obtained from American Type Culture Collection (ATCC 6303; Rockville, MD). Pneumococci were grown for 6 hours to midlogarithmic phase at 37°C using Todd-Hewitt broth (Difco, Detroit, MI), harvested by centrifugation at 1500 x *g* for 15 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at a concentration of approximately 10^5 CFUs/50µl, as determined by plating serial 10-fold dilutions on sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, The Netherlands) and 50 µl (approximately 10^5 CFU) *S. pneumoniae* were inoculated intranasally. Anti-G-CSF Ig or sheep Ig (Sigma), respectively, were administered intra-peritoneally in 200µl sterile saline 1h before and 24h after induction of pneumonia. In preliminary experiments we established that sheep Ig did not influence the host response to pneumococcal pneumonia when compared with saline. At 24 h and 48 h after infection, mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Meidrecht, the Netherlands) and sacrificed by bleeding out the vena cava inferior. Blood was collected in EDTA containing tubes.

Myeloperoxidase (MPO) assay

MPO activity was measured as described previously [41, 43]. Lung tissue was homogenized in potassium phosphate buffer using a tissue homogenizer (Biospec Products, Bartlesville,

OK), and pelleted at 4.500 x *g* for 20 min. Pelleted cells were lysed in potassium phosphate buffer pH 6.0 supplemented with hexadecyltrimethyl ammoniumbromide (HETAB) and 10mM EDTA. MPO activity was determined by measuring the H_2O_2 dependent oxidation of 3,3`5,5`tetramethylbensidine (TMB). Briefly, serial dilutions of samples in potassium phosphate buffer were mixed with tetramethylbensidine substrate N,N`dimethylformamide. The reaction was stopped with glacial acetic acid followed by OD reading at 655nm. MPO-activity is expressed as activity per gram lung tissue per reaction time. All reagents were purchased from Sigma.

Histologic examination

Lungs for histologic examination were harvested at 24h and 48h after infection, fixed in formaline and embedded in paraffin. 4 μm sections were stained with hematoxylin and eosin (H&E), and analyzed by a pathologist who was blinded for groups. To score lung inflammation and damage, the lung sample was screened for the following parameters: interstitial inflammation, intra-alveolar inflammation, edema, endothelialitis, bronchitis, pleuritis and thrombi formation. Each parameter was graded on a scale of 0 to 3 with 0: absent, 1: mild, 2: moderate, 3: severe. The total injury score was expressed as the sum of the score for all parameters, the maximum being 21.

Lung cell counts and FACS analysis

For FACS analysis and cellcounts, whole lungs were flushed with sterile saline via the pulmonary artery to eliminate blood leucocytes. Pulmonary cell suspensions were then obtained from whole lungs using an automated disaggregation device (Medimachine System; Dako, Glostrup, Denmark) and resuspended in FACS buffer (PBS supplemented with 0.5% BSA, 0.01% NaN_3, and 100mM EDTA) exactly as described previously [41, 44]. Total cell numbers were counted from each sample using a hemocytometer (Türck chamber), lung differential cell counts were done on cytospin preparations stained with Giemsa. For analysis of PMN activation, lung cells were stained with anti-CD11b-FITC and anti-Gr-1-PE for 30min at 4ºC, washed twice in FACS buffer and analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). To correct for aspecific staining, appropriate isotype controls were used. All reagents were purchased from (BD Pharmingen, San Diego, CA) and used in concentrations recommended by the manufacturer.

Preparation of lung tissue for cytokine measurements

At time of sacrifice, whole lungs were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products). 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. All reagents were purchased from Sigma.

Cytokine and Chemokine Assays

Cytokines and chemokines (G-CSF, TNF-α, IL-1β, cytokine-induced neutrophil chemoattractant (KC)) were measured using specific ELISAs (R&D Systems) according to the manufacturers` instructions. The detection limits of the assays (standard curves) were 31 pg/ml for G-CSF and TNF-α, 8 pg/ml for IL-1β and 12pg/ml for KC. Detection limits in lung homogenates were 20-fold higher due to the laboratory procedures, specified above, required to obtain these whole organ suspensions.

Determination of bacterial outgrowth

CFUs were determined from serial dilutions of lung homogenates and blood, plated on blood agar plates and incubated at 37°C for 16 h before colonies were counted.

Statistical analysis

Differences between groups were calculated by Mann-Whitney *U* test. For survival analyses, Kaplan-Meier analysis followed by log rank test was performed. Values are expressed as mean ± SE. A p-value < 0.05 was considered statistically significant.

Results

G-CSF is elevated in BALF of pneumonia patients

To obtain insight into local G-CSF levels during pneumonia, four patients with unilateral CAP underwent bilateral BAL within 12h after admission, first at the uninfected site and then in the area with the infiltrate on the chest X-ray. G-CSF was measured by ELISA in BALF and plasma; BALF from healthy subjects served as control (Table 1). G-CSF concentrations were increased in BALF obtained from infected lungs in 3 out of 4 patients, but close or below the detection limits in uninfected lungs. Plasma G-CSF concentrations were below the detection limit in all patients with CAP as were G-CSF levels in plasma and BALF of healthy controls. Hence, G-CSF was detectable locally at the site of infection in CAP patients.

Subject (Sex)	BALF culture	Infected side BALF	Healthy side BALF	Plasma
1 (M)	*S. pneumoniae*	465 pg/ml	79 pg/ml	ND
2 (F)	-	12239 pg/ml	ND	ND
3 (M)	*S. pneumoniae*	84 pg/ml	ND	ND
4 (M)	-	ND	ND	ND

Table 1: G-CSF concentrations in patients with CAP. G-CSF concentrations were measured in BALF from four patients with CAP with a unilateral infiltrate in the chest X-ray from the area of infiltrate and uninfected site and in plasma samples. ND = not detectable. BALF obtained from healthy subjects did not contain detectable G-CSF levels (not shown in table).

Endogenous G-CSF is elevated during murine pneumococcal pneumonia

We then investigated whether endogenous G-CSF concentrations increase in mice upon infection with *S. pneumoniae*. For this purpose G-CSF levels were measured in plasma and lung homogenates obtained from mice treated with sheep Ig or anti-G-CSF prior to infection with *S. pneumoniae* and compared to mice i.n. inoculated with sterile saline only. Induction of pneumonia was associated with moderately elevated plasma G-CSF levels in mice treated with sheep Ig but not in anti-G-CSF treated mice (Table 2). G-CSF could not be detected in plasma of healthy, uninfected mice (Table 2). 24 and 48h after inoculation with *S. pneumoniae*, pulmonary G-CSF concentrations were found increased in up to 50% of sheep Ig treated animals, the range being 630-770 pg/ml at 24h (3/8 mice) and 660-4630 pg/ml at 48h (4/8 mice), but below the detection limit in all anti-G-CSF treated mice as well as in mice without pneumonia.

pg/ml	24h	48h
Saline	< detection	< detection
Control	199 ± 55	176 ± 82
Anti-G-CSF	< detection	< detection

Table 2: Plasma G-CSF levels in murine pneumococcal pneumonia.Mice were treated i.p. with anti-G-CSF or control Ig at –1h and +24h relative to i.n. inoculation with 10^5 CFU *S. pneumoniae* and plasma G-CSF concentrations were measured 24h and 48h after induction of pneumonia. Mice inoculated i.n. with sterile saline served as uninfected controls. Data are mean ± SE of 6-8 mice per group, < detection indicates below detection limit.

Anti-G-CSF has no major impact on pulmonary PMN recruitment

Since G-CSF is foremost considered to contribute to the enhanced recruitment of PMN to the site of infection, we first examined whether endogenous G-CSF had an impact on pulmonary PMN influx in pneumonia. Pulmonary MPO levels and the extent of lung inflammation (as assessed by histopathology) were indistinguishable between control and anti-G-CSF treated mice 24h after induction of pneumonia (Fig. 1, Table 3). However, 48h after infection, lung MPO levels were significantly higher in control mice (Fig. 1).

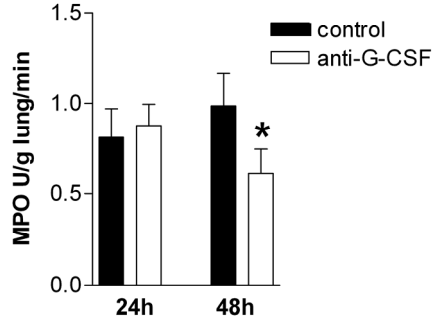

Fig. 1: Pulmonary MPO levels. Mice were treated i.p. with anti-G-CSF (open bars) or control Ig (filled bars) at –1h and +24h relative to i.n. infection with 10^5 CFU *S. pneumoniae*. Data are mean ± SE of 8 mice per group. * $p<0.05$ versus control.

Despite a similar degree of lung inflammation in both groups, the inflammatory infiltrate was predominantly composed of PMNs in control mice and mononuclear cells in anti-G-CSF treated mice (Fig. 2, Table 3). At the same time, the number of circulating PMNs was decreased in anti-G-CSF treated mice (726 ± 120/µl versus 2516 ± 427/µl in control mice at t=48h; $p<0.05$) as opposed to blood PMN counts at baseline (1418 ± 88 versus 1781 ± 186/µl in controls at t=0; n.s.).

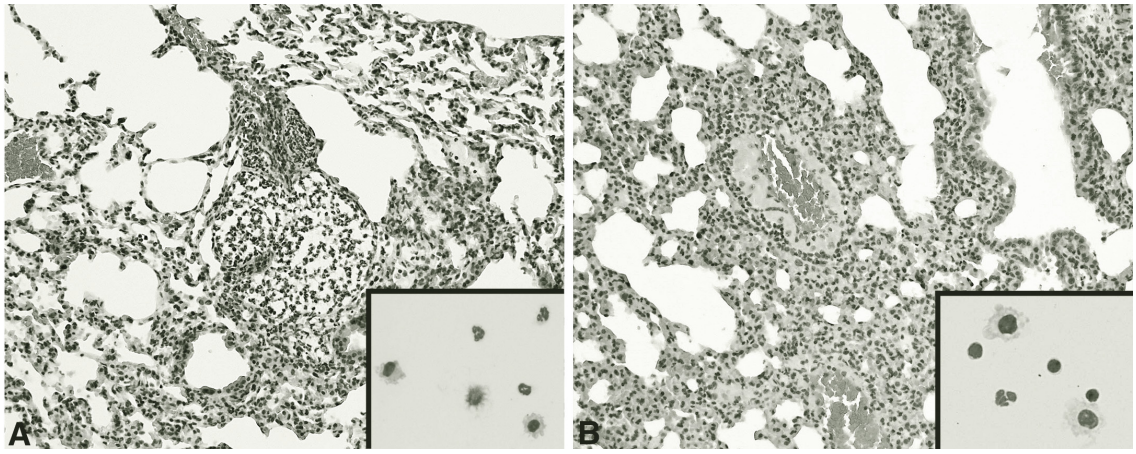

Fig. 2: Representative lung histology and cytospins (inserts) 48h after infection of mice treated i.p. with control Ig (A) or anti-G-CSF (B) at –1h and +24h relative to i.n. inoculation with 10^5 CFU *S. pneumoniae*. H&E staining, magnification x 12.5. Insets (magnification x 40) show representative cytospins of whole lung suspensions and provide more details of the composition of the inflammatory infiltrate. Slides are representative for 8 mice per group.

	24h	**48h**
Control	5.9 ± 0.4	6.3 ± 1.1
Anti-G-CSF	6.9 ± 0.7	5.8 ± 1.3

Table 3: Histological Scores of lung inflammation. Mice were treated i.p. with anti-G-CSF or control Ig at –1h and +24h relative to i.n. inoculation with 10^5 CFU *S. pneumoniae* and histopathology of lungs obtained 24h and 48h after infection was scored as described in the Methods section. Data are mean ± SE of 8 mice per group.

This prompted us to investigate the cellular composition of lungs 48h after infection with *S. pneumoniae* more precisely. To avoid that contaminating blood leukocyte influenced pulmonary cell counts, lungs were flushed thoroughly via the pulmonary artery until they appeared macroscopically pale. As shown in Table 4, total lung cell counts and PMN numbers were slightly but not statistically significantly lower in anti-G-CSF treated mice. Surprisingly, the number of macrophages was reduced in lungs of anti-G-CSF treated animals, whereas the number of lymphocytes was increased in lungs of in anti-G-CSF treated mice (both $p<0.05$ versus controls). At the same time, the number of blood lymphocytes and monocytes were unaltered in anti-G-CSF treated mice (lymphocytes: 1854 ± 164/µl versus 1570 ± 132/µl in control mice; monocytes: 3379 ± 516/µl versus 3261 ± 448/µl in control mice; t=48h, n.s.).

Thus, treatment with anti-G-CSF did not significantly impair the pulmonary PMN influx but affected the macrophage and lymphocyte recruitment to the lungs.

x 10^4/ml	Total cells	PMNs	Macrophages	Lymphocytes
Control	1135 ±169	294 ± 40	770 ± 123	71 ± 12
Anti-G-CSF	859 ± 142	199 ± 33	450 ± 85*	210 ± 38*

Table 4: Cellular composition of lungs Mice were treated i.p. with anti-G-CSF or control Ig at –1h and +24h relative to i.n. inoculation with 10^5 CFU *S. pneumoniae*. Cell counts and differentials were done on cell suspensions of flushed whole lungs 48h after induction of pneumonia, as described in the Methods section. Data are mean ± SE of 8 mice per group. * $p<0.05$ versus control.

Endogenous G-CSF contributes to PMN activation

Besides exerting chemotactic activities, G-CSF has been reported to be involved in the activation of PMNs including the upregulation of CD11b [28]. We therefore investigated the activation state of recruited lung PMNs by measuring CD11b expression on Gr-1^+ cells in whole lung cell suspension 48h after induction of pneumonia. CD11b expression was significantly elevated on Gr-1^+ PMNs of control mice, as compared to mice treated with anti-G-CSF (MFI 332.3± 57.0 and 202.1±38.0, controls versus anti-G-CSF treated mice, $p<0.05$).

Anti-G-CSF does not impair bacterial clearance

Having shown that G-CSF contributes to the activation of PMNs, we next studied whether one of the main biological tasks of PMNs, i.e. the elimination of bacteria, was impaired in anti-G-CSF treated mice. Lung CFUs were slightly higher in anti-G-CSF treated mice at 24 and 48h postinfection, but the differences with control mice did not reach significance (Fig. 3). Similarly, the number of bacteremic mice was identical in anti-G-CSF and control mice, 48h after induction of pneumonia (3/8 mice with positive blood cultures in both groups), whereas blood cultures were negative in all mice at 24h. Hence, endogenous G-CSF activity does not have a major impact on bacterial clearance in pneumoccocal pneumonia.

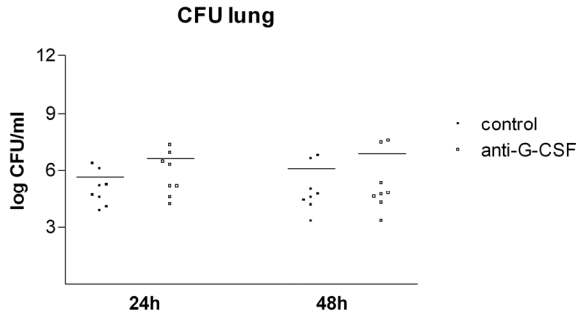

Fig. 3: Anti-G-CSF treated mice display an unaltered bacterial clearance. Mice were treated i.p. with anti-G-CSF (open bars) or control Ig (filled bars) at –1h and +24h relative to i.n. inoculation with 10^5 CFU *S. pneumoniae*. Data are mean ± SE of 8 mice per group.

Increased pro-inflammatory cytokines and CXC chemokines in anti-G-CSF treated mice

Among the pro-inflammatory cytokines, TNF-α and IL-1β are key mediators in host defense against *S. pneumoniae* pneumonia [42, 45] and anti-inflammatory properties have been attributed to G-CSF, partly by reducing the release of these mediators [26, 30, 31, 46]. Therefore, we evaluated the influence of anti-G-CSF treatment on the pulmonary concentrations of TNF-α and IL-β. At 24h postinfection, anti-G-CSF treated mice displayed elevated TNF-α and IL-β concentrations in their lungs ($p<0.05$ versus control; Fig. 4). In addition, the important murine CXC chemokine KC was markedly increased in anti-G-CSF treated mice at that time-point ($p<0.05$ versus control; Fig. 4). These differences had disappeared 48h after induction of pneumonia (data not shown). Hence, endogenous G-CSF attenuated the early pro-inflammatory cytokine and chemokine response in pneumococcal pneumonia.

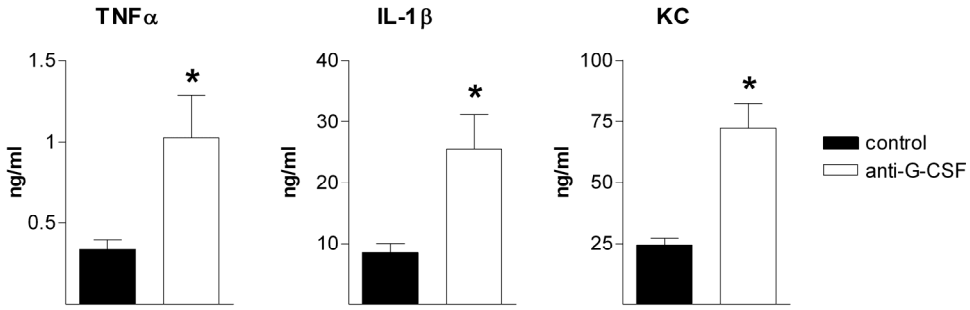

Fig. 4: Anti-G-CSF treatment results in higher TNF-α, IL-β and KC lung concentrations. Mice were treated i.p. with anti-G-CSF (open bars) or control Ig (filled bars) 1h before i.n. inoculation with 10^5 CFU *S. pneumoniae* and pulmonary cytokines/chemokines were measured 24h after induction of pneumonia. Data are mean ± SE of 8 mice per group; * $p<0.05$ versus control.

Anti-G-CSF does not influence survival in *S. pneumoniae* pneumonia

Finally, we examined the impact of endogenous G-CSF on survival. After i.n. infection with 10^5 CFU *S. pneumoniae*, 50% (6/12) of control mice and 69% (9/12) of anti-G-CSF treated mice survived (non significant; Fig.5). All mice surviving 5 days appeared no longer clinically ill.

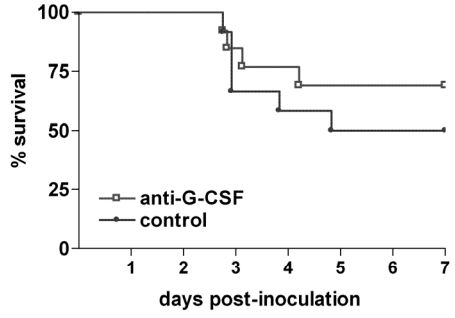

Fig. 5: Blocking endogenous G-CSF does not impair survival Mice were treated i.p. with anti-G-CSF (filled symbols) or control Ig (open symbols) at –1h and +24h relative to i.n. inoculation with 10^5 CFU *S. pneumoniae* and survival was monitored for 7 days. Data are mean ± SE of 12 mice per group.

Discussion

S. pneumoniae is the leading causative pathogen in CAP and infection with pneumococci is associated with high morbidity and mortality rates worldwide. Although G-CSF has gained much attention as an adjunct treatment modality in pneumonia [37, 38], the role of endogenous G-CSF in host defense against pneumococcal pneumonia in vivo has thus far never been investigated. We therefore decided to determine the biological significance of infection-triggered G-CSF production. We here demonstrate increased levels of G-CSF in BALF obtained from the side of infection in patients with unilateral CAP. By neutralizing endogenous G-CSF immediately prior to induction of pneumonia in mice we observed that the role of endogenous G-CSF in the host response to *S. pneumoniae* pneumonia is two-sided. On the one hand, G-CSF was involved in the activation of pulmonary PMNs, whereas on the other hand G-CSF reduced pulmonary TNF-α, IL-1β and KC concentrations during pneumonia. However, G-CSF did not influence local antibacterial defense or the dissemination of bacteria, and did not modify the clinical outcome of the infection.

Our findings in patients with CAP confirm and extend previous studies. Mice with *Escherichia coli* infection had elevated G-CSF levels in their BALF [14]. Ex vivo, alveolar macrophages from patients with respiratory tract infections were shown to release G-CSF [15]. We extend these findings and pinpoint G-CSF production to the site of infection by studying 4 patients with unilateral CAP, in 2 of whom *S. pneumoniae* was isolated. Similar to others, we observed a wide range of G-CSF concentrations in patients with infection [13], but were unable to detect G-CSF in plasma samples of CAP patients. We also observed a wide range of G-CSF levels in lungs of mice infected with *S. pneumonia*. Although the sensitivity of the G-CSF assay was reduced due to dilution of both BALF specimens from patients and lung homogenates from mice, our results clearly indicate that G-CSF is locally produced upon infection of the lower respiratory tract.

It is generally accepted that endogenous G-CSF is important for maintaining adequate numbers of circulating PMNs in healthy subjects and during infections. Indeed, G-CSF-deficient mice are neutropenic and have a diminished PMN increase upon infection, which limits their utility in infectious disease models [47]. Similarly, neutralizing G-CSF activity 3-5 days prior to infection rendered mice neutropenic and led to decreased PMN recruitment and impaired bacterial clearance with increased lethality in a peritonitis model [40]. Despite many studies on the effect of exogenously administered G-CSF, reports about the role of endogenous G-CSF during infections are confined to a few observations. One study observed an unaltered host response when endogenous G-CSF was neutralized immediately before induction of peritonitis [40], whereas more recently, Noursadeghi et al. demonstrated that an acute phase response-induced increase in endogenous G-CSF prior to induction of *E. coli* sepsis exerts beneficial effects due to improved phagocytosis and respiratory burst [48].

In the current study we pretreated mice with anti-G-CSF 1h before i.n. inoculation with *S. pneumoniae*. This approach was chosen in order to inhibit the endogenous G-CSF generated in response to the infection. Earlier administration of anti-G-CSF, i.e. several days before

infection, renders mice neutropenic [40], which is expected to impair host defense against pneumococcal pneumonia by mechanisms not directly related to G-CSF produced during respiratory tract infection. By doing so, we observed only a modest reduction in PMN recruitment to the site of infection in anti-G-CSF treated mice, as indicated by slightly less PMNs in lungs of these animals (statistically not significantly different from control mice), together with reduced lung MPO levels 48h after induction of pneumonia. Interestingly, anti-G-CSF significantly decreased the number of macrophages attracted to the pulmonary compartment, which might in part be responsible for more profound effects of this Ig on MPO levels [49]. Decreased monocyte attraction was reported earlier in G-CSF deficient mice infected with *Listeria* [47, 50], suggesting a broad role for G-CSF in multilineage cell recruitment. In addition, exogenous G-CSF administration to volunteers challenged with LPS resulted in an increase in monocyte counts in the circulation [30]. To our surprise, we observed an increased recruitment of cells with morphologic features of lymphocytes to lungs of mice pretreated with anti-G-CSF. Zhan et al. reported a similar observation in G-CSF deficient mice with *Listeria* peritonitis [51] and disclosed that these cells exhibit some characteristics of poorly differentiated macrophages, although their precise origin remains to be elucidated.

The key event in host defense against pneumococcal pneumonia is the elimination of bacteria from the lungs and prevention of systemic bacterial dissemination. Previous investigations reported improved bacterial clearance in G-CSF treated animals with pneumococcal pneumonia [35, 36]. Despite the fact that G-CSF is known to improve bactericidal properties of neutrophils and macrophages [19, 20, 52], we did not observe an impaired bacterial clearance in anti-G-CSF treated mice. This might in part be explained by the fact that lung concentrations of protective mediators such as TNF-α and IL-β were significantly higher in these mice. Indeed, in particular TNF-α and IL-1 are associated with improved bacterial clearance and synergistically enhance survival in *S. pneumoniae* pneumonia [42, 45]. Of note, Dallaire et al. did not find any effect of recombinant G-CSF administration on lung TNF-α or IL-1β concentrations in pneumococcal pneumonia [36]. However, our findings are in agreement with earlier reports on diminished TNF-α and IL-1β release from whole blood and isolated monocytes, respectively, stimulated in the presence of G-CSF [30, 31] and with in vivo findings obtained in a pneumococcal meningitis model [53]. In the present study, we also revealed an increase in pulmonary KC concentrations after anti-G-CSF administration, which might in part have compensated for the loss of the chemotactic activity of G-CSF on PMNs in animals injected with the antibody. In line herewith, Dallaire et al. reported decreased KC levels in mice with pneumococcal pneumonia treated with recombinant G-CSF [36].

In summary our results show that: 1. endogenous G-CSF inhibits the proinflammatory arm of the cytokine network in vivo, reducing the concentrations of TNF-α, IL-1β and KC at the site of infection during pneumonia. 2. endogenous G-CSF contributes to activation of PMNs recruited to the lung, and 3. The net effect of endogenous G-CSF on antibacterial defense and the clinical outcome of pneumococcal pneumonia is limited. Further studies are warranted to

investigate the role of endogenous G-CSF in other types of pneumonias, such as caused by *Klebsiella pneumoniae*, where detrimental effects of exogenous G-CSF have been reported in animal models [54].

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CHAPTER 5

Interleukin-18 impairs the pulmonary host response to *Pseudomonas aeruginosa*

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Abstract

Interleukin (IL)-18 is a potent cytokine with many different pro-inflammatory activities. To study the role of IL-18 in the pathogenesis of *Pseudomonas* pneumonia, IL-18 deficient [*IL-18* (-/-)] and wild type mice were intranasally inoculated with *P. aeruginosa*. IL-18 deficiency was associated with a reduced outgrowth of *Pseudomonas* in lungs, and a diminished dissemination of the infection. In addition, pulmonary inflammation (histopathology), and lung and plasma levels of tumor necrosis factor-α, IL-6 and macrophage inflammatory protein-2 were lower in *IL-18* (-/-) mice. Consistent with results obtained in *IL-18* (-/-) mice, treatment of wild type mice with a neutralizing IL-18 binding protein-IgG Fc fusion construct also attenuated outgrowth of *Pseudomonas* when compared with mice treated with a control protein. These results demonstrate that the presence of endogenous IL-18 activity facilitates inflammatory responses in the lung during *Pseudomonas* pneumonia, concurrently impairing bacterial clearance.

Introduction

Interleukin (IL-) 18 was originally identified as an interferon (IFN)-γ inducing factor (IGIF) (24). IL-18 is mainly produced by activated macrophages, first synthesized as a precursor protein (pro-IL-18), which requires splicing by IL-1β-converting enzyme (ICE, caspase 1) to liberate the mature active protein (4, 7). IL-18 synergistically enhances IL-12-stimulated IFN-γ production (19) and promotes cell-mediated immunity (11, 19, 26, 35). Direct pro-inflammatory effects of IL-18 include activation of nuclear factor (NF) κB (17), the induction of cytokines such as tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8 (22, 26), and activation of neutrophils (15). Hence, IL-18 can be considered a pluripotent mediator with strong pro-inflammatory properties. Endogenous IL-18 activity is negatively regulated by IL-18 binding protein (IL-18BP). For human IL-18BP four isoforms have been described resulting from mRNA splicing, of which the *a* and *b* isoforms neutralize IL-18 with high affinity (10).

Several studies have implicated IL-18 as an important mediator in the innate immune response to bacterial infection. Plasma levels of IL-18 are elevated in patients with severe sepsis (6, 14, 23), and such elevated circulating concentrations contribute to the development of a lethal systemic inflammatory response syndrome during endotoxic shock in mice (8, 21). In contrast to its apparent detrimental role during fulminant shock, IL-18 likely is required for an adequate antibacterial host defense, as indicated by a reduced resistance of IL-18 deficient or depleted mice against infections by *Salmonella typhimurium* (16), *Shigella flexneri* (28) and *Listeria monocytogenes* (20). Recently, our laboratory studied the role of IL-18 in the pathogenesis of pneumonia caused by *Streptococcus pneumoniae* (13). Although survival of IL-18 gene deficient [*IL-18* (-/-)] mice was not different from wild type mice, the absence IL-18 deteriorated host-defense, as reflected by an enhanced outgrowth of bacteria in the lungs of *IL-18* (-/-) mice relative to wild type mice. Moreover, *IL-18* (-/-) mice were more susceptible for progression to systemic infection.

While *S. pneumoniae* is the most common causative microorganism in community-acquired pneumonia (2), the most frequent pathogen involved in nosocomial pneumonia is the gram-negative bacterium *Pseudomonas aeruginosa* (25). The role of IL-18 in the pathogenesis of gram-negative bacterial pneumonia is unknown. Therefore, we compared host defense in *IL-18* (-/-) and wild type mice during respiratory tract infection with *P. aeruginosa*.

Methods

Animals

Female *IL-18* (-/-), on the C57Bl/6 background (35), and normal C57Bl/6 wild type mice (Harlan, Horst, the Netherlands), 8-10 weeks old, were used in all experiments. The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments.

IL-18BP-Fc construct

Recombinant human IL-18BP isoform *a* (kindly provided by Dr. Giorgio Senaldi, Amgen Inc.) was produced as fusion construct with human IgG_1 Fc as described previously (3). This construct, designated IL-18BP-Fc, binds and neutralizes human, mouse and rat IL-18. At the dose given in the present study (5 mg/kg), IL-18BP-Fc prevented LPS-induced IFN-γ release and lethality in mice; the inhibitory effect of IL-18BP-Fc on LPS-induced IFN-γ production was long lasting with still > 90% inhibition when IL-18BP-Fc was injected up to 6 days before the LPS challenge (3). In the current investigation IL-18BP-Fc was given as a single intraperitoneal injection 2 hours before induction of pneumonia at a dose of 5 mg/kg (100 µl). Purified human IgG_1 (Nordic Immunology, Tilburg, the Netherlands) was used as control.

Induction of pneumonia

Pneumonia was induced as described before (31-33). *P. aeruginosa* (strain PA103 or strain PA01), grown to mid-logarithmic phase in Luria broth, was harvested by centrifugation at 1500 x *g* for 15 minutes and washed twice in pyrogen-free 0.9 % NaCl. After being suspended in 10 ml 0.9 % NaCl, the number of bacteria was determined by serial dilution in sterile isotonic saline and culture on blood agar plates. Mice were lightly anaesthetized with inhaled isoflurane (Forene [Abbott, Queensborough, Kent, UK]), after which 50 µl of the bacterial solution (10^5 colony forming units [CFU]) was administered intranasally. Control mice were inoculated with 50 µl pyrogenic-free isotonic saline alone. All mice were sacrificed after 24 hours, because former experiments using this model of acute pneumonia did not show any significant changes between groups at earlier time points, and because mice died rapidly at later time points (31, 33).

Preparation of blood samples and lung homogenates

At 24 hours after inoculation mice were anesthetized with Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands). Blood was collected from the *vena cava inferior* in heparin containing vacutainer tubes; whole lungs were harvested and homogenized at 4^0C in 5 volumes of sterile 0.9% NaCl in a tissue homogenizer. After each homogenization, the homogenizer was carefully cleaned and disinfected with 70% alcohol.

Determination of bacterial outgrowth

Serial 10-fold dilutions in sterile isotonic saline were made of these homogenates and blood, and 50 µl volumes were plated onto sheep-blood agar plates and incubated at 37^0C and 5% CO_2. CFU were counted after 24 hours. For cytokine measurements lung homogenates were spun at 1500 x *g* for 15 minutes at 4^0C, supernatants were filtered through a 35 µm filter (Becton Dickenson, Lincoln Park, NJ) and frozen at -20^0C until cytokine measurement.

Histologic examination

Lungs for histologic examination were harvested 24 hours after intranasal inoculation, fixed in 10% buffered formalin and embedded in paraffin, and 4 µm sections were stained with hematoxylin and eosin. Slides were analysed by a pathologist who was blinded for groups.

Assays

Cytokine and chemokine levels were measured by ELISA's according to the manufacturers recommendations: IL-18 (R&D, Minneapolis, MN), TNF (Genzyme, Cambridge, MA), IL-6 (Pharmingen, San Diego, CA), IFN-γ (R&D), IL-12p40 (R&D), IL-12p70 (R&D), and macrophage inflammatory protein (MIP)-2 (R&D, Minneapolis, MN).

Statistical analysis

All data are expressed as means ± standard error (SE). Comparisons between means were conducted using the Wilcoxon test. Significance was set at $P < 0.05$.

Results

Induction of pneumonia and IL-18

Control mice had high levels of IL-18 concentrations in their lungs (Fig. 1). Although infection with *P. aeruginosa* slightly increased IL-18 concentrations in lung homogenates, the difference was not significant.

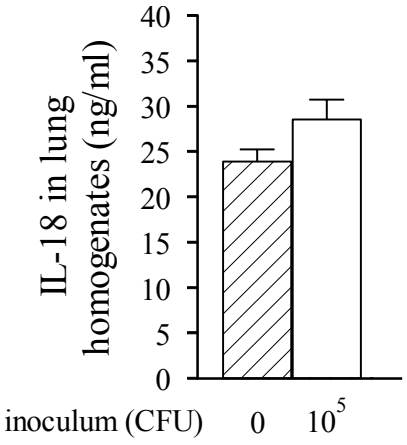

Fig. 1: IL-18 concentrations in lung homogenates of wild type mice (open bars), 24 hours after intranasal inoculation with 10^5 CFU *P. aeruginosa*, and from mice inoculated with sterile saline (///-hatched bars). Data are means (± SE); N = 10 mice per group.

Inoculation with *P. aeruginosa* induced signs of pneumonia in all mice. 24 Hours after inoculation with *P. aeruginosa*, lungs appeared swollen and reddish with multiple hemorrhages on the surface. Wet lung weights from wild type mice inoculated with *P. aeruginosa* increased by more than 150 %, compared to lungs from control mice inoculated with sterile saline ($P < 0.05$) (Fig. 2). *IL-18* (-/-) mice also demonstrated an increase in wet lung weights after induction of *Pseudomonas* pneumonia, although the increase was smaller than in wild type mice ($P < 0.05$).

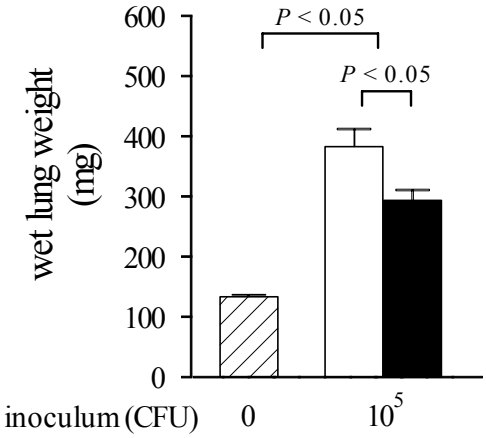

Fig. 2: Wet lung weights from wild type mice (open bars), and IL-18 (-/-) mice (solid bars) inoculated with 10^5 CFU *Pseudomonas aeruginosa*, and from mice inoculated with sterile saline (///-hatched bars). Data are means (± SE); N = 10 mice per group.

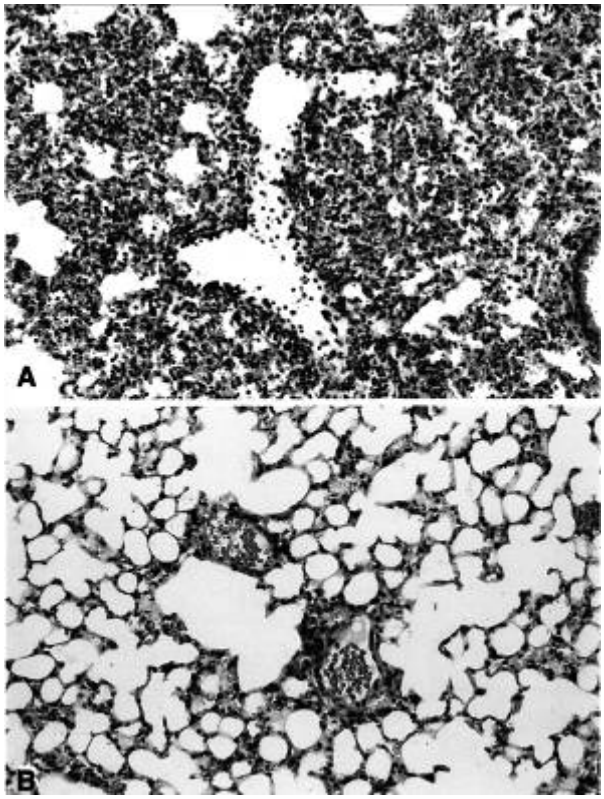

Fig. 3: Representative lung histology of wild type mice (A) and IL-18 (-/-) mice (B), 24 hours after inoculation with *P. aeruginosa*. Lungs of wild type mice (A) displayed a more dense and diffuse inflammatory infiltrate than *IL-18* (-/-) mice in which the inflammation was limited to small collections of neutrophils (B). Hematoxylin and eosin staining, magnification x 50; N = 5 per group.

Inoculation with *P. aeruginosa* induced a diffuse pneumonia in all mice. At 24 hours after inoculation with *P. aeruginosa*, lungs of wild type mice displayed pneumonia characterized by a diffuse and heavy inflammatory infiltrate mostly composed of neutrophils.

Endothelialitis was a prominent feature (Fig. 3A). In contrast, the inflammation in *IL-18 (-/-)* mice was less severe and limited to perivascular and interstitial inflammatory infiltrates (Fig. 3B).

Bacterial clearance

Next, we determined the role of endogenous IL-18 in the clearance of *Pseudomonas* from the pulmonary compartment. For this purpose, wild type and *IL-18* (-/-) mice were inoculated with *P. aeruginosa*, and CFU's were counted in lungs harvested after 24 hours (Fig. 4).

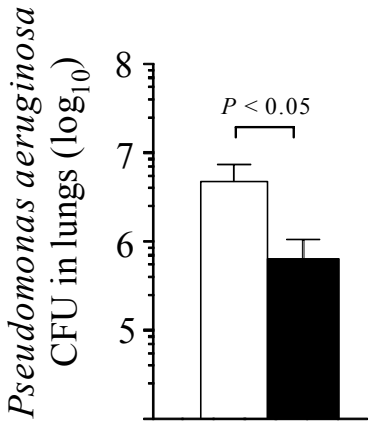

Fig. 4: Clearance of bacteria is enhanced in IL-18 (-/-) mice. Mean (± SE) *Pseudomonas aeruginosa* CFU in lungs 24 hours after intranasal inoculation with 10^5 CFU in wild type (open bars), and IL-18 (-/-) mice (solid bars).

IL-18 (-/-) mice had significantly less CFU's in their lungs at 24 hours after the induction of pneumonia than wild type mice ($P < 0.05$). In addition, the number of *IL-18* (-/-) mice that developed bacteremia was markedly lower compared with wild type mice. At 24 h after infection, 30 % of the *IL-18* (-/-) mice had positive blood cultures for *P. aeruginosa*, while 66.7 % of the wild type mice had bacteria in their blood.

Bacterial clearance in mice treated with IL-18BP-Fc

Compensatory immune mechanisms may develop in mice that genetically lack the IL-18 signaling pathway. To determine whether the differences between *IL-18* (-/-) and wild type mice were caused solely by the absence of IL-18, we inoculated wild type mice with *P. aeruginosa*, 2 hours after intraperitoneal injection of IL-18BP-Fc.

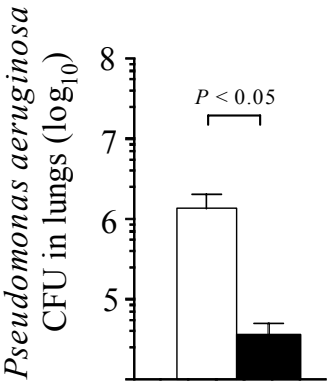

Fig. 5: Enhanced clearance of bacteria in mice treated with IL-18BP-Fc. Mean (± SE) *Pseudomonas aeruginosa* CFU in lungs 24 hours after intranasal inoculation with 10^5 CFU in mice receiving IL-18BP-Fc intraperitoneally (5mg/kg), 2 h before inoculation (solid bars), and mice receiving the control IgG_1 (open bars). N = 10 mice per group.

The results from the experiments with *IL-18* (-/-) mice could be recapitulated in this experiment, i.e., IL-18BP-Fc treatment reduced the number of CFU's recovered from lungs at 24 hours post-infection when compared to treatment with control IgG_1 (Fig. 5).

Clearance of PA01 in IL-18 (-/-) mice and wild type mice

To determine whether the differences between *IL-18* (-/-) and wild type mice were related to the *Pseudomonas* strain used in these experiments, we inoculated *IL-18* (-/-) mice and wild type mice with *P. aeruginosa* strain PA01. The results from the experiments described above could be recapitulated in this experiment, i.e., *IL-18* (-/-) mice had significantly less *Pseudomonas* strain PA01 CFU's in their lungs at 24 hours after the induction of pneumonia than wild type mice (Fig. 6).

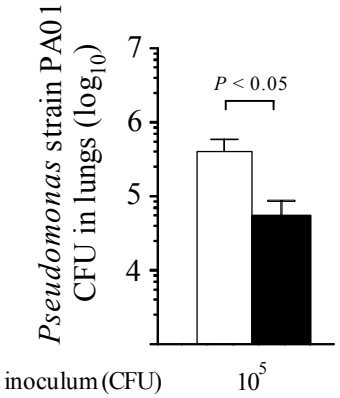

Fig. 6: Enhanced clearance of *Pseudomonas* strain PA01 in IL-18 (-/-) mice. Mean (± SE) *Pseudomonas aeruginosa* CFU in lungs 24 hours after intranasal inoculation with 10^5 CFU in IL-18 (-/-) mice (solid bars), and control mice (open bars). N = 8 mice per group.

Cytokine and chemokine levels

Local production of cytokines and chemokines within the pulmonary compartment can influence anti-bacterial host defense mechanisms during pneumonia (18, 30). Therefore, we measured the concentrations of TNF, IL-6, and MIP-2 in lung homogenates after inoculation with *P. aeruginosa* (Fig. 7).

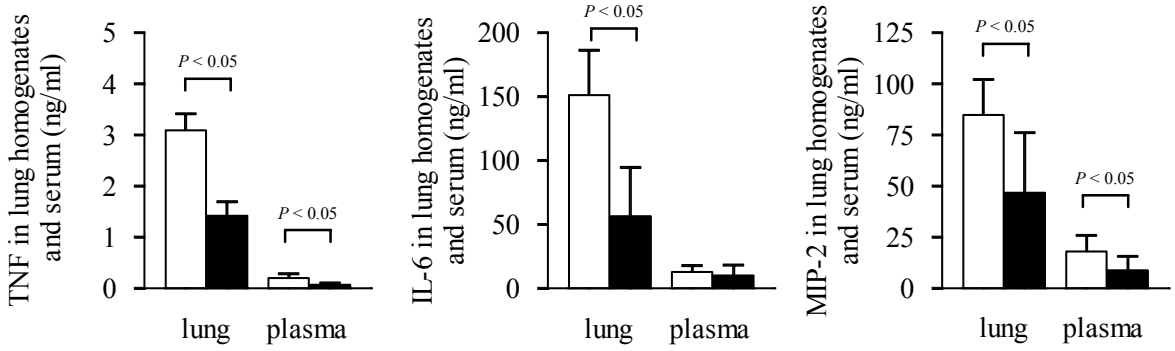

Fig. 7: Reduced local and systemic levels of TNF, IL-6, and MIP-2 in IL-18 (-/-) mice inoculated with 10^5 CFU *Pseudomonas aeruginosa.* (*IL-18* (-/-) mice, solid bars; wild type mice; open bars), at 24 hours after inoculation. Data are mean ± SE. N = 9-10 mice per group. $P < 0.05$ for mice inoculated with bacteria versus mice inoculated with sterile saline for all three mediators (data not shown).

TNF, IL-6 and MIP-2 levels were all significantly lower in lung homogenates from *IL-18* (-/-) mice, compared with wild type mice ($P < 0.05$). High plasma concentrations of TNF, IL-6, and MIP-2 were found in both *IL-18* (-/-) and wild type mice, whether bacteremic or not. At 24 hours after induction of pneumonia, higher plasma concentrations of TNF and MIP-2 were found in wild type mice compared with *IL-18* (-/-) mice ($P < 0.05$) (Fig. 7). Local concentrations of IFN-γ, IL-12p40 and IL-12p70 in *IL-18* (-/-) mice were not statistically different from those measured in wild type mice (data not shown).

Discussion

In pneumonia, the initiation, maintenance and resolution of inflammation involve the expression of the complex network of pro-inflammatory and anti-inflammatory cytokines (18, 30). Here we describe a series of experiments in which we evaluated the role of IL-18 in the innate immune response in the pulmonary compartment during pneumonia induced by *P. aeruginosa*. *IL-18* (-/-) mice were found to have an increased resistance against *Pseudomonas* pneumonia, as reflected by less bacteria in lungs and a reduced dissemination of infection, which was associated with a diminished inflammatory response upon histopathologic examination and suppressed local and systemic cytokine and chemokine concentrations. The enhanced antibacterial defense could be reproduced in normal wild type mice treated with IL-18BP-Fc, which potently neutralizes IL-18 (3), indicating that compensatory immune mechanisms that could have developed in mice that genetically lack IL-18 are unlikely to be responsible for the present findings.

Notably, IL-18 was expressed constitutively in lungs of normal mice, confirming earlier reports (1, 13, 36), and IL-18 concentrations increased only marginally during pneumonia with *P. aeruginosa*. Similarly, we recently reported a modest non-significant rise in pulmonary IL-18 levels during pneumococcal pneumonia (13). Nonetheless, both in the present and our previous investigation, IL-18 deficiency had a large impact on antibacterial defense in the pulmonary compartment. These findings suggest that either constitutively expressed IL-18 influences the innate immune response during respiratory tract infection or that the modest rise in IL-18 levels is biologically significant in the context of murine pneumonia.

We used strain PA103 because we were experienced in using this *Pseudomonas* strain in this acute pneumonia model. This strain is not a clinical isolate, but a laboratory strain that produces high amounts of *Pseudomonas* exotoxin A and reduced amounts of proteins. Although we did not consider it possible that the found differences in the first series of experiments were caused by the characteristics of the bacterium (since both *IL-18* (-/-) mice and wild type mice were infected with this strain), we determined clearance of *Pseudomonas* strain PA01 (a clinical isolate). These additional experiments showed similar results, i.e., clearance of *Pseudomonas* PA01 is hampered by IL-18.

The results of this study are in line with other reports, demonstrating a detrimental role for pro-inflammatory cytokines in host defense during *Pseudomonas* pneumonia. Indeed, we recently reported that mice deficient for either the type I IL-1 receptor or the IFN-γ receptor display an enhanced bacterial clearance of *P. aeruginosa* (31, 33). Similarly, mice deficient for the type I TNF receptor demonstrated an accelerated early clearance of *P. aeruginosa* from the lungs (34), whereas elimination of the anti-inflammatory cytokine IL-10 resulted in a diminished bacterial outgrowth (29).

While pro-inflammatory cytokines seem to impair host defense against *P. aeruginosa*, they are important for host defense in murine pneumonia models with other pathogens. In experimental pneumonia with the Gram-negative bacterium *Klebsiella pneumoniae* or the Gram-positive bacterium *S. pneumoniae*, pro-inflammatory cytokines like TNF and IL-1 (12, 27, 37) are important for the clearance of bacteria from the lungs, whereas the anti-inflammatory cytokine IL-10 impairs host defense in these models (5, 38). Importantly, we recently demonstrated that IL-18 contributes to pulmonary host defense against *S. pneumoniae* pneumonia (13). A possible explanation for the differences between the pneumonia models using different pathogens includes differences in the extent and rapidity by which these strains induce inflammation in the lung.

The absence of endogenous IL-18 activity was associated with reduced levels of TNF, IL-6 and MIP-2 at 24 hours post-inoculation. The lower bacterial load in lungs of *IL-18* (-/-) mice (providing less pro-inflammatory stimuli) could have been responsible for this finding. However, IL-18 may also be involved in cytokine and chemokine production during pneumonia in a more direct way, considering that IL-18 is capable of stimulating the secretion of these mediators by different cells *in vitro* (22, 26). Further support for this latter possibility is the recent observation that neutralization of endogenous IL-18 reduced vascular leak and the production of TNF in the lung during immune complex alveolitis in rats (9). Importantly, levels of IFN and IL-12 were not different between wild type mice and *IL-18* (-/-) mice, indicating that the found differences in clearance of *P. aeruginosa* are not caused by a compensatory change in the production of IFN or IL-12.

In conclusion we found an increased bacterial clearance in *IL-18* (-/-) mice during pneumonia caused by *P. aeruginosa*. The difference with wild type mice was associated with an attenuated inflammatory response. Together with our earlier findings of a diminished clearance of *S. pneumoniae* from the lungs of IL-18 (-/-) mice (13), these data exemplify the complex role of IL-18 in innate immunity during pulmonary infection and may have important implications for the development and use of cytokine/anti-cytokine therapies in the future.

Acknowledgement/Footnote

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CHAPTER 6

Caspase-1 is an important mediator of lung inflammation during *Acinetobacter baumannii* pneumonia

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Submitted

Abstract

Caspase-1 is considered an important mediator of inflammation. *Acinetobacter baumannii* (*A. baumannii*) is an emerging pathogen and one of the most common causative bacteria in nosocomial pneumonia. To determine the role of caspase-1 in the host response to *Acinetobacter* pneumonia, caspase-1 gene deficient and wild-type mice were intranasally infected with *A. baumannii*. Caspase-1 deficiency was associated with strongly reduced pulmonary concentrations of proinflammatory cytokines and chemokines early after infection and attentuated lung inflammation upon histopathological examination. In addition, caspase-1 deficient mice demonstrated an accelarated clearance of *A. baumannii* from their lungs. Similarly, oral treatment of wild-type mice with a selective caspase-1 inhibitor resulted in an inhibition of lung cytokine and chemokine production and an enhanced bacterial clearance. These data suggest that caspase-1 plays an important role in the regulation of lung inflammtation during *A. baumannii* pneumonia.

Introduction

Caspase-1 is an intracellular cysteine protease that is an essential processing enzyme for the release of biologically active interleukin (IL)-1β, IL-18 and IL-33 (1, 2). NALP3, also known as cryopyrin or CIAS, is a central component of the protein complex that regulates caspase-1 activation. This molecular platform, known as the NALP3-inflammasome, has been implicated in a number of host immune responses, particularly as an early sensor of endogenous danger signals and as a pivotal mediator in the initiation of an adequate innate immune response to invading pathogens (1-3). The positive contribution of caspase-1 to defense against bacteria has been demonstrated in experimental murine infections caused by *Escherichia coli, Listeria monocytogenes, Francisella tularensis* and *Salmonella typhimurium* (4-7). On the other hand, caspase-1 plays a detrimental role in overwhelming inflammation, such as induced by bolus injection of high dose lipopolysaccharide (LPS), as indicated by the fact that *caspase-1* gene deficient (caspase-$1^{-/-}$) mice are resistant against LPS induced toxicity and lethality (4, 8-11).

Knowledge of the role of caspase-1 in the regulation of inflammation in the lungs is limited. Two studies reported on the contribution of caspase-1 to acute lung inflammation induced by intrapulmonary delivery of LPS (12, 13). Whereas Rowe *et al.* found enhanced influx of neutrophils into bronchoalveolar lavage fluid (BALF) of caspase-$1^{-/-}$ mice relative to wild-type mice upon intratracheal administration of LPS (12), Noulin *et al.* did not detect differences in neutrophil recruitment to BALF between these two mouse strains after intranasal LPS inoculation (13). Sansonetti *et al*. reported attenuated lung inflammation in caspase-$1^{-/-}$ mice early after intranasal infection with *Shigella flexneri*, with enhanced inflammation and increased bacterial growth later on (14). Notably, *Shigella* is an intestinal pathogen and the role of caspase-1 in host defense against pneumonia caused by a typical respiratory pathogen has not been investigated thus far.

In recent years *Acinetobacter (A.) baumannii* has emerged as a leading pathogen causing hospital-acquired infections with pneumonia as the most common manifestation (15-17). In addition, *Acinetobacter* may cause community-acquired pneumonia, in particular in young alcoholics in tropical climates (18). Pneumonia induced by *A. baumannii* is frequently associated with a sudden and severe onset and systemic complications including septic shock have been repeatedly described (18). We recently developed a model for *A. baumannii* pneumonia to study host defense mechanisms at play during this clinically important infection (19, 20). Here we used this model to investigate the role of caspase-1 in the innate immune response to pneumonia.

Materials and Methods

Mice

Female caspase-$1^{-/-}$ mice, backcrossed to a C57BL/6 genetic background six times, were obtained from Charles River Laboratories (Wilmington, MA). Female C57BL/6 mice from Harlan Sprague-Dawley (Horst, the Netherlands) were used as wild-type controls. Age (7-9 weeks) matched mice were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments.

Induction of pneumonia:

Pneumonia was induced essentially as described (19, 20). *A. baumannii* was obtained from American Type Culture Collection (ATCC 17961; Rockville, MD). *A. baumannii* were grown to midlogarithmic phase at 37°C using Luria Bertani broth (Difco, Detroit, MI), harvested by centrifugation at 1500 x *g* for 15 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at a concentration of approximately 2 x 10^7 colony forming units (CFUs)/50µl, as determined by plating serial 10-fold dilutions on sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, the Netherlands) and 50 µl (2 x 10^7 CFU) were inoculated intranasally. In some experiments C57BL/6 wild-type mice were treated orally with the selective caspase-1 inhibitor VX-04-030 (200mg/kg; Vertex Pharmaceuticals, Cambridge, MA) immediately before infection. VX-04-030 was stored in chremophore (Sigma, St. Louis, MO) and dissolved in sterile water to a concentration of 0.1mg/ml using a sonicating water bath; control mice received chremophore diluted in sterile water.

Determination of bacterial outgrowth:

At 4 or 24 hours after infection, mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Meidrecht, the Netherlands) and sacrificed by bleeding out the vena cava inferior. Blood was collected in EDTA containing tubes. Whole lungs were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs were determined from serial dilutions of lung homogenates and blood, plated on blood agar plates and incubated at 37°C for 16 h before colonies were counted.

Preparation of lung tissue for immunoassays:

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.

Histologic examination:

Lungs for histologic examination were harvested at indicated timepoints, fixed in 10% formaline and embedded in paraffin. 4 μm sections were stained with hematoxylin and eosin (H&E), 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: interstitial inflammation, edema, endothelitis, bronchitis and pleuritis. Each parameter was graded on a scale of 0 to 4, with 0: absent, 1: mild, 2: moderate, 3: moderately severe and 4: severe. The "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 20. The presence of confluent infiltrates, or pneumonia, respectively, was evaluated in percent of the total lung surface. Per 10% pneumonia, 0.5 points were added to the lung inflammation score, resulting in the "total lung inflammation score". Neutrophil staining was done exactly as described previously using FITC-labeled anti-mouse Ly-6-G mAb (BD PharMingen, San Diego, CA)(19, 21).

Assays:

Cytokines and chemokines (TNF-α, IL-6, IL-10, IL-1β, MIP-2, and KC) were measured with commercially available ELISAs (R&D systems, Abingdon, UK). Myeloperoxidase (MPO) was measured by ELISA (Hycult, Uden, the Netherlands).

Statistical analysis:

Differences between groups were calculated by Mann-Whitney *U* test. Values are expressed as mean ± SEM. A P-value ≤ 0.05 was considered statistically significant.

Results

Caspase-1$^{-/-}$mice demonstrate reduced lung inflammation during pneumonia

To determine the role of caspase-1 in lung inflammation induced by *A. baumannii* pneumonia caspase-1$^{-/-}$ and wild-type mice were intranasally infected with *Acinetobacter* and the extent of lung inflammation was assessed by measurement of cytokine and chemokine concentrations in whole lung homogenates and histopathology of lung tissue slides. In accordance with our earlier studies (19, 20), cytokine and chemokine levels were high in particular early after infection (4 hours), whereas low levels were detected at 24 hours (Figure 1). Caspase-1 deficiency was associated with strongly reduced lung levels of proinflammatory cytokines (IL-1β, TNF-α, IL-6) and chemokines (MIP-2, KC) at 4 hours after induction of pneumonia ($P < 0.05$ versus wild-type mice). At 24 hours post infection, cytokine and chemokine levels had decreased in both mouse strains and no differences between caspase-1$^{-/-}$ and wild-type mice were detectable with the exception of IL-1β concentrations, which remained lower in caspase-1$^{-/-}$ animals ($P < 0.05$). The pulmonary concentrations of the anti-

inflammatory cytokine IL-10 showed a delayed increase, which was not altered by caspase-1 deficiency.

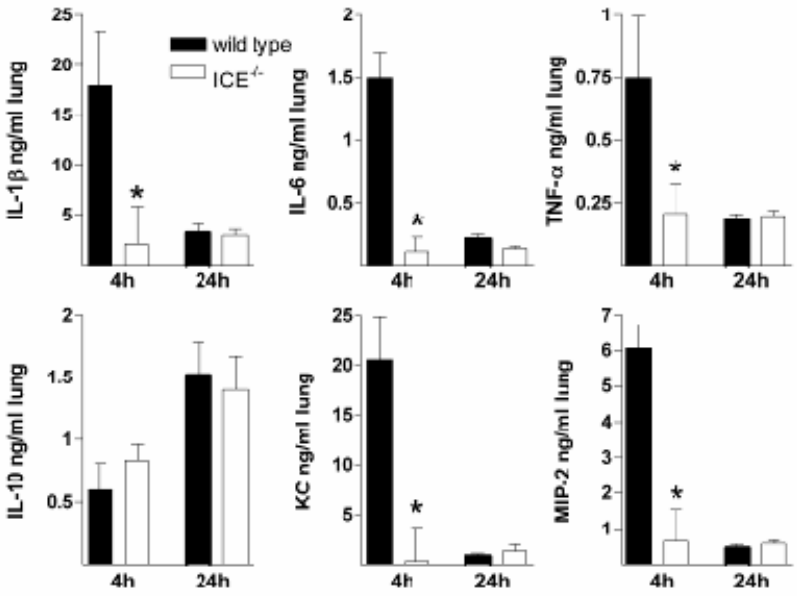

Fig. 1: Caspase-1-/- mice demonstrate reduced lung cytokine and chemokine concentrations. Caspase-1-/- (open bars) and wild type mice (closed bars) were intranasally infected with 2 x 107 CFU A. baumannii. Cytokine and chemokine levels were measured in lung homogenates obtained at 4 and 24 hours after infection. Data are means ± SEM of 5 - 7 mice per group at each time point. * $P < 0.05$ versus wild type mice.

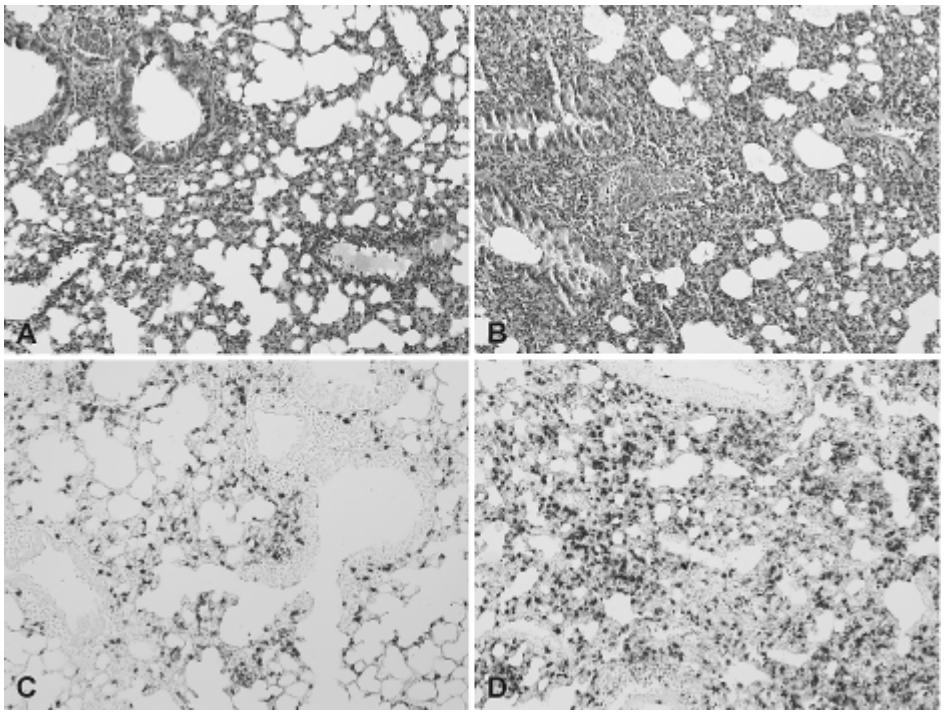

Fig. 2: Caspase-1$^{-/-}$ mice demonstrate reduced lung inflammation. Caspase-1$^{-/-}$ (open bars) and wild type mice (closed bars) were intranasally infected with 2 x 10^7 CFU *A. baumannii*. Representative tissue slides 24 hours after infection of semi-quantitatively scored lungs according to the criteria described in the Methods section. (A and C) wild type, (B and D) caspase-1$^{-/-}$ mice. H&E staing (A and B), Ly6 staining (C and D). magnification x10.

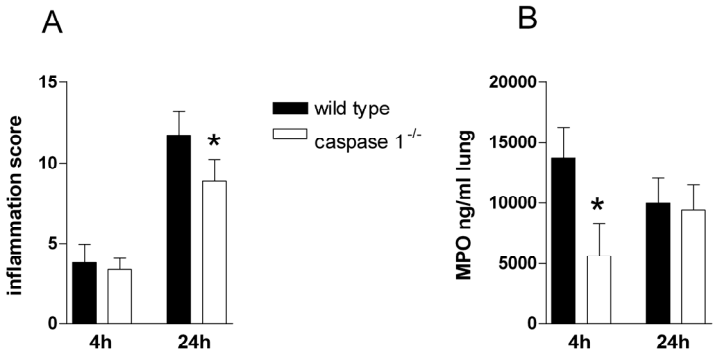

Fig. 3: Caspase-1$^{-/-}$ mice demonstrate reduced PMN influx. Caspase-1$^{-/-}$ (open bars) and wild type mice (closed bars) were intranasally infected with 2 x 10^7 CFU *A. baumannii*. Lung tissue slides at 4 and 24h after infection were semi-quantitatively scored according to the criteria described in the Methods section. (A) Pathology scores. (B) Lung MPO concentrations. Data are means ± SEM of 5 - 7 mice per group at each time point. * $P < 0.05$ versus wild type mice.

Histopathologic analyses revealed a lung inflammatory response that lagged behind the early increase in local cytokine and chemokine concentrations (Figure 2). Importantly, and in line with the attenuated early cytokine and chemokine response, caspase-1$^{-/-}$ mice displayed reduced lung inflammation as determined by the semi-quantitative pathology score outlined in the Methods section ($P < 0.05$ versus wild-type mice) (Figure 3A). In addition, caspase-1$^{-/-}$ mice had a diminished influx of neutrophils into the lungs, as reflected by neutrophil stainings of lung tissue (Figure 2, C and D) and MPO concentrations in whole lung homogenates (Figure 3).

Caspase-1 deficiency results is an accelerated clearance of *A. baumannii* from the lungs

In order to obtain insight in the consequences of the effects of caspase-1 deficiency on the inflammatory response in the lung for the clearance of *Acinetobacter*, we determined the number of CFUs in lung homogenates harvested 4 and 24 hours after infection (Figure 4). In accordance with our earlier reports (19, 20), *A. baumannii* was relatively rapidly cleared from the pulmonary compartment. Nonetheless, caspase-1$^{-/-}$ mice demonstrated an accelerated clearance when compared with wild-type mice, in particular during the early phase of the infection, as reflected by a significantly lower bacterial load at 4 hours ($P < 0.05$).

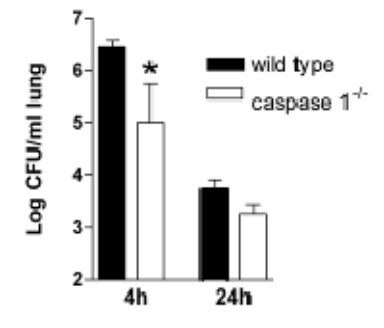

Fig. 4: Caspase-1$^{-/-}$ mice demonstrate an accelerated bacterial clearance. Caspase-1$^{-/-}$ (open bars) and wild type mice (closed bars) were intranasally infected with 2 x 10^7 CFU *A. baumannii*. The number of CFUs was determined in lung homogenates obtained at 4 and 24 hours after infection. Data are means ± SEM of 5 - 7 mice per group at each time point. * $P < 0.05$ versus wild type mice.

Effect of a selective caspase-1 inhibitor

Considering that genetically modified mice may develop mechanisms that compensate their loss of function, we next wished to confirm our data obtained with caspase-$1^{-/-}$ mice by treating wild-type mice with a pharmacologic inhibitor of caspase-1. For this, we orally administered the selective caspase-1 inhibitor VX-04-030 or vehicle immediately before induction of pneumonia and evaluated the induction of lung inflammation (Figure 5) and the clearance of *Acinetobacter* from the lungs (Figure 6).

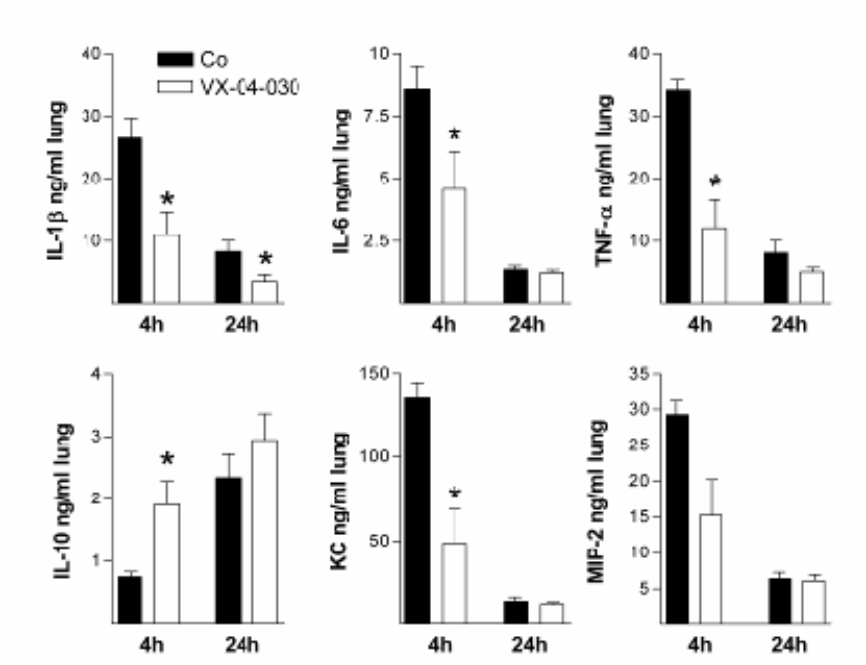

Fig. 5: VX-04-030 reduces lung cytokine and MPO levels. Mice were treated orally with VX-04-030 (200 mg/kg; open bars) or vehicle (closed bars) directly before intranasal infection with 2 x 10^7 CFU *A. baumannii*. Cytokine and chemokine levels were measured in lung homogenates obtained at 4 and 24 hours after infection. Data are means ± SEM of 8 mice per group at each time point. * $P < 0.05$ versus vehicle.

In line with the results using caspase-$1^{-/-}$ mice, VX-04-030 strongly reduced the pulmonary levels of proinflammatory cytokines (IL-1β, TNF-α, IL-6) and the chemokine KC at 4 hours after infection (all $P < 0.05$ versus vehicle). The only notable differences with the influence of genetic elimination of caspase-1 were the absence of an effect of VX-04-030 on lung MIP-2 levels and the elevated IL-10 concentrations at 4 hours post infection. Lung MPO concentrations did not differ 4 after induction of pneumonia but were significantly reduced in VX-04-030 treated mice after 24h. VX-04-030 modestly influenced the clearance of *Acinetobacter* from the respiratory tract. Whereas the bacterial loads in VX-04-030 and vehicle treated mice were similar at 4 hours after infection, VX-04-030 treatment was associated with significantly lower bacterial counts at 24 hours.

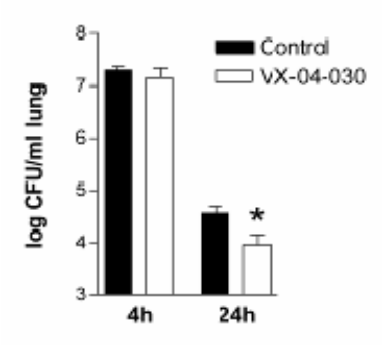

Fig. 6: VX-04-030 enhances bacterial clearance. Mice were treated orally with VX-04-030 (200 mg/kg; open bars) or vehicle (closed bars) directly before intranasal infection with 2 x 10^7 CFU *A. baumannii*. The number of CFUs was measured in lung homogenates obtained at 4 and 24 hours after infection. Data are means ± SEM of 8 mice per group at each time point. * $P < 0.05$ versus vehicle.
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Discussion

A. baumannii has emerged as a leading cause of opportunistic nosocomial infections worldwide, mostly afflicting the respiratory tract (15-17). The high rate of antibiotic resistance and widespread colonization of skin and medical equipment makes *A. baumannii* a pathogen of high concern. We here studied the role of caspase-1 in experimental pneumonia caused by *A. baumannii*, making use of a recently developed mouse model (19, 20). Caspase-$1^{-/-}$ mice demonstrated a strongly diminished early cytokine and chemokine response in their lungs together with a reduced influx of neutrophils and attenuated lung inflammation. Pharmacological inhibition of caspase-1 in wild-type mice confirmed these results, revealing markedly reduced cytokine concentrations in lungs of animals treated with a selective caspase-1 inhibitor. These data suggest that caspase-1 is an important mediator of the inflammatory response in the lungs after pulmonary infection with *A. baumannii*.

Several earlier studies have investigated the contribution of caspase-1 to host defense against bacterial infection (4-7, 14). To the best of our knowledge only one study thus far examined the role of caspase-1 during infection of the lungs (14). In line with our present investigations, caspase-$1^{-/-}$ mice displayed a strongly reduced inflammatory response in their lungs shortly after intranasal infection with *Shigella flexneri* (14). However, in contrast with our findings in *A. baumannii* pneumonia, lung infection by *Shigella* exacerbated in the absence of caspase-1, as reflected by enhanced bacterial growth and mortality (14). Clearly, *Shigella* and *Acinetobacter* are strongly different organisms and the former can not be considered a respiratory pathogen. In fact, Sansonetti *et al*. used the intranasal route of infection since mice can not be infected with *Shigella* orally and since the pulmonary inflammation evoked by intranasal administration resembles the intestinal inflammation seen in patients with dysentery (14). It is quite conceivable that in the *Shigella* model the diminished inflammatory reaction in the lungs during the early phase of the infection facilitated the subsequent growth of this pathogen. Indeed, several studies have documented an important protective role for early production of proinflammatory cytokines in host defense against bacterial pneumonia (22, 23). During airway infection by some pathogens, however, inflammation seems to impair the clearance of bacteria: elimination of TNF-α or IL-1 resulted in an accelerated clearance of

Pseudomonas aeruginosa from mouse lungs (24, 25), whereas the same interventions in murine pneumonia models with *Streptococcus pneumoniae* or *Klebsiella pneumoniae* facilitated the growth and dissemination of bacteria (26-28). In this respect it is interesting to note that – similar to what we observed here during *A. baumannii* pneumonia – caspase-1$^{-/-}$ mice showed an attenuated inflammatory reaction during corneal infection with *P. aeruginosa*, as reflected by locally reduced cytokine and chemokine levels and a diminished neutrophil influx, which was accompanied by an accelerated clearance of bacteria from the cornea (29). In addition, our own preliminary data, using neutralizing antibodies and/or knockout mice, suggest that the early production of proinflammatory cytokines inhibits the clearance of *A. baumannii* from mouse lungs, indicating that mediators like TNF-α and IL-1 may play similar roles in the pathogenesis of experimentally induced pneumonia caused by *A. baumannii* and *P. aeruginosa*. Taken together, these data suggest that caspase-1 contributes significantly to the early proinflammatory cytokine response during bacterial pneumonia, as indicated by both the *Shigella* model (14) and the experiments described here, and that this caspase-1 effect impairs the clearance of *A. baumannii* from the respiratory tract.

The results obtained with caspase-1$^{-/-}$ mice differed from the results obtained with VX-04-030 in several aspects. Importantly, the production of proinflammatory cytokines in the lungs was strongly reduced in both sets of experiments, thereby firmly establishing the pivotal role of caspase-1 in this response. The most notable differences were the lack of an effect of VX-04-030 on lung MIP-2 levels and the fact that this compound enhanced IL-10 concentrations at 4 hours post infection. In addition, VX-04-030 treatment was associated with lower bacterial loads only at 24 hours after infection, whereas caspase-1$^{-/-}$ mice had lower bacterial loads at 4 and 24 hours, significantly so only at the first time point. Although we can not provide a definitive explanation for these differences, clearly compensatory mechanisms developed in caspase-1$^{-/-}$ mice and/or incomplete inhibition of caspase-1 in VX-04-030 treated mice may have played a role.
Two previous investigations studied the role of caspase-1 in LPS-induced acute lung inflammation; one study found increased recruitment of neutrophils into BALF of caspase-1$^{-/-}$ mice after intrapulmonary delivery of LPS (12), whereas another study did not detect differences in neutrophil influx in this sterile model (13). Neither investigation extensively reported on the effect of caspase-1 deficiency on pulmonary cytokine production, although IL-1β levels were lower in caspase-1$^{-/-}$ mice beyond six hours post LPS administration (12). In our hand, neutrophil influx into the lungs was significantly reduced in caspase-1$^{-/-}$ mice. MPO-assays disclosed lower levels early after infection, which was reflected by less pronounced lung infiltrates 24h post-infection. We moreover found that elimination or inhibition of caspase-1 strongly impact in particular the production of proinflamatory cytokines and chemokines early (4 hours) after infection with *A. baumannii*. These data are in line with studies examining the role of caspase-1 in systemic inflammation elicited by intraperitoneal LPS injection (4, 8-11).

Caspase-1 is active end-enzyme of the NALP3 inflammasome, a protein complex that is considered essential for adequate induction of an innate immune response to invading pathogens (2, 3). We here established that caspase-1 is a pivotal mediator in the induction of proinflammatory cytokine and chemokine production during *A. baumannii* pneumonia and that caspase-1 deficiency results in reduced lung inflammation and an accelerated clearance of this pathogen from the airways. Further studies are warranted to determine the role of caspase-1 in pneumonia caused by other respiratory pathogens.

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We would like to thank Joost Daalhuisen and Regina de Beer for excellent technical assistance.

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CHAPTER 7

Aspiration pneumonitis primes the host for an exaggerated inflammatory response during pneumonia

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Crit Care Med. 2005; 33: 1770-1778.

Abstract

Objective: Nosocomial pneumonia is a feared complication in the critically ill patient. Aspiration pneumonitis is frequently complicated by infections. Objectives of this study was to determine the influence of aspiration pneumonitis on the host response to a common nosocomial respiratory pathogen

Design: A controlled, *in vivo* laboratory study.

Setting: Research laboratory of a health sciences university.

Subjects: Female C57Bl/6 mice.

Interventions: Mice received hydrochloric acid (HCl) or saline intratracheally followed 16 hours later by *Klebsiella pneumoniae*.

Measurements and main results: HCl induced a mild aspiration pneumonitis. Nonetheless, HCl aspiration resulted in a markedly increased inflammatory response in the lung upon infection with *K. pneumoniae*. This enhanced inflammatory reaction was accompanied by a greatly increased outgrowth of *K. pneumoniae* in lungs of mice previously exposed to HCl. Preexisting aspiration pneumonitis also triggered mouse lungs *in vivo* and alveolar macrophages *ex vivo* for enhanced release of proinflammatory mediators upon stimulation with *Klebsiella* LPS. Inhibition of TNF-α resulted in an increased inflammatory reaction and enhanced bacterial outgrowth in mice with primary *K. pneumoniae* pneumonia, whereas it had no effect in mice with preexisting aspiration pneumonitis.

Conclusions: These data indicate (1) that aspiration pneumonitis renders the host more susceptible to respiratory tract infection with *K.pneumoniae*, concurrently priming the lung for an exaggerated inflammatory response, and (2) that although TNF-a plays a major role in the host response to primary infection it does not impact on lung inflammation or defense after aspiration pneumonitis.

Introduction

Aspiration of gastric contents occurs in various hospitalized patients, in particular those with a reduced consciousness[1,2]. The associated lung injury, commonly referred to as aspiration pneumonitis, is primarily caused by gastric acid. Aspiration pneumonitis predisposes the host to development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)[3]. Although the exact underlying mechanisms for this association is unclear, it has been suggested that acid aspiration primes the lung for an enhanced inflammatory response to a subsequent challenge[4].

Nosocomial pneumonia is the leading cause of death from hospital-acquired infections, with an associated crude mortality rate of approximately 30 percent[5,6]. Interestingly, risk factors for aspiration pneumonitis and nosocomial pneumonia are partly overlapping[1,2,7], and recent studies have suggested that aspiration pneumonitis may render the host more susceptible to bacterial pneumonia[8,9]. In the present study we sought to further examine the influence of aspiration pneumonitis on the host response to subsequent pneumonia. For this, we induced pneumonia by intranasal (i.n.) inoculation with *Klebsiella (K.). pneumoniae,* a common nosocomial respiratory pathogen[7], in mice with or without preceding acid aspiration, and compared inflammatory responses and bacterial outgrowth in the lungs. In addition, arguing that tumor necrosis factor (TNF)-α has been implicated as an important mediator in various inflammatory lung diseases including aspiration pneumonitis, ALI/ARDS and pneumonia[10,11,12,13], we also evaluated the role of this pluripotent proinflammatory cytokine in lung inflammation during *Klebsiella* pneumonia in mice with or without preexisting aspiration pneumonitis.

Methods

Animals

Female C57BL/6 mice (Harlan, Horst, the Netherlands), 10-12 weeks old, were used in all experiments. The Institutional Animal Care and Use Committee of the Academic Medical Center approved the protocol.

Experimental groups & Design

Acid aspiration was done in essence as described[14,15]. Mice were anesthetized by intraperitoneal injection of 0.07 ml/g FFM (Fentanyl 0.315 mg/ml, Fluanisone 10 mg/ml, both Janssen, Beersen, Belgium; Midazolam 5 mg/ml, Roche, Mijdrecht, The Netherlands). The trachea was exposed and mice were subjected to intratracheal (i.t.) injection of 50 µl of 0.1 N hydrochloric acid (HCl, pH 1.5) (Sigma, St Louis, MO) or an equivalent volume of normal saline. In a first series of experiments mice were sacrificed 4, 8, 16 or 24 hours after i.t. instillation of HCl or saline. In subsequent experiments, in which the effect of previous exposure to HCl on the responsiveness to *Klebsiella* was investigated, mice were killed

immediately before administration of *K. pneumoniae* (16 hours after HCL or saline aspiration) or 24 hours after induction of pneumonia. *Klebsiella* pneumonia was induced exactly as described[16,17]. An inoculum of 50 µl of the bacterial solution, containing 9000 CFU, was administered intranasally (i.n.), control mice received saline. In other experiments, *Klebsiella* lipopolysaccharide (LPS, 10 µg in 50 µl saline; Sigma) or saline was administered i.n. 16 hours after i.t. injection of HCl or saline. Mice were sacrificed immediately before LPS administration or 6 hours after i.n. delivery of LPS. All time points were chosen since they are considered to be representative for an adequate assessment of lung inflammation in these models[17,16,18]. In a final study, a neutralizing anti-mouse TNFα monoclonal antibody (TN3, 0.5 mg in 200 µl saline, TN3 was a king gift Celltech, Slough, UK) was administered intraperitoneally 30 minutes before induction of *Klebsiella* pneumonia 16 hours after HCl or saline aspiration. TN3 is a well characterized antibody that at the dose given here effectively neutralized endogenous TNF-α in a variety of mouse models[19,20,21,22], including pneumonia [23].

Tissue handling

At the time of sacrifice, mice were anesthetized with FFM and blood was collected from the vena cava inferior. The lungs were removed and processed as described previously[24,17,16,25]. Lung weight was expressed as mg of lung per gram of mouse to obtain relative organ weight. In the pneumonia studies, lungs were homogenized as described previously[17]. Serial 10-fold dilutions in sterile isotonic saline were made of these homogenates, and 50 µl volumes were plated onto sheep-blood agar plates. CFU were counted after 24 hours. For measurement of cytokines and chemokines, lung homogenates were lysed in lysisbuffer (300 mM NaCl, 15 mM Tris, 2 mM MgCl, 2 mM Triton (X-100), Pepstatin A, Leupeptin, Aprotinin (20ng/ml), pH 7.4) and centrifuged at 1500 x g at 4^0C for 15 minutes.

Bronchoalveolar lavage

The trachea was exposed 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 saline. 0.9-1 ml of lavage fluid was retrieved per mouse, and total cell numbers were counted from each sample in a haemocytometer. BAL fluid (BALF) differential cell counts were carried out on Giemsa stained cytospins.

Histologic examination

Lungs were fixed in 4% paraformaldehyde and embedded in paraffin; 4 µm thick sections were stained with haematoxylin and eosin. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters as described previously[26]: interstitial inflammation, intra-alveolar inflammation, edema, endothelialitis, bronchitis, pleuritis, and thrombi formation. Each parameter was graded on a scale from 0 to 3. The total lung inflammation score was expressed as the sum of the scores for each parameter, the maximum being 21.

Assays

TNF-α, Interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1 and IL-10 were measured by Cytometric Bead Array (BD Biosciences, San Jose, CA). Macrophage inflammatory protein (MIP)-2 (R&D systems, Minneapolis, MN), keratinocyte derived chemokine (KC, R&D)) soluble TNF receptor I (sTNFr, R&D), IL-1β (R&D) and myeloperoxidase (MPO, Hycult biotechnology BV, Uden, The Netherlands) were measured in lung homogenates by ELISA. Protein levels in BALF were measured using the BCA protein kit (Pierce, Rockford, IL).

Ex vivo stimulation of alveolar macrophages

Ex vivo stimulation of alveolar macrophages was performed as described previously [26]. In short, alveolar macrophages were harvested from mice 16 hours after saline or acid aspiration by BAL (n = 8 per group). Samples of two mice were pooled and total cell numbers were counted using a hemocytometer. Immediately thereafter cell counts were differentiated using Giemsa stained cytospins. $1x10^5$ alveolar macrophages per ml in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, penicillin, streptomycin, and 10% FCS were allowed to adhere in 96 wells plates (Greiner, Alphen a/d Rijn, The Netherlands) for two hours. Adherent cells were stimulated with *K. pneumoniae* LPS for 16 h.

Statistical analysis

All data are expressed as means ± standard error (SE). Comparisons between groups were conducted using analysis of variance and Mann Whitney U test where appropriate. Significance was set at $P < 0.05$.

Results

Time course of acid aspiration lung injury

In order to establish the severity of HCl instillation in our hands, we first performed time course experiments in which we followed animals for 24 hours after i.t. administration of HCl or saline. Acid aspiration was not associated with lethality. Total cell counts in BALF showed a transient rise peaking after 4 hours, which was exclusively determined by a strong influx of neutrophils (Figures 1A and B). In addition, acid aspiration resulted in transient increases in cytokine concentrations in BALF peaking after 8 hours (shown for TNF-α and IL-6 in Figures 1C and D). Based on these results, we chose a 16-hour interval between acid aspiration and induction of pneumonia to study the effect of previous acid aspiration on the immune response to respiratory tract infection, considering that at this time point the inflammatory response to HCl was clearly lessening although still detectable (Figure 1 and Table 1).

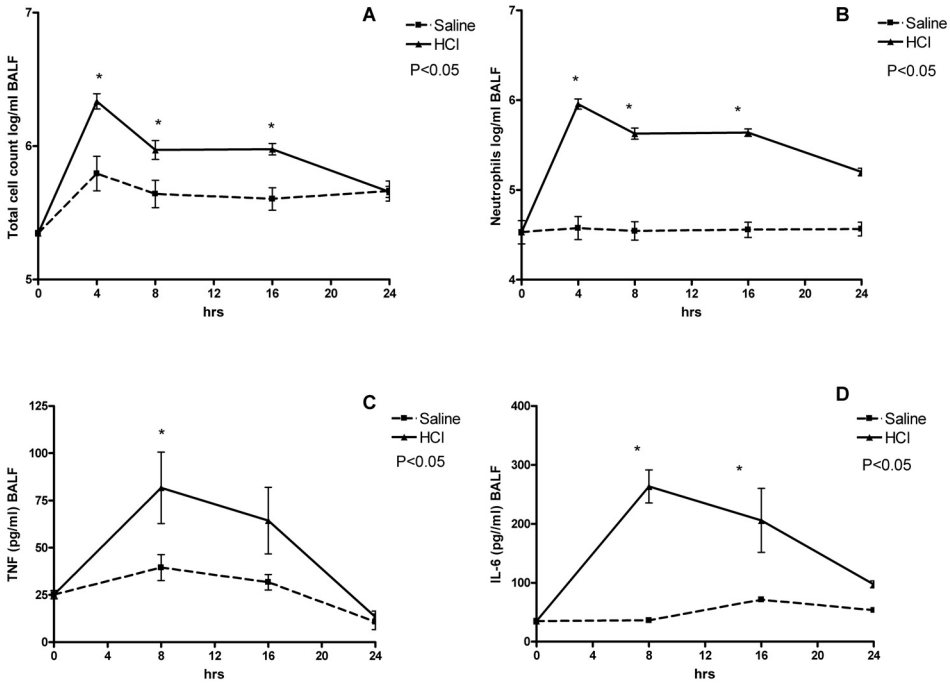


Fig. 1: Cell and neutrophil counts and TNF-α, and IL-6 levels in bronchoalveolar lavage fluid after saline or acid aspiration. Aspiration pneumonitis was induced by i.t. injection of 50 µl of 0.1 N HCl, control mice received saline. Mice were sacrificed 0, 4, 8, 16 and 24 hours after aspiration. Data are mean ± SE (n = 5 mice per group). P value indicates curve comparison using two way ANOVA; * P<0.05 at indicated time point versus saline aspiration.

Cytokines (pg/ml)	*Saline*	*HCl*
TNF-α	13 ± 1	32 ± 8*
IL-1β	193 ± 12	296 ± 46*
MCP-1	298 ± 14	714 ± 197*
IL-6	6 ± 1	119 ± 149*
IL-10	ND	ND
KC	235 ± 36	495 ± 87*
MIP-2	266 ± 104	2820 ± 56*

Table 1: Lung cytokine and chemokine concentrations 16 hours after acid or saline aspiration. Mice received either 50 µl 0.1 N HCl or an equivalent volume of saline i.t.. Cytokine and chemokine concentrations were measured in lung homogenates. Data are mean ± SE (n = 8 mice per group). ND = non detectable. * P < 0.05 versus

Acid aspiration increases pulmonary inflammation and bacterial outgrowth during *K. pneumoniae* pneumonia

Klebsiella pneumonia induced 16 hours after i.t. administration of HCl was associated with a strongly enhanced inflammatory response in the lung when compared to that observed in animals not exposed to acid. Indeed, lung concentrations of TNF-α, IL-1β and IL-6 were on average 10 times higher in mice that had received HCl, whereas chemokine levels were approximately twice as high in these animals; the pulmonary levels of the anti-inflammatory cytokine IL-10 were not different between groups (Table 2).

Cytokines (pg/ml)	*Saline/pneumonia*	*HCl/pneumonia*
TNF-α	67 ± 6	734 ± 278*
IL-1β	2014 ± 585	21786 ± 5786*
MCP-1	268 ± 17	856 ± 112*
IL-6	88 ± 7	837 ± 214*
IL-10	207 ± 41	216 ± 32
KC	898 ± 118	2078 ± 344*
MIP-2	1257 ± 295	2507 ± 331*

Table 2: Aspiration pneumonitis increases lung cytokine and chemokine levels during *K. pneumoniae* pneumonia. Mice received either 50 µl 0.1 N HCl or an equivalent volume of saline i.t. Sixteen hours later mice were i.n. inoculated with 9000 CFU *K. pneumoniae.* All mice were sacrificed 24h postinfection. Cytokine and chemokine concentrations were measured in lung homogenates. Data are mean ± SE (n = 8 mice per group). *P < 0.05 versus saline/pneumonia.

Additionally, mice previously exposed to HCl demonstrated a stronger influx of neutrophils into their BALF, increased pulmonary edema and higher BALF protein levels (Figure 2) and more pronounced inflammation upon histological analysis (Figure 3). Semiquantitative histology scores were significantly higher in HCl/pneumonia mice than in saline/pneumonia mice (13 ± 2 and 6 ± 2 respectively; inflammation, pleuritis, edema and occasional endothelialitis were observed, P<0.05). The enhanced inflammatory reaction in these mice was accompanied by an increased outgrowth of *K. pneumoniae*: the bacterial load in mice administered with HCl was 2.4 ± 0.9 x 10^8 per ml lung homogenate versus 7.6 ± 0.3 x 10^5 per ml lung homogenate in mice that had not received HCl (P < 0.05).

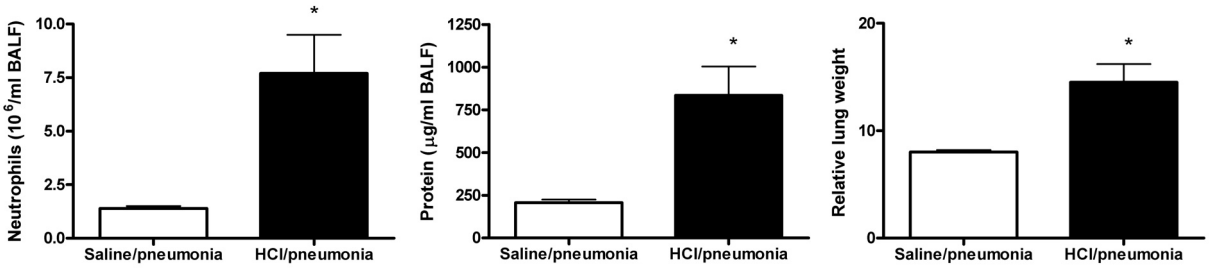


Fig. 2: Aspiration pneumonitis increases neutrophil influx, pulmonary edema and BALF protein levels are increased during pneumonia. Aspiration pneumonitis was induced by i.t. injection of 50 µl of 0.1 N HCl, control mice received saline. Sixteen hours later mice were i.n. inoculated with 9000 CFU *K. pneumoniae.* All mice were sacrificed 24 hours postinfection. Pulmonary edema was expressed as relative lung weight (mg of lung tissue/gram of mouse). Data are mean ± SE (n = 8 mice per group). *P<0.05 versus saline/pneumonia mice.

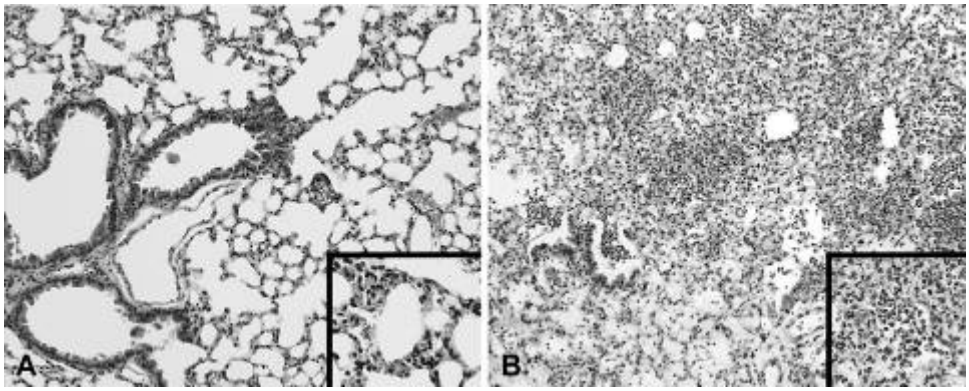


Fig. 3: Aspiration pneumonitis increases pulmonary inflammation during pneumonia. Aspiration pneumonitis was induced by i.t. injection of 50 µl of 0.1 N HCl, control mice received saline. Sixteen hours later mice were i.n. inoculated with 9000 CFU *K. pneumoniae.* All mice were sacrificed 24 hours postinfection. Representative lung histology slides from saline/pneumonia (A) and HCl/pneumonia (B) mice are shown (n = 8 mice per group). H& E staining, magnification x 10, insert x 40.

Acid aspiration increases pulmonary inflammation induced by *Klebsiella* LPS

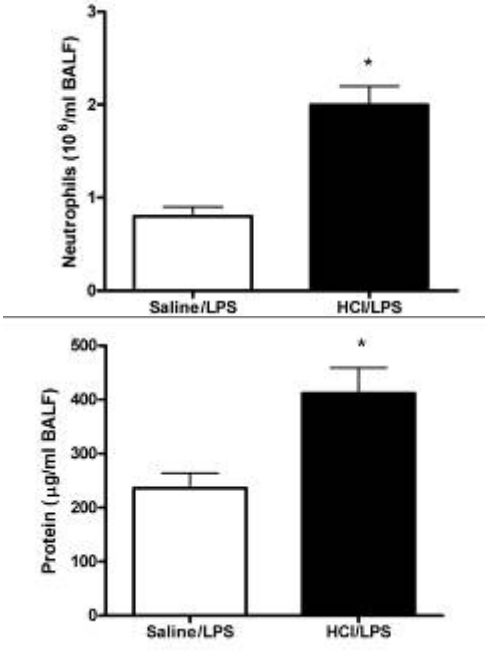
To evaluate the effects of previous HCl exposure on a subsequent sterile lung stimulus we administered *Klebsiella* LPS i.n. 16 hours after i.t. administration of HCl or saline, and evaluated the lung inflammatory reaction 6 hours later. Although the differences between the HCl and saline groups were less profound as compared with the pneumonia investigations described above, mice previously exposed to HCl displayed significantly more lung inflammation upon i.n. challenge with *Klebsiella* LPS.

Cytokine (pg/ml)	*Saline/LPS*	*HCl/LPS*
TNF-α	186 ± 21	245 ± 22*
IL-1β	2365 ± 411	3251 ± 649
MCP-1	443 ± 38	501 ± 56
IL-6	154 ± 13	313 ± 27*
IL-10	186 ± 22	282 ± 42
KC	2947 ± 154	5117 ± 898*
MIP-2	2965 ± 605	10623 ± 1144*

Table 3: Aspiration pneumonitis increases lung cytokine and chemokine concentrations induced by *Klebsiella* LPS Mice received either 50 µl 0.1 N HCl or an equivalent volume of saline i.t. Sixteen hours later mice were i.n. inoculated with 10 µg *K. pneumoniae* LPS; all mice were sacrificed 6 hours after LPS administration. Cytokine and chemokine concentrations were measured in lung homogenates. Data are mean ± SE (n = 8 mice per group). *P < 0.05 versus saline/LPS

Indeed, lung TNF-α, IL-6, MIP-2 and KC levels were higher in mice administered with HCl (Table 3), and these mice demonstrated a stronger influx of neutrophils into their BALF, increased pulmonary edema and higher BALF protein levels (Figure 4) and more inflammation upon histological analysis (Figure 5). Histology scores were higher in HCl/LPS mice as compared to saline LPS mice, 11 ± 1 versus 8 ± 1 respectively, although this did not

reach statistical significance (inflammation, pleuritis, edema and occasional endothelialitis were observed, P=0.09).



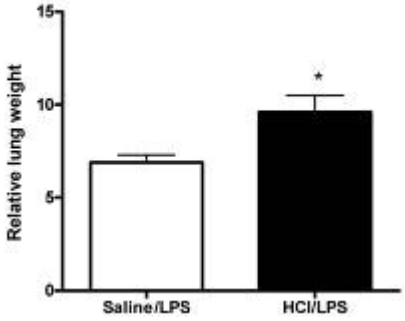


Fig. 4: Aspiration pneumonitis increases neutrophil influx, pulmonary edema and BALF protein levels during *K. pneumoniae* LPS induced lung injury. Aspiration pneumonitis was induced by i.t. injection of 50 ul of 0.1 N HCl, control mice received saline. Sixteen hours later mice were i.n. inoculated with 10 µg *K. pneumoniae* LPS. All were sacrificed 6 hours after LPS inhalation. Pulmonary edema was expressed as relative lung weight (mg of lung tissue/gram of mouse). Data are mean ± SE (n = 8 mice per group). *P<0.05 versus saline/LPS mice.

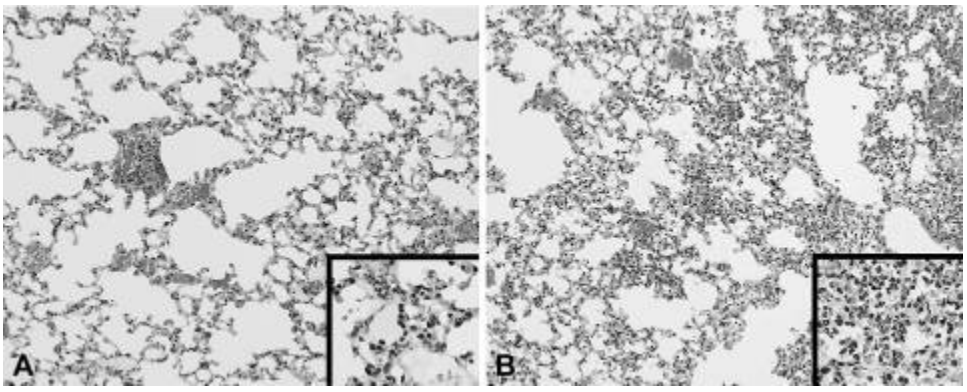


Fig. 5: Aspiration pneumonitis increases pulmonary inflammation during *K. pneumoniae* LPS induced lung injury. Aspiration pneumonitis was induced by i.t. injection of 50 ul of 0.1 N HCl, control mice received saline. Sixteen hours after aspiration mice were i.n. inoculated with 10 ug *K. pneumoniae* LPS. All were sacrificed 6 hours after LPS inhalation. Representative lung histology slides from saline/LPS (A) and HCl/LPS (B) mice are shown (n = 8 mice per group). H& E staining, magnification x 10, insert x 40.

Acid aspiration primes alveolar macrophages for enhanced TNF-α release

Alveolar macrophages are a major source for cytokines in the bronchoalveolar space[12,13]. We therefore considered it of interest to determine the influence of acid aspiration on the production of cytokines by alveolar macrophages. For this mice received either HCl or saline i.t.; after 16 hours their alveolar macrophages were harvested and stimulated “ex vivo” with *Klebsiella* LPS. In line with the in vivo data described above, alveolar macrophages obtained

from mice exposed to HCl released significantly more TNF-α than alveolar macrophages harvested from mice administered with saline (Figure 6).

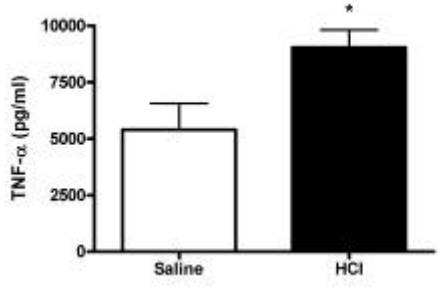


Fig. 6: Aspiration pneumonitis primes alveolar macrophages for enhanced TNF-α release. Aspiration pneumonitis was induced by i.t. injection of 50 µl of 0.1 N HCl, control mice received saline. Sixteen hours later mice were subjected to broncho alveolar lavage. Freshly isolated AM e (n = 8 per group: cells from two mice were pooled yielding four samples per group) were incubated with *K. pneumoniae* LPS (10 µg/ml) for 16 h before measurement of TNF-α. *P<0.05 versus saline aspiration mice.

Role of TNF-α in lung inflammation and bacterial outgrowth during primary and secondary *Klebsiella* pneumonia

TNF-α has been implicated as an important mediator in the innate immune response to *Klebsiella* pneumonia. Inhibition of TNF-α by a soluble TNF-α receptor-immunoglobulin construct has been found to reduce lung inflammation while facilitating bacterial outgrowth during *K. pneumoniae* pneumonia in previously healthy mice[27]. We here investigated whether this role for TNF-α is maintained during *Klebsiella* pneumonia preceded by acid aspiration. For this mice administered i.t with HCl or saline 16 hours before i.n. infection with *K. pneumoniae* received a neutralizing anti-mouse TNF-α antibody 30 minutes prior to induction of pneumonia.

	Control	*Anti-TNF-α*	*Control*	*Anti-TNF-α*
Cytokines (pg/ml)	**Saline/ pneumonia**	**Saline/ pneumonia**	**HCl/ pneumonia**	**HCl/ pneumonia**
IL-1β	2105 ± 312	3026 ± 625	26568 ± 4457*	32743 ± 5627 *
MCP-1	385 ± 122	436 ± 165	955 ± 224*	1460 ± 302*
IL-6	123 ± 10	391 ± 122*	1574 ± 736*	1604 ± 447*
IL-10	352 ± 27	297 ± 36	392 ± 38	283 ± 21
sTNF-RI	223 ± 18	246 ± 21	198 ± 16	216 ± 12
KC	1157 ± 947	6655 ± 1605*	2602 ± 837*	2906 ± 849*
MIP-2	2434 ± 415	5519 ± 1788*	4501 ± 879*	5854 ± 1892*
MPO (ug/ml)	15.5 ± 1.7	22.3 ± 3.9*	21.9 ± 2.6*	22.0 ± 1.8*
Rel.lung weight	8.3 ± 0.4	11.8 ± 1.9*	12.6 ± 1.3*	14.6 ± 2.3*

Table 4: Effect of anti-TNF-α during primary *K. pneumoniae* and pneumonia following aspiration pneumonitis Mice received either 50 µl 0.1 N HCl or an equivalent volume of saline i.t.. Sixteen hours after aspiration mice were i.n. inoculated with 9000 *K. pneumoniae* CFU.Groups of saline/pneumonia and HCl/pneumonia mice were pretreated with a neutralizing anti-TNF antibody 30 mins prior to induction of pneumonia. All mice were sacrificed 24 hours postinfection. Cytokine and chemokine concentrations were measured in lung homogenates. Data are mean ± SE (n = 8 mice per group). *P<0.05 versus control saline/pneumonia mice.

In mice that had received saline, anti-TNF-α treatment increased the inflammatory response during *Klebsiella* pneumonia, as reflected by higher IL-6, MIP-2 and KC concentrations in lung homogenates, higher relative lung weights (Table 4) and more inflammation, as reflected by MPO levels (Table 4) as well as upon histological analysis (Figure 7, A versus B) with histology scores of 6 ± 1 in control and 13 ± 2 in anti TNF-α treated mice ($P<0.05$).
Of note, the anti inflammatory response was unaffected by anti-TNF-α treatment, independent of previous acid or saline aspiration, since levels of IL-10 and sTNF-R were comparable between all groups (Table 4). The increased inflammation in anti-TNF-α treated mice was accompanied by an approximately 100-fold higher bacterial load in the lungs of these animals ($3.0 \pm 1.0 \times 10^5$ versus $375 \pm 166 \times 10^5$ per ml lung homogenate respectively, $P < 0.05$). By contrast, in animals previously exposed to HCl, anti-TNF-α did not influence lung inflammation (Table 4, Figure 7, C versus D), histology scores 14 ± 2 in control and 13 ± 2 in anti TNF-α treated mice ($P>0.05$), nor bacterial outgrowth ($8 \pm 4 \times 10^7$ per ml lung homogenate in anti-TNF-α treated mice versus $13 \pm 6 \times 10^7$ per ml lung homogenate respectively in control mice, $P = >0.05$). Of note, in these experiments acid aspiration increased lung inflammation during *Klebsiella* pneumonia in mice not treated with anti-TNF-α, confirming the results presented in Table 2 and Figures 2 and 3.

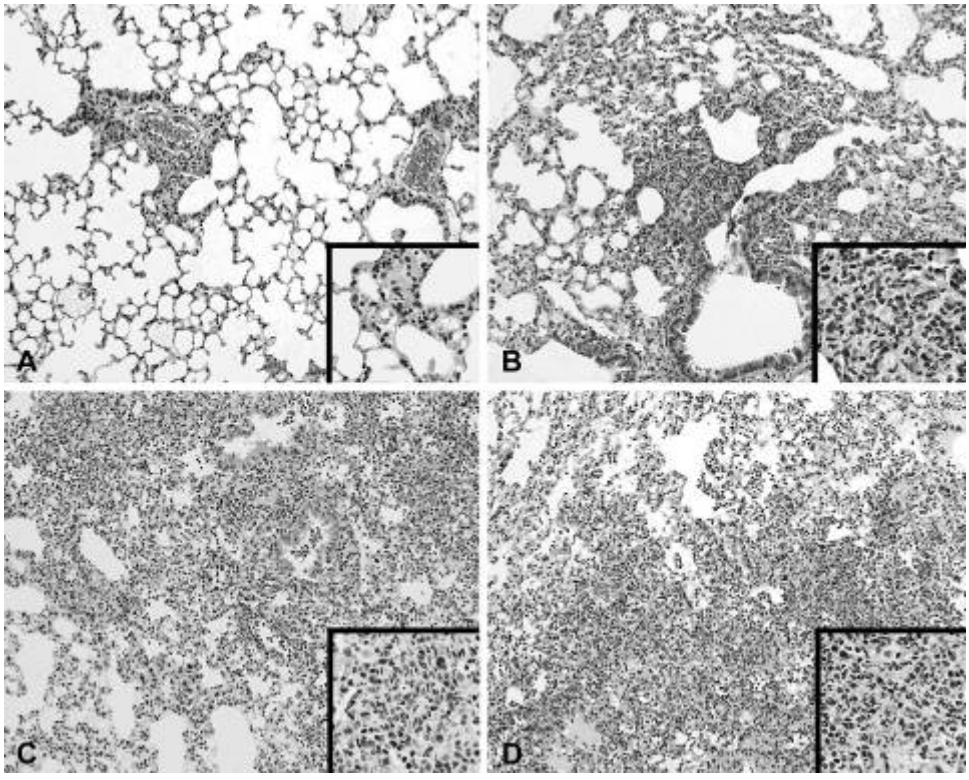


Fig. 7: Anti TNF-α treatment increases inflammation during pneumonia preceded by saline aspiration but not during pneumonia preceded by aspiration pneumonitis. Aspiration pneumonitis was induced by i.t. injection of 50 µl of 0.1 N HCl, control mice received saline. Sixteen hours after aspiration mice were i.n. inoculated with 9000 CFU *K. pneumoniae.* Groups of saline/pneumonia (A and B) and HCl/pneumonia (C and D) mice were pretreated 30 minutes before induction of pneumonia with a neutralizing anti-TNF antibody (B and D). All mice were sacrificed 24 hours postinfection. H& E staining, magnification x 10, insert x 40.

Discussion

Nosocomial pneumonia is a common and severe complication in critically ill patients. Although some patients with nosocomial pneumonia do not suffer from preexisting lung disease, many display an inflammatory reaction in their pulmonary compartment, caused by various clinical conditions such as ventilator-induced lung injury, ARDS and aspiration pneumonitis, prior to developing pneumonia. The main objective of this study was to determine whether a mild and transient form of aspiration pneumonitis, induced by a single i.t. administration of HCl, influences the host response to subsequent *K. pneumoniae* pneumonia. We here show that previous exposure to HCl results in an exaggerated inflammatory reaction during pneumonia caused by both an increased bacterial outgrowth and priming of alveolar macrophages for the effect of *Klebsiella*. Inhibition of TNF-α, generally considered an important mediator of lung injury, did not reduce pulmonary inflammation in mice with pneumonia that had inhaled acid and even enhanced lung inflammation in mice with primary *K. pneumoniae* pneumonia.

Two earlier experimental studies investigated the pathogenesis of pneumonia in the host with aspiration pneumonitis[8,9]. Mitsushima et al.[8] infected mice with *Pseudomonas aeruginosa* five minutes after i.t. delivery of HCl or saline and found an increased bacterial load in the lungs of acid treated animals 24 hours later; the associated lung inflammatory response was not evaluated in that study. Rotta et al.[9] infected rats with *Escherichia coli* one minute after i.t. administration of gastric aspirate and observed higher bacterial counts and more lung injury in these animals 24 hours later when compared with rats that had received saline earlier. Our study differs from these earlier investigations not only in that we used a different respiratory pathogen, but in particular in that we chose an interval of 16 hours between the acid and bacterial challenges. It is not unexpected that concurrent administration of HCl [8] or gastric aspirate[9] with bacteria results in an enhanced inflammatory response when compared with administration of bacteria only. Indeed, acid aspiration perse causes neutrophil recruitment and proinflammatory cytokine release into the bronchoalveolar space [28,29,9,30,31] (and the present study) and it can be anticipated that the simultaneous instillation of another proinflammatory stimulus (*i.e*. bacteria) will elicit even more lung inflammation. This markedly differs from our study, in which the acid-induced inflammatory response was weaning at the time pneumonia was induced and lung histology was normal (data not shown). Our data show that even if the introduction of bacteria into the respiratory tract is delayed for 16 hours after induction of a mild aspiration pneumonitis, the lungs respond with an exaggerated inflammatory response. Considering that previous acid exposure resulted in a much stronger increase in lung inflammation after infection with live *K.pneumoniae* than after instillation of *Klebsiella* LPS, and considering that bacterial loads in mice administered with HCl were 1000-fold higher than in saline treated mice, it is likely that the enhanced lung inflammation during post-aspiration pneumonitis pneumonia is caused by a combination of the presence of a stronger proinflammatory stimulus (*i.e*. more bacteria) and triggering for an increased inflammatory reaction to a given stimulus. At least part of this augmented

inflammatory response is caused by priming of alveolar macrophages; indeed, our results suggest that aspiration pneumonitis renders alveolar macrophages more sensitive for subsequent inflammatory stimuli. Previous reports have indicated that severe sterile injury renders the host more susceptible to secondary infection due to subsequent deactivation of the innate and adaptive immune system, which is usually referred to as `immunoparalysis" or endotoxin tolerance[32,33,34,35]. It is known that systemic inflammatory responses caused by endotoxin or other related TLR stimuli in uninjured hosts can cause hyporesponsiveness to restimulation. However, endotoxin tolerance is a site specific process since in contrast to other residential macrophages alveolar macrophages do not develop endotoxin tolerance after intravenous injection with endotoxin[36]. In this study, we add a novel observation and show that when two intra pulmonary stimuli are combined aspiration pneumonitis induces hyper responsiveness of alveolar macrophages ex vivo as well as increased cytokine release in vivo. Of note, in early experiments as discussed above, we evaluated the kinetics of saline and HCL induced injury. In these studies we found that 24 hours after aspiration all parameters of lung injury had returned to normal, showing that this model is completely reversible within 24 hours (unpublished data from our group show that no recurrent lung injury is observed between 24 and 48 hours after HCL inoculation). On the basis of these findings we anticipated that in our subsequent studies where mice are sacrificed 40 hours after saline/HCL aspiration, lung injury due to HCL or saline aspiration was completely reversed. Therefore, we decided that in these later studies the yield of including saline/saline and HCL/saline control groups would be very low and, to minimize the number of animals in this study, these control groups were not included in experiments where saline and HCL aspiration were followed by pneumonia.

The mechanisms by which aspiration pneumonitis results in a reduced resistance against respiratory pathogens have not been fully elucidated. Likely, direct damage of the epithelial barrier lining the respiratory tract by a caustic effect of acid plays a role herein. In addition, acid exposure may stimulate the adherence of bacteria to airway epithelial cells[8,37]. It should be noted that an adequate inflammatory response in the lung to invading bacteria is important for an effective host defense. Indeed, inhibition of the activity of proinflammatory cytokines has been found to facilitate the outgrowth of *K. pneumoniae* and other respiratory pathogens in mouse models of primary pneumonia[12,13]. Apparently, the overexuberant inflammation in mice with preexisting aspiration pneumonitis does not help the host in mounting a stronger defense against infection.

TNF-α has been implicated as an important factor in the pathogenesis of various inflammatory lung diseases[10,11,12,13]. TNF-α concentrations have been found elevated in BALF of patients with ARDS or pneumonia[38,39,40,41,42], and in experimentally induced aspiration pneumonitis inhibition of TNF-α attenuated lung inflammation[43,44]. Moreover, several lines of evidence indicate that this pluripotent cytokine is of eminent importance for an adequate host response to *K. pneumoniae* pneumonia. In mice with primary *Klebsiella* pneumonia, inhibition of endogenously produced TNF-α with a soluble TNF receptor-immunoglobulin construct resulted in an enhanced outgrowth of bacteria[27]. Moreover

transient transgenic expression of TNF-α within the murine respiratory tract, using a recombinant adenoviral vector containing TNF-α cDNA, enhanced the clearance of *K. pneumoniae* from the lung[45]. In our current investigation we found > 10-fold higher TNF-α concentration in the lungs of mice with *K. pneumoniae* pneumonia and preexisting aspiration pneumonitis when compared with mice with primary pneumonia. In line with the study by Laichalk et al.[27], we found that anti-TNF-α treatment strongly enhanced the outgrowth of *K. pneumoniae* in mice that received saline i.t. 16 hours before induction of pneumonia, which was accompanied by increased lung inflammation. However, in mice in which *K. pneumoniae* infection followed aspiration pneumonitis, anti-TNF-α did neither influence lung inflammation nor bacterial outgrowth. Together these data suggest that the relatively low TNF-α concentrations detected after primary *Klebsiella* pneumonia are of utmost importance for an appropriate immune response to the invading bacteria. In contrast, in mice with preexisting aspiration pneumonitis the strongly elevated TNF-α during pneumonia is part of an exaggerated overall proinflammatory response in the lung and by itself TNF-α does not impact on inflammation or antibacterial defense.

In conclusion, we here demonstrate that aspiration pneumonitis triggers the host to an enhanced inflammatory response to *K. pneumoniae* concurrently facilitating bacterial outgrowth. Although TNF-α plays a pivotal role in host defense against primary *Klebsiella* pneumonia, in the host with preexisting aspiration pneumonitis the strongly elevated TNF-α concentrations in the lungs are not involved in the enhanced lung inflammation or the increased bacterial outgrowth. Since neutralization of TNF-α in this setting was clearly not helpful one might wonder whether other anti-inflammatory strategies are likely to fail in this proinflammatory setting as well. It is conceivable that, although TNF-α is considered essential for the host response in this model, in the setting of an overall exaggerated proinflammatory response, as shown in aspiration + pneumonia mice, the relative importance of TNF-α is reduced. Therefore, subsequent studies evaluating the effects of anti-inflammatory strategies with broader anti-inflammatory potential than mere TNF-α neutralization are of considerable interest in this setting. The different roles of TNF-α in primary and secondary *K. pneumoniae* pneumonia underline the need to study the pathogenesis of pneumonia and potential new treatment targets not only in healthy animals but also in animals with preexisting disease.

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Part II

The Role of Pattern Recognition Receptors in the Innate Immune Response

CHAPTER 8

The importance of CD14, LBP and Toll-like Receptors in acute infections

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Introduction

The innate immune system mediates the first line of defense against invading pathogens and contains the infection prior to the induction of the adaptive immune response. The most important effector cells of the innate immune system are neutrophils and macrophages, which are able to phagocytose and engulf pathogens, secrete a vast array of cytokines and chemokines and thereby coordinate additional host response mechanisms. The innate immune system simultaneously controls the activation of the adaptive immune response and determines the appropriate effector response to a given pathogen.

The first task of innate immune cells is to recognize and discriminate potential pathogens from self. To meet this challenge, phagocytes recognize conserved microbial patterns via a restricted number of so-called pattern recognition receptors (PRR). Although a number of PRR involved in opsonization, complement activation and phagocytosis have been described earlier, the very crucial receptors responsible for the induction of inflammatory genes have been unknown until recently.

The discovery of Toll-like receptors (TLRs) changed this situation dramatically. Named after the *Drosophila* protein Toll, which plays a role in the antifungal immune response of the fruitfly, the mammalian homologues of TLRs recognize distinct microbial patterns and subsequently initiate the production and secretion of cytokines and chemokines that are important for an effective host defense (1). At present, the TLR family is known to consist of 10 members (TLR1-10) and much has been learned about the interaction of specific pathogens with TLRs, although the ligands for some TLRs are still unknown. In this chapter we will give a comprehensive overview of the current knowledge about this newly discovered family of microbial PRR. In addition, we will discuss the importance of the myeloid lipopolysaccharide-receptor CD14 and the lipid transfer protein, LPS-binding protein (LBP), in the innate immune response to bacteria.

The role of LBP and CD14 in the innate immune response

The initial event triggering the innate immune response to invading pathogens pertains to the recognition of microbes. To accomplish this goal, innate immune cells express receptors that recognize highly conserved microbial molecules, also called pathogen associated molecular patterns (PAMPs). LPS is a prominent example of these PAMPs and one of the most potent bacterial agonists known. As a major constituent of the outer cell wall of Gram-negative bacteria, LPS serves as the principal mediator of the inflammatory response to these pathogens. The understanding of essential features of the innate immune response to LPS has increased dramatically with the discovery of LBP and CD14 (2, 3). LBP is an acute phase protein that is predominantly produced by hepatocytes upon stimulation with cytokines like IL-1β and IL-6 (4). As a consequence, elevated LBP serum levels can be measured in severe sepsis (5, 6). LBP acts as a lipid-transfer protein that captures LPS out of aggregates that are formed by LPS monomers due to its amphiphilic properties and transfers it to CD14 (3, 7) (Fig. 1).

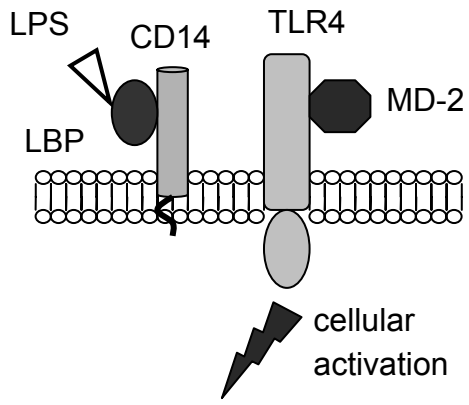

Fig. 1: The LPS-Receptor Complex: Lipopolysaccharide (LPS) monomers are captured by LPS-binding protein (LBP) and transferred to membrane bound CD14. CD14 that lacks a transmembrane domain in turn interacts with Toll-like receptor (TLR) 4 and MD-2, which eventually leads to signal transduction and the synthesis of proinflammatory cytokines.

The spontaneous diffusion of LPS to the cell-associated CD14 is very slow and transfer by LBP augments the cellular response to LPS up to 1.000-fold (8). The biological importance of LBP and CD14 has been illustrated by the fact that LBP or CD14 gene-deficient mice are resistant to endotoxin shock (9, 10) and highly susceptible to Gram-negative infections such as *Salmonella* peritonitis (9, 11, 12). We recently demonstrated, that LBP is an indispensable factor in the initiation-phase of the immune response to *Escherichia coli* peritonitis in mice (13). LBP-deficient mice succumbed rapidly to an intraperitoneal challenge with *E. coli*, due to diminished early cytokine and chemokine release leading to a delayed attraction of neutrophils to the peritoneal cavity with consecutively increased bacterial outgrowth and systemic dissemination of the infection (Fig. 2).

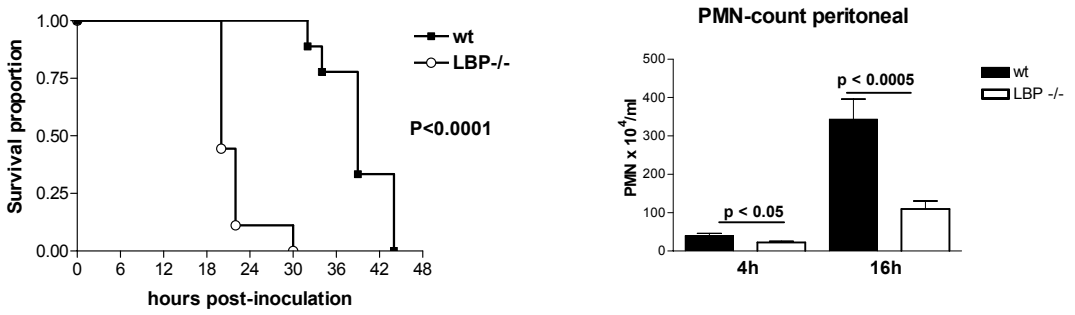

Fig. 2: The role of LBP in *E. coli* peritonitis. LBP gene-deficient ($LBP^{-/-}$) mice showed an accelerated mortality when compared to wild type (wt) mice following induction of *E. coli* peritonitis (left graph). The absence of LBP greatly impaired the influx of polymorphonuclear cells (PMN) to the peritoneal cavity during *E. coli* peritonitis (right graph). Adapted from Ref. 13.

A similar observation of impaired neutrophil recruitment in LBP-deficient mice was made in a murine *Klebsiella pneumoniae* pneumonia model (14). In addition, neutralizing LBP or CD14 with blocking antibodies led to a diminished tumor necrosis factor (TNF)-α production and impaired outcome in systemic *Klebsiella pneumoniae* infection (15). Hence, LBP plays an essential role in the initiation of the inflammatory response to Gram-negative infections *in vivo*.

Evidence exists, that LBP and CD14 are also involved in the immune response to Gram-positive bacteria and increased LBP and soluble CD14 plasma levels have been described in Gram-positive sepsis (6, 16). *In vitro* studies revealed that the inflammatory response to lipoteichoic acid (LTA) from various Gram-positive bacteria, e.g. *Bacillus subtilis, Staphylococcus aureus* and *Streptococcus pneumoniae* is enhanced in the presence of LBP and/or CD14 (17, 18). Moreover, peptidoglycan binds to CD14 *in vitro* and, highly interesting, an important *in vivo* role for LBP in the induction of the inflammatory response to pneumococcal peptidoglycan could be established in a murine meningitis model (19). Regarding CD14, there is no clear *in vivo* evidence supporting the notion that CD14 is a crucial receptor in host defense against Gram-positive pathogens. The only study investigating the *in vivo* role of CD14 in *Staphylococcus aureus* sepsis failed to prove a crucial role for CD14 (20).

Beside its clear role in facilitating the presentation of LPS to its receptor complex, LBP has also been demonstrated to be involved in the neutralization of LPS. Earlier reports illustrated that LBP does so by transferring LPS to HDL whereas more recently the association of LBP with LDL and VLDL lipoproteins has been revealed (21, 22). Of importance, the association of LBP with LDL/VLDL strongly enhanced the binding of LPS to these lipoproteins and, moreover, the association of LBP with lipoproteins was found to be even more pronounced in serum of septic patients, indicating an important role for LBP in the scavenging process of LPS during sepsis (22). After having demonstrated that LBP is also produced by intestinal epithelial cells, Vreugdenhil et al. could show that LBP associates with chylomicrons and mediates the detoxification of LPS, suggesting an important defense mechanism against the

toxic effects of translocated intestinal bacteria (23, 24). Another report revealed that high LBP levels, as found in septic patients, impair the inflammatory response of monocytes and macrophages *in vitro* and that the exogenous administration of high doses of LBP protected mice from lethality during *E. coli* peritonitis (though the exact mechanism has not been described) (25, 26). Beside LBP's properties to neutralize LPS via transfer to lipoproteins, a very recent report suggested that moderate to high LBP levels inhibit the inflammatory response to LPS by inhibiting the LPS transfer from CD14 to the TLR4/MD-2 signaling receptor (27). Together, the attractive possibility exists, that low LBP concentrations are indispensable for the induction of an appropriate inflammatory response at the onset of infection, whereas higher LBP concentrations, found at later stages during infection and in septic patients, neutralize LPS and thereby help to prevent overwhelming inflammation.
CD14 in its membrane-bound form (mCD14) is clearly involved in the induction of the inflammatory response as illustrated by the fact that the administration of a recombinant antibody directed against CD14 attenuated clinical symptoms and strongly reduced the production of proinflammatory cytokines in the human endotoxemia model (28). However, soluble CD14 (sCD14), that enables CD14 negative cells (e.g. endothelial cells) to mount an inflammatory response to LPS, has also been reported to participate in anti-inflammatory pathways. Soluble CD14 that is elevated in septic patients contributed to the neutralization of LPS by enhancing the transfer of cell-bound LPS to lipoproteins, which in turn diminished the LPS response of monocytes in septic patients (29).

Toll-like Receptor 4

Since CD14 is a glycosylphosphatidylinositol (GPI) anchored molecule that lacks a transmembrane domain and is incapable of transducing signals, other receptors must be responsible for LPS signaling. The missing link was identified in 1997 when the mammalian Toll analogue TLR was discovered (1) (Fig. 1). Positional cloning of the locus responsible for LPS hyporesponsiveness in C3H/HeJ and C57BL/10ScCr mice as well as the generation of TLR4 gene-deficient mice identified TLR4 as the receptor responsible for LPS signaling (30, 31). The interaction of LPS with TLR4 requires the association with MD-2. There is strong evidence that LPS does not directly interact with TLR4 but instead binds to MD-2 and that this process is facilitated in the presence of CD14 (32). The requirement for MD-2 is unique to TLR4 mediated responses and MD-2 gene-deficient mice are resistant to endotoxin shock, just like TLR4 gene-deficient animals (33).
Besides LPS, a number of other, structurally unrelated TLR4-ligands have been discovered over the last years. Clinically of high importance is the finding that pneumolysin, one of the crucial virulence factors of *S. pneumoniae*, signals via TLR4. Moreover, the recognition of pneumolysin by TLR4 has been demonstrated to correlate with the invasiveness of pneumococcal disease *in vivo* (34). This study also exemplified the fact that not just bacterial cell wall components but also bacterial toxins are capable of influencing inflammation via interaction with TLRs.

In addition, a number of endogenous molecules such as surfactant protein A, heat-shock proteins, fibronectin, fibrinogen and hyaluronan have been demonstrated to signal via TLR4 (35).

Toll-like Receptor 2

TLR2 is a quite promiscuous PRR that mediates the inflammatory responses to various bacterial molecules such as LTA, peptidoglycan, lipopeptides, zymosan from yeast, lipoarabinomannan from mycobacteria, porins from *Neisseria meningitidis*, or GPI-anchors from *Trypansomoma cruzi* (Table 1) (18, 36-38).

Receptor	Ligand	Origin	Ref.
TLR4	LPS	Salmonella, E. coli	(31)
	Pneumolysin	Strep. pneumoniae	(34)
	RSV fusion protein	Respiratory syncytial virus	(70)
TLR2	LTA	Staph. aureus, Strep. pneumoniae	(18)
	Peptidoglycan	Staph. aureus	(36)
+ TLR1	Lipopeptides	Bacteria	(45)
+ TLR1	Lipopeptides	Mycobacteria	(46)
+ TLR6	Lipopeptides	Myoplasma	(45)
+ TLR6	Zymosan	Yeast	(44)
	Lipoarabinomannan	Mycobacteria	(71)
	Porins	Neisseria meningitidis	(38)
	LPS	Legionella, Rhizobium	(72)
	LPS	Porphyromonas gingivalis	(48)
	LPS	Leptospira	(47)
	LPS	Helicobacter pylori	(49)
TLR3	dsRNA	Viral RNA	(55)
TLR5	Flagellin	Flagellated Gram-negative bacteria	(50)
TLR7	Imiquimod	Antiviral compounds	(56)
	ssRNA	Viral RNA	(58)
TLR8	ssRNA	Viral RNA	(57)
TLR9	CpG	Bacterial DNA	(61)
	CpG	Herpes simplex virus 2	(62)

Table 1. Toll-like Receptor Ligands

The fact that TLR2 signals the presence of LTA, peptidoglycan and lipopeptides, all cell wall components of Gram-positive bacteria, led to the postulation that TLR2 might be "the" PRR mediating the presence of Gram-positive bacteria. Accordingly, TLR2 gene-deficient mice were found highly susceptible to *S. aureus* sepsis *in vivo* (39). More recently, two studies investigated the importance of TLR2 in pneumococcal meningitis in mice and found an only moderate role for this receptor with respect to the bacterial clearance and the induction of an inflammatory response in this model (40, 41). In addition, we established that TLR2 is involved in the early inflammatory response in the lung during pneumococcal pneumonia, but is not important for the clearance of pneumococci from the pulmonary compartment or for survival (42). Another study reported that the inflammatory response induced by the Gram-positive pathogen *Listeria monocytogenes* only partially depended on the presence of TLR2 (43). Together, although these data indicate that TLR2 contributes to the host response to Gram-positive bacteria, the additional involvement of TLR2-independent pathways is likely.
There is abundant evidence that TLR2 has to functionally cooperate with other TLRs to signal the presence of pathogens. Analysis of TLR6 gene-deficient mice and the expression of a dominant negative TLR6 revealed that TLR2 associates with TLR6 to signal the presence of zymosan and mycoplasma lipopeptides (44, 45). In contrast, the response to bacterial and mycobacterial lipopeptides required the cooperation of TLR2 with TLR1 (46).
Noteworthy, some LPS that are structurally different from enterobacteria, have been demonstrated to signal via TLR2. These include LPS from *Leptospira interrogans*, *Porphyromonas gingivalis* and *Helicobacter pylori* (47-49).

Toll-like Receptor 5

Flagellin, the monomeric protein component from flagellum, acts as a very potent soluble immunostimulatory and proinflammatory factor. In 2001 TLR5 was identified as the receptor signaling the presence of flagellin from flagellated Gram-negative bacteria (50). TLR5 is preferentially expressed on monocytes, immature dendritic cells and epithelial cells and systemic flagellin administration induced a strong iNOS expression in intestinal epithelial cells (51). Other studies investigated the role of TLR5 in the gastrointestinal tract. Flagellin is secreted by commensal and pathogenic bacteria in the intestines but induces an inflammatory response only when translocated to the basolateral side. The finding that TLR5 is exclusively expressed on the basolateral surface of intestinal epithelial cells could explain this phenomenon (52). Of clinical importance and in contrast to commensal bacteria, *Salmonella typhimurium* can translocate flagellin independent of bacterial invasion and thus may induce intestinal inflammation and diarrhea via involvement of basolateral TLR5. Quite recently it has been shown that gastric epithelial cells recognize and respond to the presence of *Helicobacter pylori* via TLR5 and TLR2 (49).
High TLR5 expression has also been described in lung tissue and stimulating alveolar epithelial cells with flagellin induced a very strong inflammatory response *in vitro* (51). *In vivo* studies in mice treated with flagellin intravenously demonstrated that flagellin has the

capacity to cause acute lung inflammation. In accordance, it could be shown that plasma flagellin concentrations correlate with the severity of lung inflammation in septic patients (51). Therefore, flagellin may be involved in the development of acute respiratory failure during Gram-negative sepsis.

Very recently two groups generated TLR5 gene-deficient mice and studied the importance of this receptor during bacterial infections *in vivo* (53, 54). Of great interest, during systemic infection with flagellated, pathogenic *Salmonella typhimurium*, TLR4 is able to compensate for the lack of TLR5 (53). While no survival difference was observed between $TLR5^{-/-}$ and wild-type mice, $TLR4^{-/-}$ mice died faster; TLR4/TLR5 double knock-out animals were even more susceptible that $TLR4^{-/-}$ mice (53). Likewise, while the inflammatory response to intranasal flagellin strongly depended on the presence of TLR5, survival after infection with the flagellated bacterium Pseudomonas aeruginosa was only impaired in TLR4/TLR5 double knock-out animals (53). Another group reported that TLR5 is not expressed on macrophages and dendritic cells but preferentially expressed on $CD11c^{+}$ lamina propria cells (54). Using a model of oral Salmonella infection they nicely demonstrate that $TLR5^{-/-}$ are protected from lethality and display reduced bacterial loads in spleens and liver (54). However, they also observed no difference in survival when using an i.p. infection route. Of great interest, the bacterial load in mesenteric lymph nodes was greatly reduced in $TLR5^{-/-}$ animals after oral infection with *Salmonella typhimurium.* In other words, TLR5 expressed on $CD11c^{+}$ lamina propria cells recognizes intestinal Salmonella *in vivo* (54).

Toll-like Receptors 3, 7 and 8

TLR3, TLR7 and TLR8 are receptors that are most closely related to the host defense against viruses. TLR3, which is preferentially expressed on dendritic cells, was first identified as the receptor signaling the presence of viruses by recognizing double stranded RNA (55). In addition, signaling via TLR3 provides a strong adjuvant stimulus to dendritic cells, illustrated by upregulation of the costimulatory molecules CD80 and CD86 (55). In 2002 imiquimod was identified as a ligand for TLR7 (56). Imiquimod belongs to the family of imidazoquinolines, which are potent antiviral substances that induce the synthesis of interferons. Imiquimod is now approved for treatment of genital warts. TLR7 is expressed on both myeloid and plasmacytoid dendritic cells and stimulation leads to maturation and IL12p70 and/or interferon production. In 2004 the natural ligands for TLR7 and TLR8 have been identified as ssRNA (57, 58). Interestingly, while TLR7 signals the presence of ssRNA in mice, TLR8 does so in humans (57). Just like TLR3, TLR7 and TLR8 are localized within endosomes inside the cells, where they recognize the presence of viral infections (59). The finding that stimulation of dendritic cells with TLR7 ligands and the additional exposure to cytomegalovirus or HIV-1 enhances the virus-specific memory T-cell responses indicates that TLR7 might be an important receptor for adjuvants in vaccine development (60).

Toll-like Receptor 9

It has been know for quite a while that unmethylated bacterial DNA sequences are potent immunomodulatory molecules that induce a strong T-helper 1 response. However, the exact mechanism was poorly understood until the cellular receptor for these unmethylated CpG dinucleotides (CpG) was discovered to be TLR9 (61). Plasmacytoid dendritic cells express TLR9 and are known to produce type I interferons in response to viral infections. Moreover, the detection of viral CpG motifs from Herpes simplex virus 2 (HSV-2) by plasmacytoid dendritic cells was demonstrated to depend on TLR9 (62). In addition, the interferon production during HSV-2 infection *in vivo* required the presence of TLR9. TLR9 has also been implicated in the recognition of human IgG-chromatin complexes in B- lymphocytes (63). This latter finding implies an important role for TLR9 in autoimmunity.

Signaling pathways of Toll-like Receptors

TLRs are members of a larger superfamily of Interleukin-1 receptors (IL-1R) that share significant homology in their cytoplasmatic region, known as the Toll/IL-1R domain (TIR). Moreover, the TLR and IL-1R family signal via shared downstream signaling molecules that include the adaptor molecule myeloid differentiation protein 88 (MyD88), IL-1R associated protein kinases (IRAKs) and tumor necrosis factor receptor associated factor 6 (TRAF6) (Fig. 3). This association eventually causes activation of TAK1 that in turn leads to IKK activation, followed by IκB degradation and consequent release of NF-κB (64). Activation of TAK1 also results in the activation of mitogen activated protein kinases (MAPK).

MyD88 is considered a central element in TLR and IL-1R signaling. MyD88 deficient macrophages are completely unresponsive to peptidoglycan (TLR2), flagellin (TLR5), imiquimode (TLR7) and CpG DNA (TLR9), demonstrating the essential role of MyD88 in the response to these TLR ligands (64). However, the TLR3 dependent response did not depend on MyD88. The cytokine synthesis in response to LPS was almost abolished in MyD88 deficient animals whereas NF-κB activation was delayed but not completely abrogated. These data suggested that additional, MyD88-independent, pathways must be involved in TLR4 and TLR3 signaling. The search for MyD88-independent pathways first established another TIR domain-containing adaptor protein (TIRAP) that associates with MyD88 and is important for TLR4 and TLR2 signaling (65). More recently another adaptor molecule named TIR domain-containing adaptor inducing interferon-β (TRIF) has been described and data from TRIF deficient animals established that TRIF is responsible for MyD88-independent signaling in response to TLR4 and TLR3 ligands (66, 67). TRIF activates two distinct transcription factors, NF-κB and interferon regulatory factor 3 (IRF-3), and TRIF deficient mice are defective in both TLR3 and TLR4-mediated expression of INF-β and activation of IRF-3 (67, 68).

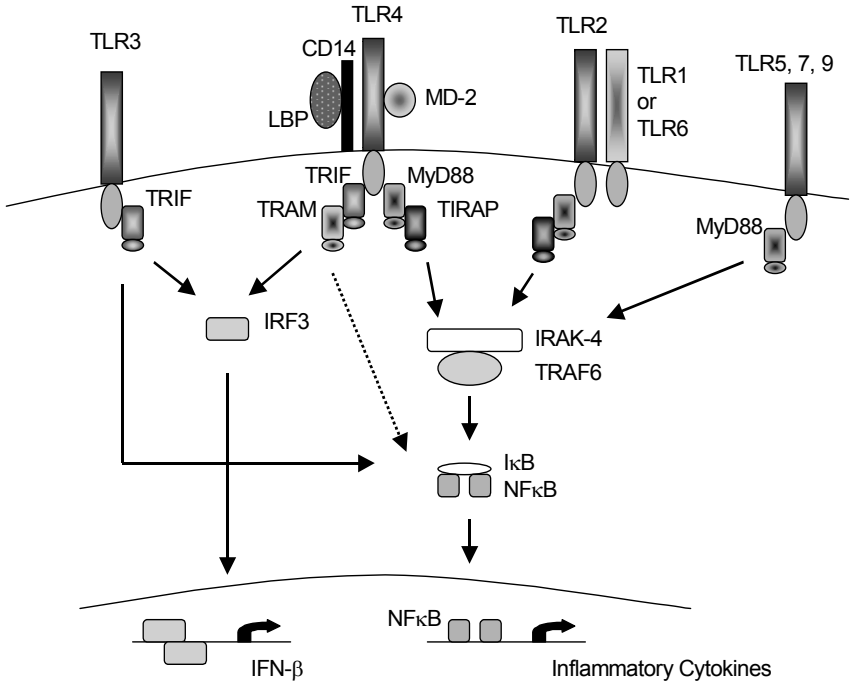

Fig. 3: The signaling pathway of the Toll-like Receptor family. Depicted are the TLR family members with known ligands. Signaling via most TLRs involves the adaptor protein MyD88 and eventually leads to NF-κB activation and the production of proinflammatory cytokines. A MyD88 independent pathway has been described for TLR4 and TLR3, which involves the adaptor protein TRIF and results in interferon-β production and NF-κB activation. Further details are explained in the text. Abbreviations: TIR: Toll/Interleukin-1 receptor domain; MyD88: myeloid differentiation protein 88; TIRAP: TIR domain containing adaptor protein; TRIF: TIR domain containing adaptor inducing interferon; TRAM: TRIF related adaptor protein; IRAK: IL-1R associated protein kinase; TRAF6: tumor necrosis factor receptor associated factor 6; IRF-3: interferon regulatory factor 3.

NF-κB activation in TRIF deficient mice was completely abolished in response to TLR3 ligands. In addition, cytokine production in response to TLR4 ligands, but not to any other ligand, was severely impaired in TRIF deficient animals while NF-κB activation was only abolished in MyD88/TRIF double knock-out mice. In other words, TLR4 signaling via MyD88 induces the early NF-κB activation whereas signaling via TRIF leads to late NF-κB activation and blocking both pathways abrogates any signals. The latest member of the TIR adaptor proteins described to day is called TRIF-related adaptor protein (TRAM). TRAM is involved in the TLR4-mediated MyD88-independent (TRIF-dependent) INF-β production and late NF-κB activation whereas TLR3 signaling was not affected by TRAM (69).

Together, MyD88 is a crucial adaptor protein in response to TLR2, 4, 5, 7 and 9 ligands. The additional requirement for TIRAP is characteristic for TLR2 and TLR4 only. The MyD88-independent pathway is mediated via TRIF and associated with type I IFN production and restricted to TLR3 and TLR4. TLR4 signaling via TRIF requires the additional involvement of TRAM (Fig. 3).

Conclusion

The inflammatory responses initiated by TLRs are crucial in the defense against pathogens. Ideally the inflammatory response to bacterial infections is effective, self-limiting and targeted towards the elimination of bacteria. However, uncontrolled and overwhelming inflammation - even in the absence of bacteria - can cause death from sepsis and multi organ failure. The exact factors responsible for the prolonged and excessive inflammation during sepsis are not entirely understood. It is quite evident, that the ultimate therapeutic goal is to preserve an effective immune response while preventing overwhelming inflammation. But how can we achieve this goal?

The discovery of TLRs and their complex signaling pathways will most likely help us to understand the multifaceted mechanisms involved in inflammation and may provide the basis for new therapeutic targets. Take, as an example, a patient with Gram-negative sepsis. The inflammatory response to a flagellated Gram-negative bacterium involves multiple TLRs such as TLR4 (signaling the presence of LPS), TLR5 (flagellin), TLR2 (lipopeptides) and TLR9 (CpG DNA). The resulting immune response will eventually lead to the elimination of the pathogen, but overwhelming, systemic inflammation might cause the symptoms of sepsis and acute respiratory distress. If flagellin, which is highly expressed on respiratory epithelial cells, is indeed a major factor responsible for acute pulmonary inflammation during sepsis, the selective inhibition of TLR5 might help prevent ARDS and may therefore prove beneficial to the patient without interfering with the antibacterial defense.

Future research will most likely help us to identify the multitude of factors involved in the elimination of pathogens and reasons for uncontrolled, overwhelming inflammation. This knowledge may eventually support the development of better pathogen specific therapies.

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CHAPTER 9

Lipopolysaccharide binding protein is an essential component of the innate immune response to *Escherichia coli* peritonitis in mice

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Abstract

LPS binding protein (LBP) is an acute phase protein that enhances the responsiveness of immune cells to LPS by virtue of its capacity to transfer LPS to CD14. To determine the role of LBP in the innate immune response to peritonitis, LBP gene deficient ($LBP^{-/-}$) and normal wild type mice were intraperitoneally infected with *Escherichia coli*, the most common causative pathogen in this disease. LBP was detectable at low concentrations in peritoneal fluid of healthy wild type mice; these local LBP levels increased rapidly upon induction of peritonitis. $LBP^{-/-}$ mice were highly susceptible to *E.coli* peritonitis, as indicated by an accelerated mortality, earlier bacterial dissemination to the blood, impaired bacterial clearance in the peritoneal cavity and more severe remote organ damage. $LBP^{-/-}$ mice displayed a diminished early TNF-α, IL-6, KC and MIP-2 production, and an attenuated recruitment of PMNs to the site of infection, indicating that acute inflammation was promoted by LBP. Locally produced LBP is an essential component of an effective innate immune response to *E. coli* peritonitis.

Introduction

Acute bacterial peritonitis is a life-threatening condition characterized by the presence of bacteria in the otherwise germ-free peritoneal cavity, almost invariably caused by perforation of intestines. Consequently, the most commonly encountered pathogens are enteric Gram-negative bacteria, among which *Escherichia coli* (*E. coli*) can be found in up to 60% of cases (14). Despite advances in surgery and antimicrobial therapy the mortality rate of peritonitis ranges between 30 and 50%. Above all, in sepsis that originates from peritonitis mortality rates can be as high as 80% (9). The acute course of peritonitis makes the instant initiation of host defense mechanisms mandatory.

The innate immune system enables the host to mount an immediate response to invading pathogens (17). Hence, the innate immune system is the central element of host defense in peritonitis. To recognize the presence of pathogens, innate immune cells express receptors that identify highly conserved pathogen associated molecular structures (16). LPS is the major constituent of the outer cell wall of Gram-negative bacteria and the principal mediator of inflammatory responses to these pathogens. CD14, Toll-like Receptor (TLR) 4 and MD-2 make up the LPS receptor complex involved in the cellular recognition of and signaling by LPS (6, 10, 19, 25). The earliest events after LPS release require the transfer of LPS to immune cells. Spontaneous diffusion of LPS to cellular binding sites is very slow as a result of the amphiphile structure of LPS and its tendency to form aggregates in aqueous solution. LPS binding protein (LBP) greatly enhances the transfer of LPS from aggregates to the CD14/TLR4 receptor complex (23, 25). As a lipid transfer protein, LBP recognizes and binds the lipid A portion of LPS and augments the immune response to LPS up to 1000-fold (15, 22, 26). LBP is an acute phase protein that is synthesized principally in hepatocytes and its production is greatly increased upon stimulation with interleukin (IL) -1β or IL-6 (20). Consequently, elevated LBP serum levels have been described in severe sepsis (2).

The biological significance of LBP was established when LBP gene deficient ($LBP^{-/-}$) mice were shown to be resistant to LPS toxicity and highly susceptible to *Salmonella typhimurium* peritonitis (5, 11). Similar observations in *Salmonella* peritonitis were made in mice lacking CD14 or TLR4, emphasizing the importance of these 3 components of the LPS recognition machinery in this Gram-negative infection (1). However, although these reports provide valuable information, these studies made use of an animal model of low clinical importance since *Salmonella* infections are food-born and almost never cause peritonitis. Attempts to investigate the *in vivo* contribution of LBP in the clinically relevant oral *Salmonella* infection could not reveal an important role for LBP (5). In addition, as an intra-cellular pathogen *Salmonella* relies on different host defense mechanisms than the extra-cellular pathogen *E. coli*, which is exemplified by the fact that $CD14^{-/-}$ mice are protected against lethality in *E. coli* peritonitis but succumb to *Salmonella* peritonitis (1, 6). According to these reports, CD14 deficiency was associated with an impaired polymorphonuclear leucocyte (PMN) influx in *Salmonella* peritonitis whereas an intense and early recruitment of PMN protected mice in *E. coli* peritonitis (7, 27).

Knowledge of the contribution of LBP to host defense against *E. coli* infection in general, and against *E. coli* peritonitis in particular, is highly limited. Fierer *et al.* reported no difference in survival of $LBP^{-/-}$ and wild type mice after high dose i.p. injection with *E. coli*, but did not show the actual data; the influence of LBP deficiency on local and systemic host responses was not reported (5). Le Roy *et al.* did not observe an effect on bacterial loads or cytokine release in mice treated with a blocking anti-LBP mAb and intravenously challenged with either high or low dose of *E. coli* (13). Nonetheless, evidence exists that LBP does play a role in *E. coli* peritonitis. Indeed, the administration of high doses of exogenous LBP (100μg) protected mice from lethality during *E. coli* peritonitis due to a proposed anti-inflammatory mechanism (12). In the present study we sought to determine the role of endogenously produced LBP in the early local and systemic host response to abdominal sepsis caused by *E. coli*, making use of $LBP^{-/-}$ mice.

Materials and Methods

Mice

$LBP^{-/-}$ mice were generated as described previously and backcrossed to C57BL/6 background 11 times (26). Pathogen-free 9-11 week old male C57BL/6 wild type mice were purchased from Harlan Sprague-Dawley (Horst, The Netherlands). Age and sex matched mice were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments.

Stimulation of peritoneal macrophages and whole blood

Wild type mice (n=8) were anesthetized by inhalation of isoflurane (Abbott Laboratories, Kent, U.K.) and peritoneal lavage was performed with 5ml of sterile saline using an 18-gauge needle. Lavage fluid was collected in sterile tubes and put on ice. Peritoneal macrophages were washed, counted and resuspended in RPMI 1640 containing 1mM pyruvate, 2mM L-glutamine, penicillin and streptomycin in a final concentration of $1x10^6$ cells/ml. Cells were then cultured in 96-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) for 2h and washed with RPMI 1640 to remove non-adherent cells. Adherent monolayer cells were stimulated with heat-killed *E. coli* (O18:K1, $1x10^8$ CFU/ml), or RPMI 1640 for 16h. Stimulations were carried out in the presence (10%) or absence of FCS (Gibco, Detroit, MI). Supernatants were collected and stored at –70°C until assayed for TNF-α (assay described below). Blood was drawn from $LBP^{-/-}$ and wild type mice after induction of deeper anesthesia by i.p. injection of 0.07ml/g FFM (Fentanyl 0.315mg/ml, Fluanisone 10mg/ml (both Janssen, Beersen, Belgium), and Midazolam 5mg/ml (Roche, Mijdrecht, The Netherlands). Whole blood was diluted in FCS-free RPMI 1640 1:5 and plated in 96-well plates, and stimulations were carried out as described for peritoneal macrophages.

Induction of peritonitis

Peritonitis was induced as described previously (21). In brief, *E.coli* O18:K1 was cultured in Luria Bertani medium (LB, Difco, Detroit, MI) at 37°C, harvested at mid-log phase and washed twice before inoculation. Mice were injected i.p. with 2 x 10^4 CFU *E. coli* in 200µl sterile saline. The inoculum was plated on blood agar plates to determine viable counts.

Monitoring of mortality and enumeration of bacteria

In survival studies, 9 mice per treatment group were inoculated with *E.coli* and mortality was assessed every 2 h. In separate studies, mice were sacrificed 4h or 16h after infection; at these time-points, mice were anesthetized by inhalation of isoflurane and peritoneal lavage was performed with 5ml of sterile isotonic saline using an 18-gauge needle. Lavage fluid was collected in sterile tubes and put on ice. After collection of peritoneal fluid deeper anesthesia was induced by i.p. injection of 0.07ml/g FFM (as described above). After opening of the abdomen blood was drawn from the lower caval vein and collected in sterile tubes containing heparin and immediately placed on ice. Liver lobes were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs were determined from serial dilutions of peritoneal lavage, liver homogenates and blood, plated on blood agar plates and incubated at 37°C for 16 h before colonies were counted.

Cell counts and differentials

Cell counts, determined on each peritoneal lavage sample stained with Türck's solution (Merck, Darmstadt, Germany), were counted in a hemocytometer (Türck counting chamber). The cells were then diluted to a final concentration of 10^5 cells/ml and differential cell counts were performed on cytospin preparations stained with Giemsa.

Assays

Murine LBP was measured using a commercially available ELISA (HyCult Biotechnology, Uden, the Netherlands) according to the manufacturer's instructions; the detection limit was 0.4 ng/ml. Cytokines and chemokines (TNF-α, IL-6, cytokine-induced neutrophil chemoattractant (KC) and macrophage inflammatory protein-2 (MIP-2)) were measured using specific ELISAs (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The detection limits were 31 pg/ml for TNF-α, 16 pg/ml for IL-6, 12 pg/ml for KC and 94 pg/ml for MIP-2. Alanine aminotransferase (ALAT) and creatinine were determined with commercially available kits (Sigma, St. Louis, MO) using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Histology

Lung and liver for histology were harvested at 4 or 16 h after infection, fixed in 4% formaline and embedded in paraffin. 4 µm sections were stained with hematoxylin and eosin (H&E), and analyzed by a pathologist who was blinded for groups. To score lung inflammation and

damage, the entire left lung was screened for the following parameters: interstitial inflammation, intra-alveolar inflammation, edema, endothelialitis, bronchitis, pleuritis and thrombi formation. To score liver injury, the following parameters were analyzed: formation of thrombi, hepatocellular necrosis, portal inflammation and endothelialitis. Each parameter was graded on a scale of 0 to 3 with 0: absent, 1: mild, 2: moderate, 3: severe. The total injury score was expressed as the sum of the score for all parameters, the maximum being 21 for the lung and 12 for the liver.

Statistical analysis

Differences between groups were calculated by Mann-Whitney *U* test. For survival analysis, Kaplan-Meier analysis followed by log rank test was performed. Values are expressed as mean ± SEM. A p-value < 0.05 was considered statistically significant.

Results

LBP is detectable in peritoneal lavage fluid

To investigate whether constitutively produced LBP is detectable in peritoneal fluid of healthy uninfected mice and whether peritonitis induces the local and systemic increase of this protein, we measured LBP concentrations in peritoneal lavage fluid (PLF) and plasma. In PLF, LBP was measurable at low levels in uninfected mice and increased 5-fold (4h) and > 30-fold (16h) after induction of *E. coli* peritonitis (Table 1). Plasma LBP concentrations remained merely unaltered 4h after induction of *E. coli* peritonitis and were modestly increased after 16h, when compared to uninfected mice (Table 1). LBP was not detectable in PLF or plasma of $LBP^{-/-}$ mice. Hence, peritoneal LBP concentrations showed an earlier and more pronounced rise than plasma LBP during *E. coli* peritonitis.

LBP concentrations	***PLF (ng/ml)***	***Plasma (μg/ml)***
Uninfected control	19.1 ± 2.7	8.6 ± 0.8
4h post *E. coli* i.p.	95.3 ± 27.5 $^{+}$	10.8 ± 1.0
16h post *E. coli* i.p.	645.5 ± 117.1* $^{+}$	30.9 ± 6.0 $^{\#}$

Table 1: LBP concentrations in peritoneal lavage fluid and plasma Mice (n=8-9) were infected with 2 x 10^4 CFU *E. coli* i.p. and sacrificed after 4 and 16h. LBP concentrations were measured with ELISA in peritoneal lavage fluid (PLF) and plasma. Samples of uninfected mice served as controls. Data are mean±SEM, $^{+}$ indicates $p<0.005$ and $^{\#}$ $p<0.05$ versus uninfected control, * indicates $p<0.0001$ versus 4h.

LBP is essential for responsiveness of peritoneal macrophages to heat-killed *E. coli in vitro*

To obtain a first insight into the requirement for LBP in the peritoneal host response to *E. coli* we determined the responsiveness of peritoneal macrophages to heat-killed bacteria. Freshly

isolated peritoneal macrophages from wild type mice, incubated under serum-free conditions, released lower amounts of TNF-α upon stimulation with heat-killed *E. coli* than wild type peritoneal macrophages supplemented with FCS (as a source of LBP) ($p<0.05$, Fig. 1). To ensure that these results are related to LBP and not to other proteins present in FCS, we repeated these experiments using whole blood derived from wild type and LBP$^{-/-}$ mice, stimulated in their own serum. The *E. coli* induced TNF-α release was diminished in blood from LBP$^{-/-}$ mice ($p<0.05$, Fig. 1). Hence, the responsiveness to *E. coli* depended on the presence of LBP.

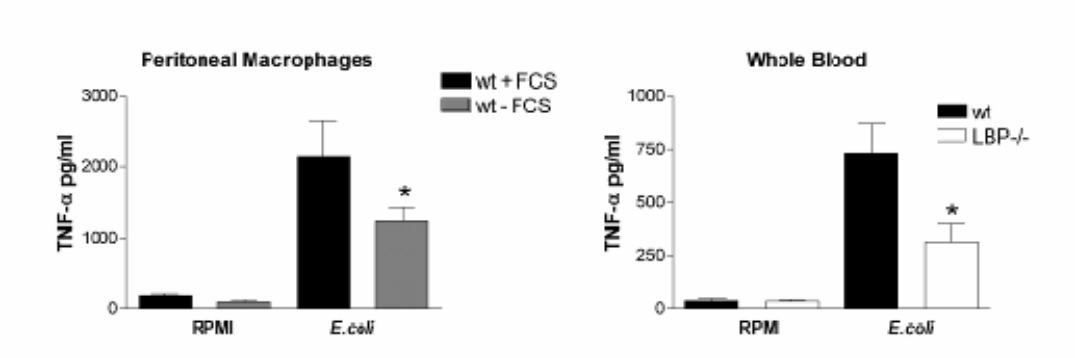

Fig. 1: Cellular responsiveness to *E. coli* depends on LBP. Peritoneal macrophages from wild type mice were stimulated in the presence or absence of 10% FCS with heat-killed *E. coli* O18:K1 ($1x10^8$ CFU/ml) for 16h. Blood was collected from wild type and LBP$^{-/-}$ mice (n=8) and stimulated for 16h. Depicted are mean ± SEM, * indicates $p<0.05$ versus wild type (whole blood) or versus wild type supplemented with FCS (peritoneal macrophages).

The absence of LBP renders mice more susceptible to *E. coli* peritonitis

To study the contribution of endogenous LBP to the outcome in peritonitis *in vivo*, wild type and LBP$^{-/-}$ mice (n=9 per strain) were inoculated with (2×10^4 CFU) *E.coli* i.p. and followed for 5 days (Fig. 2). LBP$^{-/-}$ mice died earlier than their wild type counterparts. Indeed, all LBP$^{-/-}$ mice succumbed to the bacterial challenge within 30 hours, a time-point at which all wild type mice were still alive. The last wild type mouse died as late as 44 hours after inoculation ($p<0.0001$ LBP$^{-/-}$ versus wild type mice). Thus, the lack of LBP rendered mice more susceptible to peritoneal infection with *E. coli*.

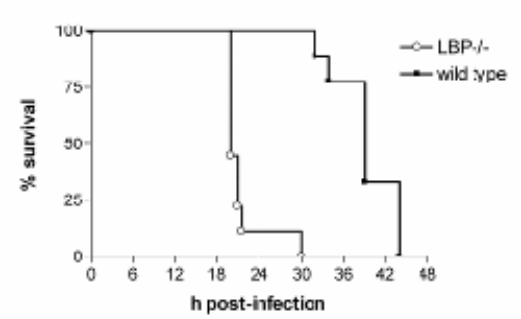

Fig. 2: Accelerated mortality in LBP$^{-/-}$ mice. Survival was monitored in wild type (filled symbol) and LBP$^{-/-}$ mice (open symbol) (n=9 per strain) after i.p. infection with 2×10^4 CFU *E.coli*. $P<0.0001$ by log rank test.

LBP is important to prevent early bacterial dissemination

To investigate how LBP contributes to host defense in acute peritonitis, we quantified the number of bacteria in PLF, liver and blood 4h and 16h after i.p. infection with *E. coli*.

Already 4h after induction of peritonitis significantly higher numbers of bacteria were obtained from blood samples of LBP$^{-/-}$ mice as compared to wild type animals (3.2 ± 0.7 x 10^4 CFU/ml in wild type and 11.2 ± 2.3 x 10^4 CFU/ml in LBP$^{-/-}$ mice, p=0.007, Fig. 3). CFU counts from both PLF and liver samples did not differ between the 2 strains at this early time-point (Fig. 3). Sixteen h after induction of peritonitis a considerably higher bacterial burden in peritoneal lavage fluid, liver, and – analogous to early findings – blood samples of LBP$^{-/-}$ animals was found (peritoneal lavage fluid: 2.2 ± 0.3 and 13.7 ± 3.7 x 10^8 CFU/ml, p=0.0002; liver: 0.9 ± 0.2 and 7.4 ± 3.3 x 10^8 CFU/ml, p<0.0001; blood: 0.4 ± 0.1 and 3.6 ± 1.4 x 10^8 CFU/ml, p=0.006; wild type and LBP$^{-/-}$ mice, respectively; Fig. 3). Therefore, LBP contributes to the host's capacity to control early bacterial dissemination and local, peritoneal bacterial clearance.

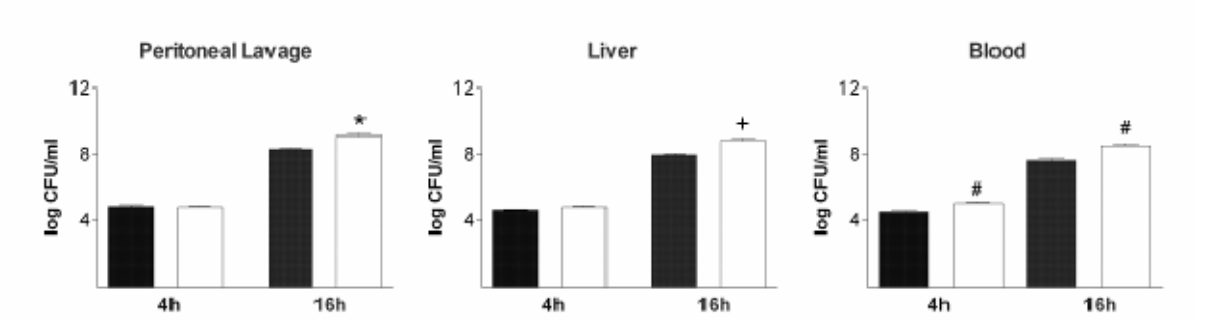

Fig. 3: Impaired bacterial clearance in LBP$^{-/-}$ animals. PLF, liver and blood CFUs 4 and 16h after i.p. infection with *E.coli* of wild type (filled bars) and LBP$^{-/-}$ mice (open bars). Data are mean ± SEM of n=8-9 mice per strain at each time-point. Results are representative of 2 independent experiments. $^{+}$ indicates p<0.0001, * p<0.0005 and # p<0.01 versus wild type mice.

LBP-deficiency is associated with impaired PMN influx

Having shown that LBP contributes to containment of the infection within the peritoneal cavity, we then asked which factors might be involved in the early spread of *E. coli*. Given that PMN influx to the site of infection is a hallmark of the innate immune response, we next determined leukocyte counts in PLF. Four h after the induction of peritonitis PMN influx was found moderately diminished in LBP$^{-/-}$ mice (p<0.05 versus wild type, Table 2). This difference became even more evident at later time-points, when a remarkably reduced number of PMN was observed in LBP$^{-/-}$ animals (p<0.0005 versus wild type, Table 2).

Time	***Mice***	***Total Cells*** x 10^4/ml	***PMN*** x 10^4/ml	***Macrophage*** x 10^4/ml
4h	Wild type	52±10.2	39.2±6.9	12.8±3.5
4h	LBP$^{-/-}$	47±4.8	22.2±3.6 *	24.8±4.6 *
16h	Wild type	409±56.1	343.7±52.3	53.8±11.2
16h	LBP$^{-/-}$	143±30.3 *	109.8±21 *	41.9±8.7

Table 2: Peritoneal cell counts and differentials in wild type and LBP$^{-/-}$ mice with *E.coli* peritonitis. Cell counts from peritoneal lavage samples 4 and 16h after i.p. infection with *E. coli*. Values are mean ± SEM of 8-9 mice per strain at each time-point; * p<0.05 versus wild type mice.

LBP is important for appropriate early cytokine/chemokine responses

Factors accountable for PMN attraction to the site of infection involve cytokines and chemokines produced by both macrophages and PMN. To investigate which mediators are influenced by the absence of LBP, we measured IL-6, TNF-α, KC and MIP-2, all factors known to play an important role in host defense against peritonitis. In accordance with impaired PMN attraction, peritoneal KC and MIP-2 concentrations were markedly reduced in $LBP^{-/-}$ mice 4h after induction of peritonitis (Fig. 4A). Likewise, IL-6 levels in peritoneal fluid were lower in $LBP^{-/-}$ animals (Fig. 4A). These mediators did not differ between the two mouse strains 16h after induction of peritonitis (data not shown). Peritoneal TNF-α concentrations, which were undetectable at 4h post-infection, were significantly higher in $LBP^{-/-}$ mice after 16h, which probably reflected the severe systemic infection mice suffered from by then (116 ± 22.1 pg/ml in wild type and 254 ± 44.8 pg/ml in $LBP^{-/-}$ mice, $p<0.05$). As a measure of the early systemic inflammatory response, TNF-α and IL-6 concentrations were quantified in plasma 4h after induction of peritonitis and found strongly decreased in $LBP^{-/-}$ animals (Fig. 4B).

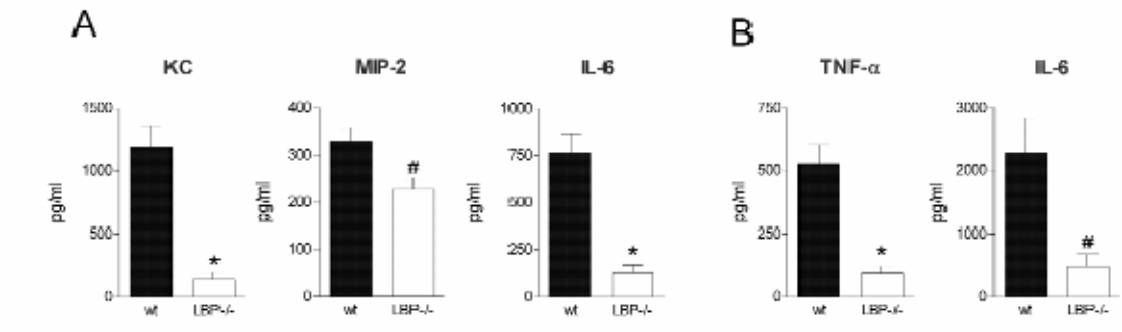

Fig. 4: Decreased CXC chemokine and cytokine concentrations in $LBP^{-/-}$ mice. A) Protein levels of KC, MIP-2 and IL-6 were measured in PLF and B) TNF-α and IL-6 concentrations in plasma of wild type (filled bars) and $LBP^{-/-}$ (open bars) mice (n=8-9 per strain) 4h after i.p. infection with *E. coli* (2 x 10^4 CFU). Data are presented as mean ± SEM, * indicates $p<0.0005$ and # $p<0.005$ versus wild type mice.

More severe multiple organ failure in $LBP^{-/-}$ mice

In line with the findings of earlier and increased bacterial dissemination in $LBP^{-/-}$ mice, these mice displayed more severe remote organ damage, as assessed by histology and clinical chemistry. The number of thrombi, extend of hepatocellular necrosis and portal inflammation in the liver as well as the degree of inflammation in lungs were higher in $LBP^{-/-}$ mice (3.5 ± 0.5 in wild type and 7.5 ± 0.7 in $LBP^{-/-}$ mice for liver samples ($p=0.002$) and 5.0 ± 0.5 in wild type and 6.8 ± 0.5 in $LBP^{-/-}$ mice for lung samples ($p<0.05$), according to inflammation scores described in M&M, Fig. 5). Multiple organ failure is a serious complication of severe sepsis. To investigate whether septic peritonitis was associated with multiple organ failure, we measured biochemical parameters for liver injury (ALAT) and kidney failure (creatinine) 16h after i.p. infection with *E. coli*. Higher elevations of ALAT and creatinine were detected in

LBP$^{-/-}$ animals, indicating more severe organ damage in these mice ($p<0.05$ versus wild type, Fig. 6).

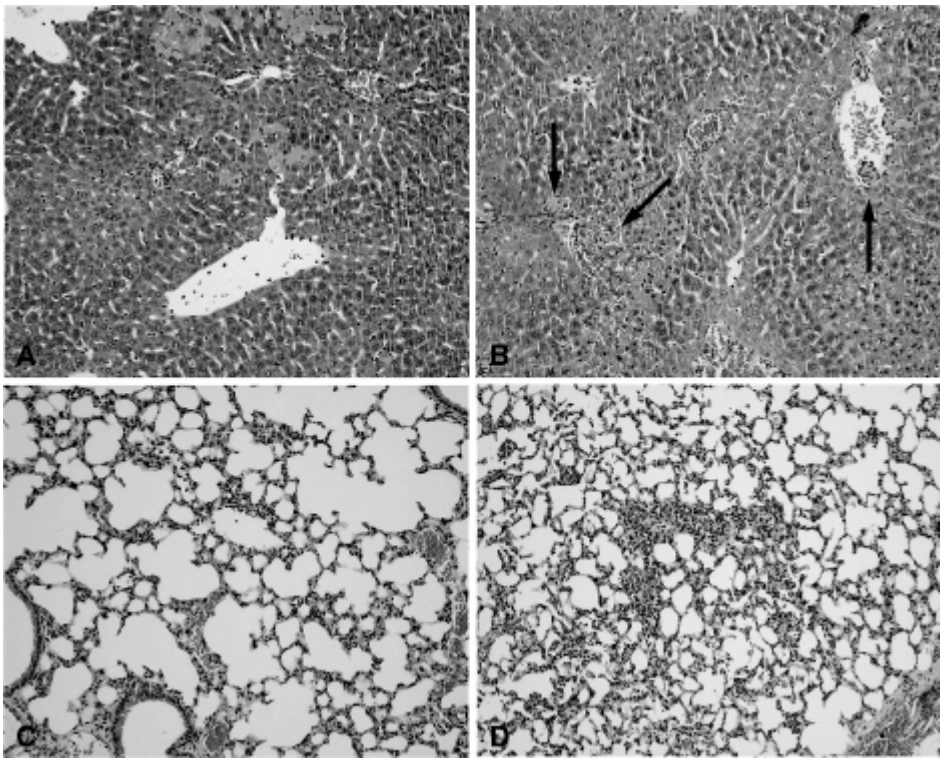

Fig. 5: More severe liver and lung injury in LBP$^{-/-}$ mice. Representative histological pictures of liver (A and B) and lung (C and D) from wild type (A and C) and LBP$^{-/-}$ (B and D) mice 16h after i.p. infection with *E. coli* (2 x 10^4 CFU). In the liver of LBP$^{-/-}$ mice (B) numerous thrombi (indicated by arrows) are observed together with inflammation and hepatocellular necrosis. Lung inflammation is also more pronounced in LBP$^{-/-}$ mice (D) compared to wild type mice. Representative of 8-9 mice per strain. H&E staining, magnification x 10.

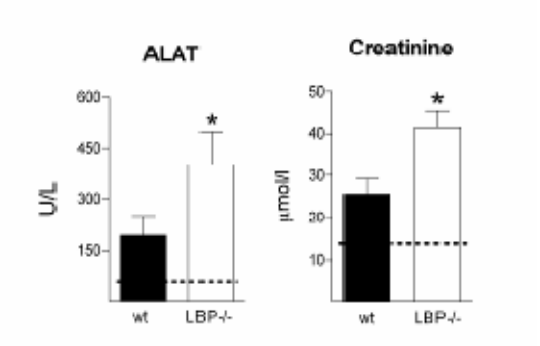

Fig. 6: **More pronounced liver and kidney injury in LBP$^{-/-}$ mice**. ALAT (liver injury) and creatinine (kidney failure) concentrations were measured in plasma of wild type (filled bars) and LBP$^{-/-}$ mice (open bars) 16h after i.p. infection with *E. coli* (2 x 10^4 CFU). Dotted lines represent the mean values obtained from plasma of uninfected mice. Data are mean ± SEM of 8-9 mice per strain; * indicates $p<0.05$ versus wild type mice.

Discussion

Peritonitis is a life threatening condition that is frequently associated with systemic dissemination of bacteria and septic shock. Host defense in peritonitis is a classical domain of the innate immune system as the rapid response to invading pathogens is essential for the host to survive. In this study we show that LBP is a crucial component in the early phase of host defense against *E. coli* peritonitis. We demonstrate that endogenous LBP is essential for the

clearance of *E. coli*, presumably via LBP's contribution to the early initiation of the inflammatory response, such as the production of cytokines, chemokines and (subsequent) attraction of PMNs to the peritoneal cavity. The delayed inflammatory response in the absence of LBP led to more severe signs of systemic infection including multiple organ failure and accelerated mortality.

Low levels of LBP were readily detectable in peritoneal fluid of uninfected wild type mice, establishing the local presence of LBP within the peritoneal cavity. Intra-peritoneal infection with *E. coli* induced a faster increase in peritoneal than plasma LBP concentrations. We hypothesized that this local, endogenous LBP might exert biological functions in peritoneal host defense and found an association with the initiation of the inflammatory response to *E. coli*. Lamping *et al.* reported a protective, anti-inflammatory role of exogenously administered high dose LBP (100μg) in D-galactosamine-sensitized mice injected with LPS and in *E. coli* peritonitis by showing reduced mortality rates in these mice (12). Since high-dose LBP was associated with reduced serum IL-6 and TNF-α levels upon LPS injection, they postulated that high concentrations of LBP exert protective effects by diminishing LPS toxicity. However, it is questionable whether such high LBP concentrations are ever reached under physiological conditions. Moreover, the beneficial mechanism of high dose LBP administration in *E. coli* peritonitis was not investigated. In our present study we found advantageous effects of physiological LBP levels in *E. coli* peritonitis, and attribute this to LBP's contribution to an effective pro-inflammatory host response. We believe that our model adequately reflects the biological situation found at the onset of peritonitis. High, acute phase LBP levels arise later, when the IL-1 and IL-6 induced LBP synthesis has been initiated, and usually peaks after 24h. It is tempting to speculate that low concentrations of LBP are essential for the initiation of an inflammatory response, whereas higher LBP concentrations represent an important anti-inflammatory mechanism to ameliorate the overwhelming inflammation seen at later time-points during septic peritonitis. Focusing on initial events following i.p. infection with *E. coli* we observed LBP $^{-/-}$ mice to be less capable to clear bacteria from the site of infection together with an earlier and more pronounced systemic dissemination. To determine how LBP is related to antimicrobial host defense in *E. coli* peritonitis we first investigated the cellular influx into the peritoneal cavity and found markedly reduced numbers of infiltrating PMNs in LBP$^{-/-}$ mice. It has been shown in peritonitis that bacterial clearance depends on the attraction of PMNs to the site of infection and that the CXC chemokines KC and MIP-2 are major contributors to PMN recruitment (5, 7, 24). Accordingly, we found significantly lower KC and MIP-2 concentrations in the PLF of mice lacking LBP, which might explain the inadequately low number of PMNs in LBP$^{-/-}$ mice infected with *E. coli*. In line with our findings, Fierer *et al.* observed a similar reduction in peritoneal CXC chemokines and a decreased PMN influx in LBP$^{-/-}$ mice 3h after i.p. infection with *Salmonella* (5). Since i.p. administration of casein or the Gram-positive pathogen *S. aureus* induced a comparable chemokine release and PMN influx in wild type and LBP$^{-/-}$ mice, it is likely that the lack of LBP specifically impairs the Gram-negative bacteria / LPS induced chemokine release and subsequent PMN attraction.

During *Salmonella* peritonitis TNF-α is another factor contributing to the attraction of PMNs to the peritoneal cavity, and $LBP^{-/-}$ mice could be rescued from *Salmonella*-induced lethality by exogenously administered TNF-α (8, 27). In accordance, in the absence of LBP, we found a reduced capacity of peritoneal macrophages and whole blood to release TNF-α upon stimulation with *E. coli in vitro*. Moreover, plasma TNF-α concentrations were substantially lower in $LBP^{-/-}$ mice after induction of peritonitis *in vivo*. These observations add to the notion that LBP contributes considerably to an effective early inflammatory response during *E. coli* peritonitis.

Our finding that LBP promotes acute inflammation in *E. coli* peritonitis matches with studies obtained in *Salmonella* peritonitis (5, 8, 11). Although it remains to be elucidated how PMNs exactly contribute to host defense against *Salmonella*, an intracellular pathogen, these cells seem to play a role in the model of *Salmonella* peritonitis – with LBP as an important effector molecule. However, previous studies performed in *E. coli* peritonitis could not disclose a vital role for LBP (5, 13). Differences in the *E. coli* strain used may have played a role. Whereas most investigators made use of *E. coli* O111:B4, we used the more virulent, encapsulated *E. coli* O18:K1 strain. Differences in host response to these two strains have been reported earlier. Although the peritoneal PMN influx depended on LBP when mice were challenged with heat-killed *E. coli* O111:B4, a protective role for LBP against lethality could not be demonstrated after intravenous or i.p. administration of live *E. coli* O111:B4 *in vivo* (5, 13). In addition, anti-CD14 antibodies did not impair outcome in *E. coli* O111:B4 infected mice but worsened bacterial clearance in *E. coli* O18:K1 challenged rabbits (13, 18). Moreover, C3H/HeJ mice, which are unresponsive to LPS due to a point mutation in the tlr4 gene, are highly susceptible to *E. coli* O18:K1 but not to *E. coli* O111:B4 infection (4, 7). Pretreatment with murine TNF-α/IL-1 led to restored bacterial killing and could rescue *E. coli* O18:K1 treated C3H/HeJ mice from lethality (3, 4). Together with our present observations, these data imply different host response mechanisms against these two *E. coli* strains. The strong dependency of *E. coli* O18:K1 on LBP, CD14 and TLR4 seems to rely on the greater need for additional stimuli like TNFα or IL-1 in order to enable phagocytes to kill bacteria. Accordingly we found an impaired bacterial clearance and attenuated TNF-α production in $LBP^{-/-}$ mice.

Host defense in peritonitis is a delicate balance between pro-inflammatory pathways intended to eliminate bacteria and anti-inflammatory pathways intended to prevent systemic inflammation (21). Any imbalance in pro- or anti-inflammatory mediators might prove harmful. In the current study, the inadequate onset of inflammation in $LBP^{-/-}$ mice led to early systemic dissemination and increased bacterial outgrowth, which in turn instigated systemic inflammation and multi organ failure. We therefore conclude that LBP is indispensable for the innate immune response in *E. coli* O18:K1 peritonitis and that its presence at physiological levels is mandatory for an adequate initiation of host response mechanisms.

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CHAPTER 10

Oxidized phospholipids inhibit phagocytosis and impair outcome in Gram-negative sepsis *in vivo*

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Submitted

Abstract

Oxidized phospholipids that are generated during inflammation exert anti-inflammatory properties and prevent death during murine endotoxemia. Oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (OxPAPC) inhibits the interaction of lipopolysaccharide (LPS) with LPS-binding protein (LBP) and CD14. We here determined the functional properties of OxPAPC and potential interference with CD14 during abdominal sepsis caused by *Escherichia (E.) coli*. Administration of OxPAPC rendered mice highly susceptible to *E.coli* peritonitis, as indicated by an accelerated mortality and enhanced bacterial outgrowth and dissemination. $CD14^{-/-}$ mice also displayed increased mortality and bacterial outgrowth and OxPAPC did not further impair host defense in these animals. The mechanisms by which OxPAPC and CD14 deficiency impaired the immune response differed: whereas $CD14^{-/-}$ mice demonstrated a strongly reduced recruitment of phagocytes to the site of the infection, OxPAPC did not influence the influx of inflammatory cells but strongly diminished the phagocytosing capacity of neutrophils and macrophages by a CD14 independent mechanism. Furthermore, OxPAPC potently inhibited uptake of fluorospheres as well as receptor-mediated endocytosis and fluid-phase pinocytosis. These data suggest that oxidized phospholipids such as produced during inflammatory reactions may contribute to mortality during Gram-negative sepsis *in vivo* via impairment of the phagocytic properties of professional phagocytes.

Introduction

Acute bacterial peritonitis is a life-threatening infection characterized by the presence of bacteria in the normally germ-free peritoneal cavity. Almost invariably caused by perforation of intestines, the most frequently encountered pathogens are enteric Gram-negative bacteria such as *Escherichia coli* (*E. coli*), which can be found in up to 60% of the cases (1). Mortality rates of peritonitis range between 30 and 50% despite advances in surgery and antimicrobial therapy. A serious complication originating from peritonitis is systemic inflammation and sepsis with mortality rates of up to 80% (2).

The prompt initiation of host defense mechanisms is essential for the host to survive. The innate immune system, that is responsible for mounting an immediate response to invading pathogens, is considered the central element of host defense in peritonitis. Lipopolysaccharide (LPS) is a major constituent of the outer cell wall of Gram-negative bacteria, such as *E. coli*, and the principal inducer of inflammatory responses to these pathogens. CD14, Toll-like Receptor (TLR) 4 and MD-2 make up the LPS receptor complex involved in the cellular recognition of and signaling by LPS (3-8). LPS-binding protein (LBP) greatly augments the transfer of LPS to the CD14/TLR4 complex. Recently, we demonstrated the pivotal role of LBP during murine *E. coli* peritonitis: the rapid recruitment of polymorphonuclear cells (PMNs) to the site of infection critically depended on the presence of LBP and mice lacking LBP displayed a greatly increased bacterial outgrowth and dissemination that led to early death (9).

PMNs are key phagocytes required for the immediate elimination of bacteria. One of the most powerful weapons generated by activated PMNs are reactive oxygen species that are utilized for antibacterial defense. However, in parallel with the killing of bacteria, free radicals may damage host molecules, and in particular induce lipid peroxidation. Oxidized phospholipids (OxPL) are generated *in vivo* at sites of acute and chronic inflammation (10-12). Accumulating evidence suggests that OxPL are not merely by-products of the inflammatory response, but can actively regulate inflammation (13). Most previous studies focused on the pro-inflammatory effects of OxPL, which are thought to play a role in initiating and maintaining chronic inflammation such as atherosclerosis (13-15). However, it is increasingly recognized that OxPL at the same time possess potent anti-inflammatory properties, which include the direct antagonism of LPS recognition by cells of the innate immune system. Indeed, OxPL effectively inhibit the interaction of LPS with LBP, CD14 and TLR4 (16). The biological significance of this finding was further underlined by the observation that the exogenous administration of OxPL could prevent mortality of mice exposed to high doses of LPS; in this respect OxPL reproduced the LPS resistant phenotype of mice lacking LBP, CD14 or TLR4 (16-20).

The biological role of OxPL during Gram-negative infection with viable bacteria has not been studied so far. We therefore decided to investigate the effects of OxPL during *E. coli* induced abdominal sepsis *in vivo* and the role of CD14 herein.

Materials and Methods

Mice

Pathogen-free 9-11 week old male C57BL/6 wild type mice were purchased from Harlan Sprague-Dawley (Horst, The Netherlands). CD14 gene deficient ($CD14^{-/-}$) mice were obtained from Jackson laboratories (Bar Harbor, ME) (21) and backcrossed to C57BL/6 background 6 times. Age and sex matched mice were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments.

Phospholipids

1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC) and dimyristoyl-phosphatodylcholine (DMPC) were purchased from Sigma Aldrich (Vienna, Austria). Oxidized PAPC (OxPAPC) was generated by air oxidation as described recently and stored in chloroform at -70C (16). Directly before use, OxPAPC were dried in glass tubes under the stream of N_2 and resuspended in NaCl by vortexing.

Induction of peritonitis

Peritonitis was induced as described previously (9, 22, 23). In brief, *E.coli* O18:K1 was cultured in Luria Bertani medium (LB, Difco, Detroit, MI) at 37°C, harvested at mid-log phase and washed twice before inoculation. Mice were injected i.p. with 1-2 x 10^4 colony forming units (CFU) *E. coli* in 200µl sterile saline. The inoculum was plated on blood agar plates to determine viable counts. OxPAPC (12.5mg/kg) or control lipids (DMPC) (12.5mg/kg in 200µl NaCl) or the same volume of carrier were injected i.p. immediately before bacterial inoculation.

Monitoring of mortality and enumeration of bacteria

In survival studies, 8-12 mice per treatment group were inoculated with *E.coli*. Since mortality occurs primarily between 24 and 48 hours after infection in this model, mortality was assessed every 2 hours in this period; thereafter mortality was monitored every 6 hours. In separate studies, mice were sacrificed 4h or 20h after infection; at these time-points, mice were anesthetized by inhalation of isoflurane and peritoneal lavage was performed with 5ml of sterile isotonic saline using an 18-gauge needle. Lavage fluid was collected in sterile tubes and put on ice. After collection of peritoneal fluid, deeper anesthesia was induced by i.p. injection of 0.07ml/g FFM mixture (Fentanyl 0.315mg/ml, Fluanisone 10mg/ml (both Janssen, Beersen, Belgium), and Midazolam 5mg/ml (Roche, Mijdrecht, The Netherlands)). After opening of the abdomen blood was drawn from the lower caval vein and collected in sterile tubes containing heparin and immediately placed on ice. Liver lobes were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs were determined from serial dilutions of peritoneal lavage,

liver homogenates and blood, plated on blood agar plates and incubated at 37°C for 16 h before colonies were counted.

Cell counts and differentials

Cell counts, determined on each peritoneal lavage sample stained with Türck's solution (Merck, Darmstadt, Germany), were counted in a hemocytometer (Türck counting chamber). The cells were then diluted to a final concentration of 10^5 cells/ml and differential cell counts were performed on cytospin preparations stained with Giemsa.

Cytokine/chemokine assays

Cytokines and chemokines (tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, monocyte chemoattractant peptide (MCP)-1) were measured using the cytometric bead array (CBA) multiplex assay (Becton Dickinson, San Diego, CA) according to the manufacturer's instructions. The detection limits were 5 pg/ml. Keratinocyte-derived chemokine (KC), macrophage-inflammatory protein (MIP)-2 and IL-1β concentrations were determined using commercially available ELISAs (R&D Systems, Minneapolis, MN).

Bacterial killing assay

Bacterial killing was determined according to a protocol published recently (24). In brief, RAW 264.7 cells (ATCC, Manassas, VA) were mobilized using 5mg/ml lidocaine in PBS, washed and plated in 24-well plates at a density of $2x10^5$ cells/well. Cells were allowed to adhere for 2h at 37C and washed thoroughly with serum-free (SF) RPMI. *E. coli* O18:K1 were added at a multiplicity of infection (MOI) of 100 and spun onto cells at 2000rpm for 5min, after which plates were placed at 37C for 10min. Each well was then washed 5 times with ice-cold PBS to remove extracellular bacteria. To determine bacterial uptake after 10min, triplicate of wells were lysed with sterile H_2O and designated as t=0. Pre-warmed SF-RPMI with or without 50μg/ml OxPAPC was added to remaining wells and plates were placed at 37C for 5, 10, 30, 60 or 90min after which cells were again washed 5 times with ice-cold PBS and lysed as described above. Cell-lysates were plated in serial-fold dilutions on blood agar plates and bacterial counts were enumerated after 16h. Bacterial killing was expressed as the percentage of killed bacteria in relation to t=0 (percent killing = 100 – {(# CFU at time x / # CFU at time 0) x 100}).

Phagocytosis assays

Phagocytosis was evaluated in essence as described before (25, 26). Peritoneal lavage was performed in wild type and $CD14^{-/-}$ mice (n=8 per strain) using 5ml of sterile saline. Lavage fluid was collected in sterile tubes and put on ice. Peritoneal macrophages were washed, counted and resuspended in RPMI 1640 at a final concentration of $1x10^6$ cells/ml. Cells were then allowed to adhere in 12-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) over night. Adherent monolayer cells were washed thoroughly with Hanks' balanced salt solution (HBSS) and incubated with FITC-labeled heat-killed *E. coli* (O18:K1,

$1x10^8$ CFU/ml) in the presence or absence of 50μg/ml OxPAPC at 37C or 4C for 30min. Immediately thereafter cells were put on ice, washed in PBS, suspended in "Quenching" solution (Orpegen, Heidelberg, Germany) and analyzed using a FACSCalibur (Becton Dickinson). To obtain primary PMNs, wild type mice (n=8) were injected i.p. with 4% proteose peptone (Difco, Detroit, MI). The next day mice were anesthetized by inhalation of isoflurane (Abbott Laboratories, Kent, U.K.) and peritoneal lavage was performed with 5ml of sterile saline using an 18-gauge needle. Lavage fluid was collected in sterile tubes, washed twice and cells were seeded in tissue culture plates to get rid of adherent macrophages. Non-adherent cells were counted after 2 hours and consisted of >95% PMNs. Phagocytosis-assay was performed as described above. The phagocytosis index of each sample was calculated: (mean fluorescence x % positive cells at 37C) minus (mean fluorescence x % positive cells at 4C). The same procedure was followed when using RAW 264.7 cells with the exception of different incubation times (up to 240min) and an additional proteinase K (Promega, Leiden, the Netherlands) step (250μg/ml at RT for 30min) to remove extracellular bacteria before FACS analysis. In some experiments LPS (100ng/ml; from E.coli O55:B5, Sigma) was added during overnight adherence. When indicated anti-SR-A antibody (clone 2F8, Serotec, Kidlington, UK) or isotype control (Serotec) were added at 10μg/ml. Phagocytosis of fluorescent polystyrene microspheres (FluoroSpheres® 1μm, Molecular Probes, Eugene, OR) was performed with RAW 264.7 cells that were co-incubated with increasing doses of OxPAPC (10-50-100μg/ml), DMPC (50μg/ml) or carrier at 37C. Uptake was analyzed by FACS after 120min and related to 4C controls as described above.

Endocytosis/Pinocytosis assays

Cellular uptake of horseradish peroxidase (HRP; Sigma), FITC-dextran (Molecular Probes), Lucifer Yellow (LY; Molecular Probes) or mannosylated BSA-FITC (Sigma) was determined as reported earlier (27). RAW 264.7 cells were cultured in 12-well plates at a concentration of $0.5x10^6$ cells/well and allowed to adhere over night in RPMI supplemented with 10%FCS. The next day, cells were washed thoroughly in serum-free RPMI and FITC-dextran (1mg/ml), LY (1mg/ml) or mannosylated BSA-FITC (10μg/ml) were added and cells were incubated at 37C for indicated times. In some experiments excess mannan (from *Saccharomyces cervisiae*, Sigma) was added at a concentration of 3mg/ml. At indicated time-points, cells were put on ice, washed thoroughly with cold PBS and analysed by FACS analysis. Cells pulsed at 4C were used to determine background uptake. The uptake index of each sample was calculated: (MF * % positive cells at 37C) minus (MF * % positive cells at 4C). To determine uptake of HRP, cells were cultured as described above, HRP was added at indicated concentrations and incubated for 3h at 37C. Next, cells were washed 4 times, lysed with 0.05% Triton X-100 in 10mM Tris buffer, pH 7.4 for 30min. The enzyme activity of the lysate was measured using o-phenzlendiamine and H_2O_2 as substrate.

Statistical analysis

Differences between groups were calculated by Mann-Whitney U test or one-way ANOVA. For survival analysis, Kaplan-Meier analysis followed by log rank test was performed. Values are expressed as mean±SEM. A p-value <0.05 was considered statistically significant.

Results

OxPL impair survival during E.coli peritonitis *in vivo*

OxPL have been shown to improve survival during murine endotoxemia due to their capacity to reduce the bioavailability of LPS, thereby attenuating overwhelming systemic inflammation (16). We consequently were interested in studying the effect of OxPL such as oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholin (OxPAPC) on the course of septic peritonitis induced by viable Gram-negative bacteria, i.e. microorganisms that express LPS. Mice were inoculated i.p. with *E. coli* together with OxPAPC or vehicle and observed for 5 days. As depicted in Fig. 1A, OxPAPC treated mice started to succumb as early as 20h after infection while all control mice remained alive till t=38h. In total 92% (11/12) of OxPAPC treated mice died versus only 42% (5/12) of the control animals (p=0.004). Hence, the administration of OxPAPC rendered mice more susceptible to *E. coli* peritonitis.

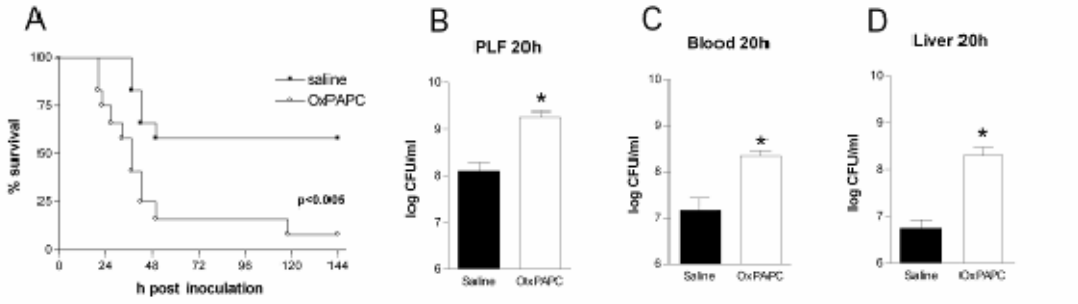

Fig. 1: OxPAPC increases mortality and facilitates bacterial outgrowth and dissemination. Mice received either OxPAPC (12.5 mg/kg i.p.; open symbols) or carrier (saline; closed symbols) and were i.p. infected with 1.2 x 10^4 CFUs *E.coli*. (A) Survival data are representative of two independent experiments of n=12 per group; p value indicates the difference between survivals by log rank test. (B) In separate experiments, mice were treated as above and PLF, blood and liver CFUs were determined 20 hours after infection. Data are mean±SEM of n=8 mice per group. Results are representative of 2 independent experiments. * indicates p<0.05 versus control mice.

OxPL facilitate bacterial growth and dissemination

To obtain insight in the mechanism underlying the accelerated and higher mortality of mice that received OxPAPC, we repeated this experiment and sacrificed mice 4 or 20h after infection to enumerate bacterial counts in peritoneal lavage fluid (PLF, the primary site of infection), blood and liver (to evaluate to which extent the infection became systemic). Already 4h after induction of peritonitis bacterial counts in OxPAPC-treated mice were up to one log higher than in controls, but the differences between groups did not reach statistical

significance (control versus OxPAPC (x 10^3 CFU/mL): PLF: 2.0±0.6 vs. 10.2±3.6; blood: 1.8±0.8 vs. 20.9±9.6; liver: 8.8±6.0 vs. 27.8±12.5). Thereafter, *E. coli* grew exponentially in all body compartments but much faster in OxPAPC treated animals and significantly increased CFU counts were recovered from PLF, blood and liver from these mice 20h after infection (Fig. 1B to D). Therefore, the i.p. administration of OxPAPC is associated with an increased bacterial outgrowth and dissemination during *E. coli* peritonitis.

OxPL transform wild type mice into a CD14-deficient phenotype

Because earlier findings pointed towards the fact that OxPL inhibit the interaction of LPS with LBP and CD14 (16) and since LPS is a major immunogenic component of *E. coli* (28), we next aimed to clarify whether the effects of OxPAPC described above could be explained by a functional CD14-blockade in the presence of these phospholipids. For this purpose $CD14^{-/-}$ and wild-type mice were treated with OxPAPC or control lipids (DMPC) and infected i.p. with *E. coli*. Survival studies disclosed that $CD14^{-/-}$ mice were highly susceptible to *E. coli* peritonitis and succumbed quickly (Fig. 2A).

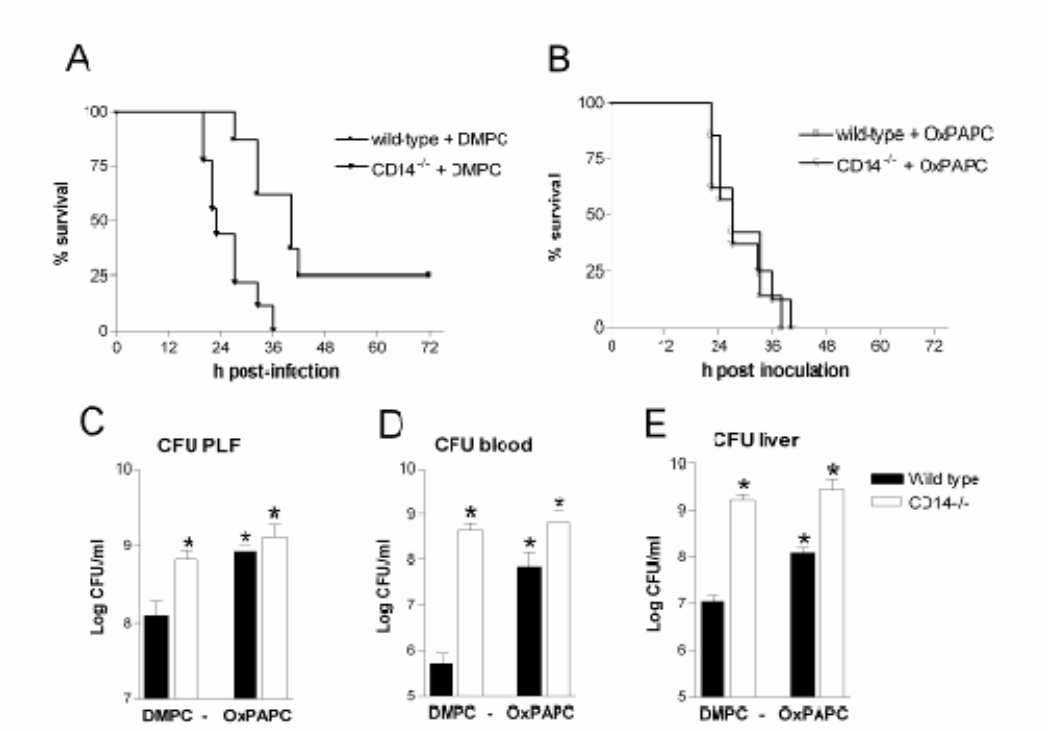

Fig. 2: OxPAPC does not further increase the enhanced mortality of $CD14^{-/-}$ mice. Wild type and $CD14^{-/-}$ mice were i.p. infected with $2x10^4$ CFUs *E. coli* in addition to administration of 12.5mg/kg control lipids (DMPC; A) or OxPAPC (B). All four groups of mice were infected simultaneously and survival curves were separated for reasons of clarity (all $CD14^{-/-}$ and/or OxPAPC treated mice succumbed at the same pace with $p<0.05$ versus wild-type mice that received DMPC). Survival was monitored over 1 week, n=8 per group; P value indicates the difference between survivals by log rank test. Experiments were repeated and mice were infected i.p. with $1.2x10^4$ CFUs *E. coli* 20h before bacterial counts were enumerated in PLF (C), blood (D) and liver (E). * $p<0.05$ versus wild type DMPC mice.

The administration of OxPAPC transformed wild-type mice into a $CD14^{-/-}$ phenotype whereas no additional effect on lethality was observed in $CD14^{-/-}$ animals treated with OxPAPC (Fig. 2B). Of note, to exclude potential non-specific effects of lipids, we used control lipids (DMPC) instead of carrier (NaCl) in this set of experiments. As expected, DMPC treated mice behaved exactly like mice that received carrier in earlier experiments, i.e. they showed an improved survival when compared to OxPAPC treated animals. Mortality rates were slightly higher in this experiment (compared to Fig. 1A), due to the – in retrospect – higher number of bacteria mice were infected with. We then repeated this experiment and enumerated bacterial counts 20h after infection. $CD14^{-/-}$ mice treated with control lipids (DMPC) displayed significantly increased bacterial counts in PLF, liver and blood when compared with DMPC treated wild type mice (Figure 2C-E). OxPAPC treatment enhanced bacterial outgrowth in wild type mice, confirming the experiments shown in Figure 1 (again illustrating the fact that DMPC treatment results in identical results as carrier treatment). Importantly, OxPAPC did not further increase bacterial loads in $CD14^{-/-}$ mice (Fig. 2C, D, E). In addition, although the numbers of *E.coli* CFU tended to be higher in blood and livers of OxPAPC treated $CD14^{-/-}$ mice than in OxPAPC treated wild type mice (Figure 2D and E), the differences between groups did not reach statistical significance. Together, these findings underline the importance of CD14 during Gram-negative peritonitis and lay emphasis on CD14 as a potential target molecule that might explain the detrimental effects induced by OxPL *in vivo*.

OxPL do not influence early cytokine/chemokine responses

Having shown that OxPL impaired outcome, we then asked which factors might be involved in the outgrowth and spread of *E. coli*. Responses accountable for an appropriate innate immune response during peritonitis include the local production of proinflammatory cytokines and chemokines at the site of the infection (26, 29, 30). Since OxPL can inhibit LPS bioavailability (16), we considered it possible that OxPAPC would impair the early cytokine/chemokine response to *E. coli* thereby enhancing susceptibility to abdominal sepsis. To address this issue we measured IL-6, TNF-α, IL-1β, MCP-1, KC, MIP-2 and IL-10 in PLF and plasma. As shown in Table 1, OxPL did not impair the early induction of pro-inflammatory cytokines after i.p. infection with *E. coli*. Peritoneal IL-6, TNF-α, MCP-1 and KC concentrations even were higher in OxPL treated mice at t=4h, which indicates that a suppressed early immune response cannot explain differences in outcome and bacterial elimination. Since PMN influx to the peritoneal cavity is a critical component of the innate immune response during peritonitis, we next determined leukocyte counts in PLF. However, 4h after induction of peritonitis we did not find any indication for an impaired leukocyte influx (Table 2).

	PLF		*Plasma*	
(pg/ml)	**Co**	**OxPAPC**	**Co**	**OxPAPC**
IL-6	28.4±6.7	256.6±77.7 ^{A}	1,535±423	2,559±791
TNF-α	8.8±0.5	14.3±2.0 ^{A}	436±173	639±201
IL-1β	136±51	358±38 ^{A}	ND	ND
MCP-1	152.6±29.1	548.9±137.8 ^{A}	6,230±1,431	6,732±1,386
KC	25.5±0.5	273.5±104 ^{A}	24,183±5,897	6,963±3,198 ^{A}
MIP-2	125.6±0.6	149.9±19.9	845±173	763±228
IL-10	89.4±5.2	98.1±4.1	4.9±1.3	19.6±3.1 ^{A}

Table 1: **OxPAPC does not impair the early cytokine/chemokine response**. Wild type mice (n=8 per group and time-point) were inoculated i.p. with $1.2x10^4$ CFU *E. coli* and 12.5mg/kg OxPAPC or carrier (NaCl, control mice). PLF and plasma was obtained after 4h and cytokines/chemokine concentrations were assayed as described in M&M. Data are mean±SEM, ^{A} indicates $p<0.05$ versus NaCl treated control mice; ND indicates: not detectable.

	4h (cellsx10^4/ml)		
	Total	**PMN**	**PM**
NaCl	37.2 ± 11.4	23.0 ± 6.6	14.2 ± 5.2
OxPAPC	108.0 ± 37.9	57.0 ± 22.8	51.0 ± 15.7 ^{A}

Table 2: OxPAPC does not attenuate early cell recruitment. Wild type mice (n=8) were infected i.p. with $1.2x10^4$ CFU *E. coli* and 12.5mg/kg OxPAPC or carrier (NaCl) and PLF was obtained after 4h and 20h. Total cell counts were determined and differentials done on cytospin preparations stained with Giemsa. Data are mean ± SEM, ^{A} indicates $p<0.05$ versus NaCl treated mice.

On the contrary, OxPAPC treated mice had the tendency to attract more leukocytes than control mice, likely as a result of the higher bacterial load in these mice. Thus, the number of PMN at the site of infection does not explain differences in bacterial clearance and outcome. When examining the local peritoneal and systemic cytokine/chemokine response in wild-type and $CD14^{-/-}$ mice at t=20h, we identified an enormously increased release of IL-6, TNF-α and MCP-1 in PLF and plasma of $CD14^{-/-}$ animals after 20h (Fig. 3), whereas MIP-2 and KC levels were similar in all groups (data not shown). These values by far exceeded the levels measured in wild-type mice treated with OxPAPC. Of interest, $CD14^{-/-}$ control mice (i.e. DMPC treated) had highest cytokine/chemokine concentrations, whereas $CD14^{-/-}$ animals that received OxPAPC showed less pronounced elevations (Fig. 3). Hence, OxPAPC modestly reduced the cytokine response in $CD14^{-/-}$ but not in wild type mice *in vivo*. On the other hand, $CD14^{-/-}$ animals displayed a severely impaired ability to attract PMNs and macrophages to the peritoneal cavity, irrespective of OxPAPC treatment (Table 3). Even 20h after the induction of peritonitis when vast amounts of bacteria were encountered in all mice, the number of

PMNs and macrophages was significantly reduced in the absence of CD14. Hence, while OxPAPC did not impair the attraction of inflammatory cells to the site of infection, CD14 crucially contributes to this important host defense mechanism.

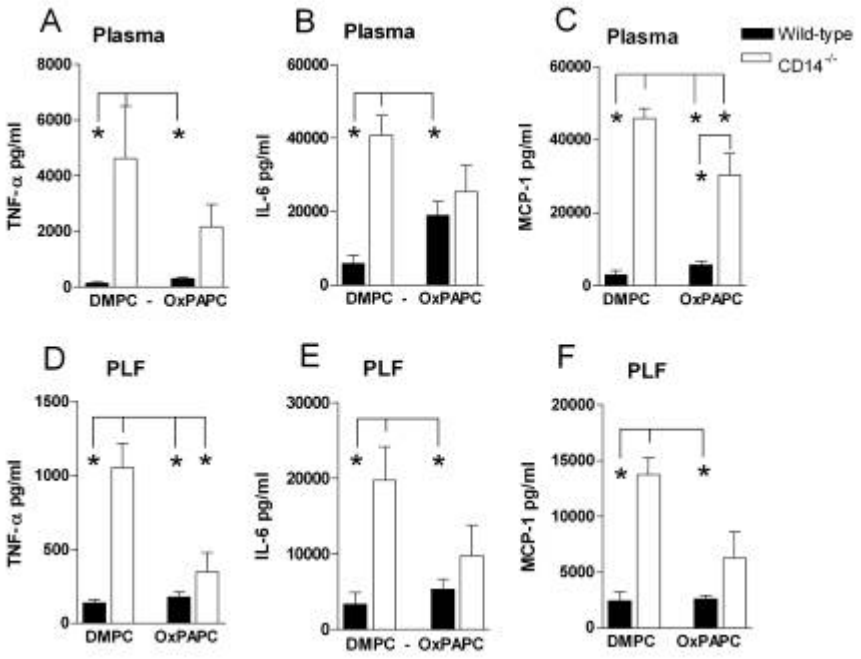

Fig. 3: Cytokine/Chemokine response in wild-type and CD14$^{-/-}$ mice treated with OxPL. Wild type (filled bars) and CD14$^{-/-}$ (open bars) mice were treated with 12.5mg/kg control lipids (DMPC) or OxPAPC and infected with 1.2x10^4 CFUs *E. coli* i.p.. PLF and plasma TNF-α, IL-6 and MCP-1 concentrations were measured after 20h. Mean±SEM; * p<0.05 versus indicated group.

Group	20h (cells x 10^6/ml)		
	Total	**PMN**	**PM**
Wt DMPC	23.3±1.6	18.9±1.3	7.1±1.2
CD14$^{-/-}$ DMPC	2.9±0.4 ^{A}	2.6±0.3 ^{A}	0.3±0.1 ^{A}
Wt OxPAPC	16.2±2.7	13.6±2.1	2.6±0.7
CD14$^{-/-}$OxPAPC	2.7±0.7 ^{A}	2.4±0.6 ^{A}	0.3±0.1 ^{A}

Table 3: Differential effects of CD14 deficiency and OxPAPC on cell recruitment. Wild type (Wt) and CD14$^{-/-}$ mice (n=8 per strain and treatment) were inoculated i.p. with 1.2x10^4 CFU *E. coli* and 12.5mg/kg OxPAPC or DMPC, respectively, and PLF was obtained after 20h. Total cell counts were determined in a Tuerck chamber and differentials done on cytospin preparations stained with Giemsa. Data are mean ± SEM, ^{A} indicates p<0.05 versus wild type mice that received the same lipid preparation.

OxPAPC impairs the phagocytic capacity of peritoneal macrophages and PMN

Having shown that OxPAPC affects CD14-independent pathways of the innate immune response, we next aimed to investigate the functional and cellular alterations induced by OxPAPC. Microscopic evaluation of infiltrating leukocytes in PLF of OxPAPC-treated mice revealed a high proportion of cells covered by enormous amounts of bacteria (Fig. 4A).

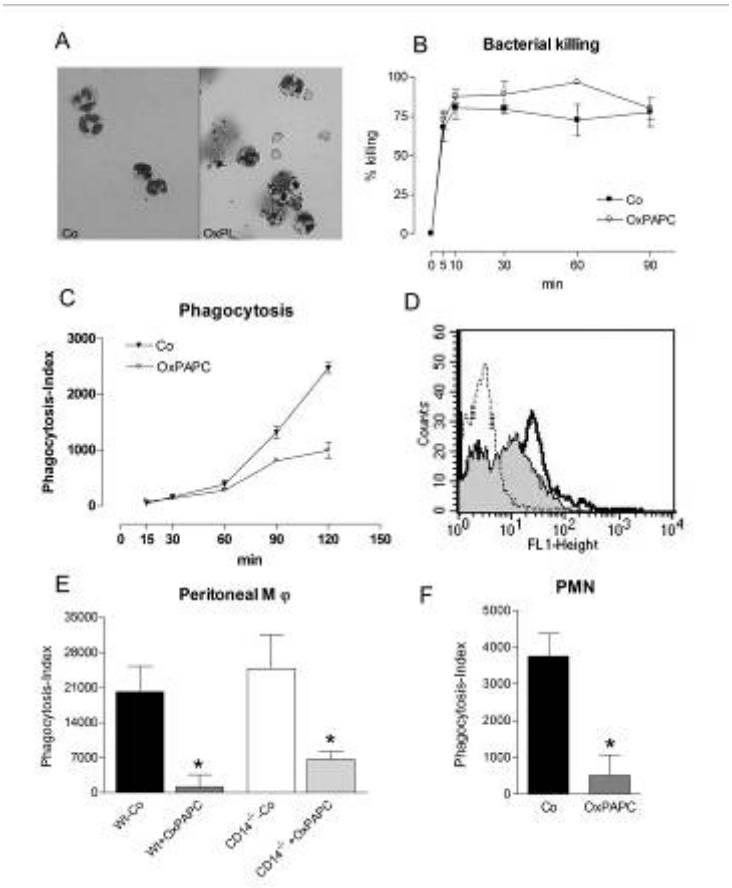

Fig. 4: Impact of OxPAPC on bacterial killing and phagocytosis. (A) Representative cytospin preparations of peritoneal lavage cells stained with Giemsa 20h after infection with $1.2x10^4$ CFU *E. coli*. (left: control mice, right: OxPAPC-treated animals). (B) RAW 264.7 cells were incubated with viable *E. coli* (MOI 100) and bacterial killing was assessed in the presence of carrier (Co) or OxPAPC (50µg/ml) over time. (C and D) RAW 264.7 cells ($1x10^6$/ml) were incubated at 37C with $1x10^8$ CFU/ml FITC-labeled *E. coli* and time-dependent phagocytosis was quantified in the presence or absence of OxPAPC (50µg/ml) by FACS analysis. (D) Representative histogram at t=120min: hatched line: 4C control; black line: 37C control group; filled grey: 37C OxPAPC group. Depicted are results from at least 2 independent experiments performed in triplicate. Primary peritoneal macrophages (E) or primary PMNs (F) of wild-type and $CD14^{-/-}$ mice (both $1x10^6$/ml) were incubated with $1x10^8$ CFU/ml FITC-labeled *E. coli* for 30min. Phagocytosis was quantified in the presence or absence of OxPAPC (50µg/ml) by FACS analysis. Data are mean±SEM of n=4 (E) or n=8 (F) per group; * indicates $p<0.05$ versus controls.

Although bacteria were also visible in samples from control mice, the number and close association with leukocytes was much less impressive. This led us to examine whether OxPAPC influences functional properties of leukocytes, such as killing or phagocytosis of bacteria. Using primary peritoneal macrophages and PMNs from C57/BL6 mice or RAW 264.7 peritoneal macrophages, we studied both phagocytosis and bacterial killing in the presence of absence of OxPAPC. As depicted in Fig. 4B, the addition of OxPAPC following uptake of bacteria did not affect the killing properties of macrophages. However, OxPAPC impaired the ability of phagocytes to internalize *E. coli* in a time-dependent manner (Fig. 4C and D). In addition, both primary peritoneal macrophages and PMNs showed an impaired phagocytosis of FITC-labeled *E.coli* in the presence of OxPAPC (Fig. 4E and F). Of

importance, CD14 did not interfere with the phagocytic properties of macrophages or PMNs, as indicated by unaltered phagocytosis rates of primary cells from $CD14^{-/-}$ mice (Fig. 4E). Together, these data showed that OxPAPC inhibits phagocytosis of *E.coli* but does not influence bacterial killing.

OxPAPC impairs endocytosis by macrophages

In order to further understand the impact of OxPL on phagocytosis, we then asked whether our finding of impaired phagocytosis in the presence of OxPAPC is specific for pathogens such as *E. coli*, or reflects a more general phenomenon. Since we performed all phagocytosis assays (Fig. 4) under serum-free conditions using non-opsonized bacteria, the possibility that OxPAPC interferes with Fc-receptor or complement-mediated phagocytosis is unlikely. However, scavenger receptors (SR-)A have been identified as important receptors that specifically contribute to Fc-receptor independent phagocytosis of *E.coli* (31). We therefore investigated the potential contribution of SR-A and found, in accordance with earlier reports (31), that blocking SR-A with anti-SR-A antibodies significantly reduced uptake of *E. coli* by macrophages (Fig. 5A).

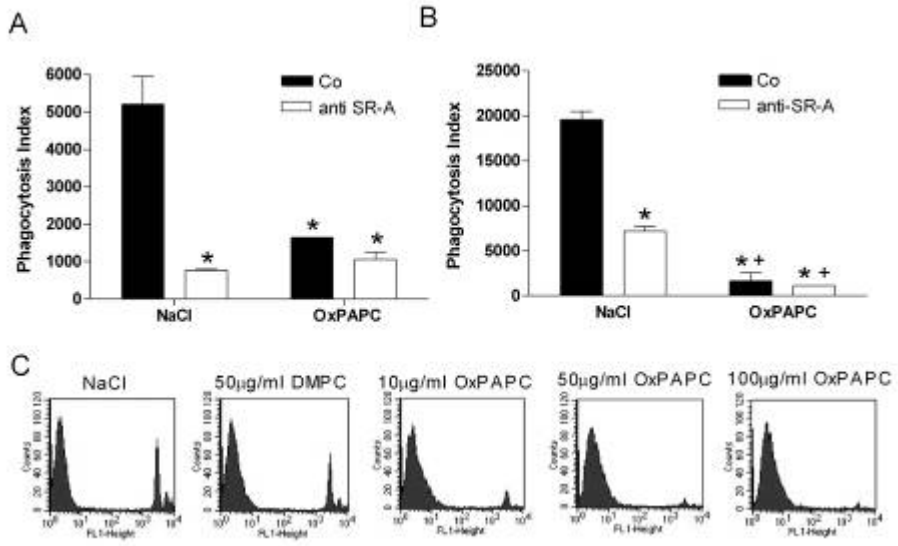

Fig. 5: OxPAPC impair phagocytosis of *E.coli* and Fluorospheres. Unstimulated (A) or LPS-stimulated (B) RAW 264.7 cells were incubated with $1x10^8$ CFU/ml FITC-labeled *E. coli* in the presence of carrier or OxPAPC (50µg/ml) and/or anti-SR-A (10µg/ml) or isotype control (Co) and uptake was assessed by FACS. Data are mean±SEM; * indicates $p<0.05$ versus carrier+isotype control, + indicates $p<0.05$ versus carrier+anti-SR-A. (C) RAW 264.7 cells were incubated with Fluorospheres in the presence of carrier, DMPC or indicated amounts of OxPAPC and uptake at t=120min was measured by FACS.

Addition of OxPAPC alone showed exactly the same inhibitory effect as anti-SR-A alone and no additive effect was observed when the combination of both reagents was tested (Fig. 5A). We then tried to more closely imitate the *in vivo* situation seen during peritonitis, when peritoneal macrophages encounter LPS or bacteria that activate them before they phagocytose whole bacteria, and pre-incubated macrophages with LPS prior to the addition of *E. coli*. As expected, phagocytosis of *E.coli* was tremendously increased when macrophages were pre-

activated by LPS (Fig. 5B; compare Y-axes of panels A and B). In addition, when anti-SR-A or OxPAPC were added to these activated macrophages, the inhibitory action of OxPAPC clearly exceeded that of anti-SR-A (Fig. 5B). Together these findings indicate that OxPAPC either interferes with phagocytosis receptors other than SR-A, or that OxPAPC utilizes additional (possibly activation-dependent) pathways. To assess SR-A-independent phagocytosis and the role of OxPAPC herein, we next investigated the uptake of polystyrene microspheres and identified that OxPAPC dose-dependently inhibited the uptake of these microspheres (Fig. 5C). Importantly, control lipids (DMPC) did not show this effect.

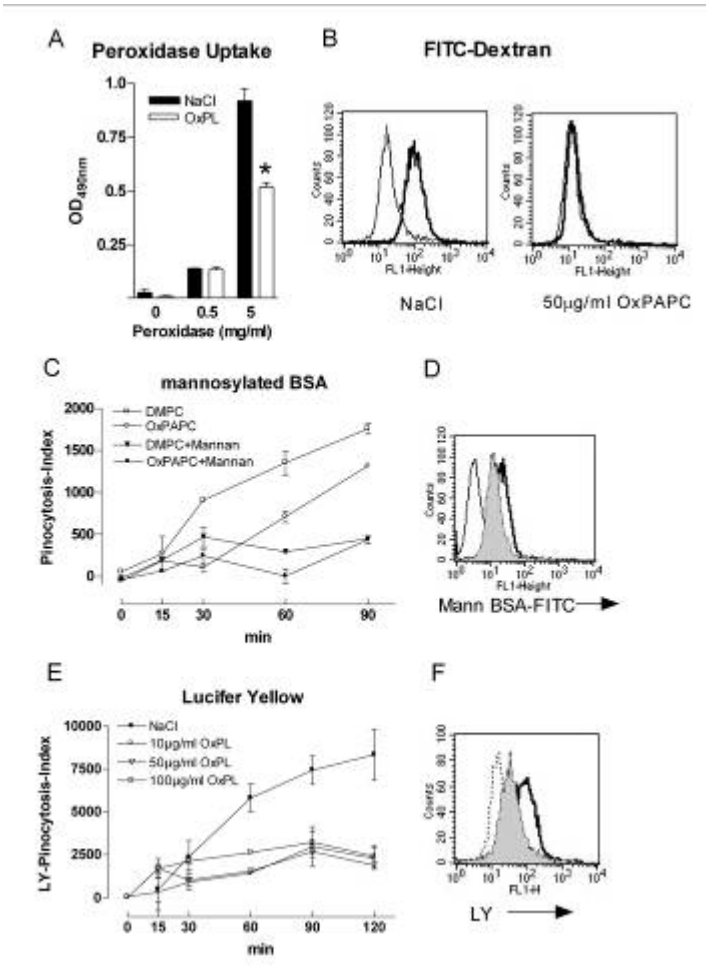

Fig. 6: OxPAPC inhibit receptor-mediated and fluid-phase endocytosis. (A) RAW 264.7 cells were incubated with indicated amounts of HRP in the presence of OxPAPC (50µg/ml) or carrier. HRP uptake was measured photometrically (490nm) in cell lysates after addition of OPD. RAW 264.7 cells were incubated with FITC-Dextran (B), mannoslyated BSA-FITC (C and D) or LY (E and F) for indicated times in the presence of carrier or OxPAPC and uptake was measured by FACS. (B) Depicts a representative histogram after 60min incubation with FITC-Dextran. (C) Time-dependent reduction of mann-BSA uptake in the presence of OxPAPC (50µg/ml, open circles) as compared to DMPC (open squares). Excess mannan (3mg/ml) inhibits uptake of mann-BSA-FITC irrespective of OxPAPC (C, filled symbols). (D) Representative histogram after incubation with mann-BSA-FITC at t=30min (thin line: 4C control, thick line: 37C carrier, filled grey: 37C OxPAPC). (E) Fluid phase pinocytosis of LY is inhibited in the presence of OxPAPC (10, 50 or 100 µg/ml, open symbols) as compared to carrier (closed symbola). A representative histogram at t=120min is depicted in F (thin line: 4C control; thick line: 37C carrier; filled grey: 37C 10µg/ml OxPAPC). Mean±SEM; * $p<0.05$ versus carrier.

Considering the fact that macrophages are quite unique in their capacity to not only phagocytose but also ingest particles via fluid phase pinocytosis and macropinocytosis, the latter also involving actin remodeling (32), we next examined whether these properties were also influenced by OxPAPC. For this purpose we first analyzed the uptake of peroxidase (HRP) by macrophages and found reduced internalization when OxPAPC was added (Fig. 6A). To study effects on a single cell level, we then chose to test the uptake of FITC-dextran, which is internalized via macropinocytosis, representing an uptake mechanism that is quite uniquely found in macrophages, DCs and epithelial cells (32). For this purpose, macrophages

were incubated with FITC-Dextran and OxPAPC clearly diminished the macropinocytotic uptake of FITC-dextran (Fig. 6B). Because HRP and dextran uptake has been shown to partly depend on mannose-receptor (MR) (27), we then investigated the uptake of a well-defined, pure MR ligand, namely mannosylated BSA-FITC (27). The addition of OxPAPC reduced receptor-mediated endocytosis of mannosylated BSA when compared to DMPC (Fig. 6C) or carrier (Fig. 6D). Moreover, addition of excess mannan abolished the uptake of mannosylated BSA thus confirming the specificity of this mechanism (Fig. 6C). We finally studied whether OxPAPC also interferes with receptor-independent, fluid-phase endocytosis. Lucifer yellow (LY) has been described as a receptor-independent ligand that is taken up by cells via pinocytosis (27, 33). We found that even small amounts of OxPAPC diminished the uptake of LY (Fig. 6E and F). Together, OxPAPC inhibits phagocytosis as well as receptor-mediated and receptor-independent pathways of endocytosis/pinocytosis by macrophages.

Discussion

Gram-negative peritonitis is a life threatening condition frequently associated with systemic dissemination of bacteria and septic shock. Host defense in peritonitis is an established domain of the innate immune system as the rapid response to invading pathogens is essential for the host to survive. OxPL are endogenous mediators of inflammation and products of oxidative stress that have been shown to also exert anti-inflammatory effects (11, 16, 34). Inhibition of LPS-LBP and LPS-CD14 interactions by OxPAPC protected mice from overwhelming inflammation during endotoxemia (16). In the present study we examined the functional *in vivo* role of OxPAPC and its potential interaction with CD14 during murine *E. coli* peritonitis. Our key finding was that OxPAPC, in sharp contrast to its protective effect during LPS-induced shock, rendered mice highly susceptible to abdominal sepsis induced by viable *E. coli* by a mechanism that at least in part is CD14 independent. Although both administration of OxPAPC and CD14 deficiency resulted in increased mortality and bacterial growth after i.p. infection with *E. coli*, the mechanisms accounting for these findings differed: whereas OxPAPC interfered with crucial functional properties of recruited phagocytes reducing their phagocytic capacity, CD14 deficiency resulted in an attenuated recruitment of phagocytes to the site of the infection. The present data reveal for the first time that OxPL may impair host defense against Gram-negative infection.

Host defense in peritonitis is a delicate balance between pro-inflammatory pathways aimed at the rapid elimination of bacteria and anti-inflammatory pathways intended to prevent systemic inflammation (35). Any imbalance in pro- or anti-inflammatory mediators might prove harmful. We initially considered it conceivable that OxPAPC, due to their anti-inflammatory properties described in models of acute inflammation (16), might represent an endogenous mediator that assists preventing overwhelming inflammation and therefore would reduce mortality in mice suffering from severe peritonitis. Our findings, however, could not confirm this hypothesis but rather disclosed an important, though detrimental, role for OxPAPC in host defense against *E. coli in vivo*. In the presence of OxPAPC a higher proportion of mice

succumbed and this was accompanied by an increased bacterial load in all organs tested. The important attraction of phagocytes to the peritoneal cavity was not compromised by OxPAPC. It has been reported earlier, that OxPL themselves induce the production of chemokines such as IL-8, KC and MCP-1 *in vitro* as well as *in vivo* (11, 14, 36). In our hands, the addition of OxPAPC during *E. coli* peritonitis *in vivo* was accompanied by increased peritoneal MCP-1 and KC concentrations, which may have contributed to the elevated number of PMNs and monocytes/macrophages in peritoneal fluid early (t=4h) after induction of peritonitis.

OxPAPC can inhibit the interaction of LPS with CD14 (16). Since CD14 is a major component of the LPS-signaling complex on innate immune cells and an important player in host defense against Gram-negative bacterial infections (3, 37-41), we were interested to study whether our observations of increased bacterial outgrowth and mortality in OxPAPC-treated mice were linked to an inhibitory action of OxPL on the interaction of LPS with CD14. Using CD14 gene-deficient mice, we first were able to demonstrate an important role of CD14 in the innate immune response during *E. coli* peritonitis *in vivo*. Analogous to our earlier studies, where we investigated the role of LBP in this infection model and in line with earlier reports in *Salmonella* peritonitis, an inadequate onset of inflammation in $CD14^{-/-}$ mice led to early systemic dissemination and increased bacterial outgrowth (9, 42). Although bacterial dissemination and survival rates were identical in $CD14^{-/-}$ and OxPAPC-treated mice, the underlying mechanisms differed. OxPAPC administration had no influence on the number of PMN attracted to the peritoneal cavity but recruited PMNs imposed covered by bacteria. This inspired us to investigate the functional properties of PMNs and macrophages and led us to discover an impaired phagocytosis by professional phagocytes in the presence of OxPAPC. Indeed, RAW cells as well as primary PMNs and peritoneal macrophages were less capable of effectively phagocytosing *E. coli* in the presence of OxPAPC. Although CD14 was attributed to phagocytosis of Gram negative bacteria by an earlier report (43), we could not disclose a role for this receptor. In line, our laboratory previously found no effect of a blocking anti-CD14 antibody intravenously administered to human subjects on phagocytosis by blood monocytes and PMNs (41). However, CD14 is known to participate in the elimination of apoptotic cells and OxPAPC – due to its capacity to interfere with CD14 - is certainly a candidate molecule that might interact with this process (44). In fact, one report showed an impaired phagocytosis of apoptotic cells in the presence of minimally modified LDL that bind to CD14 and concurrently enhance the uptake of OxLDL (45). Nevertheless, the potential impact of OxPL on phagocytosis of bacteria has not been investigated thus far and we hereby report for the first time that OxPAPC interfere with the elimination of bacteria *in vivo* and that this mechanism does not depend on CD14.

Another receptor that crucially contributes to opsonin-independent phagocytosis of *E.coli* is SR-A (31). SR-A recognizes a wide range of polyanions including LPS from Gram-negative bacteria. Although not known so far, the possibility exists that OxPAPC interferes with the binding of polyanions to SR-A, thereby preventing the phagocytosis of Gram-negative bacteria. However, our data from activated macrophages clearly demonstrate that OxPAPC inhibits phagocytosis to a greater degree than blocking Ab against SR-A. Addition of

OxPAPC to anti-SR-A further reduced the uptake of *E.coli*, thus indicating different inhibitory pathways. Of note, although the class B scavenger receptor CD36 has been shown to bind OxPAPC (46), CD36 is not involved in the phagocytosis of *E. coli* (47), which precluded us from investigating CD36 as a target receptor that might explain the effects of OxPAPC observed in this study.

Our data indicate that the inhibitory action of OxPAPC is not restricted to phagocytosis of *E. coli*. Considering the multitude of endocytotic pathways elicited by macrophages, we found OxPAPC to not only impair phagocytosis of bacteria but also polystyrene particles as well as receptor-mediated and fluid-phase endocytosis. These findings definitely underline the broad, and potentially harmful, impact of oxidation products generated during bacterial infections or chronic inflammation *in vivo*. Although the pathophysiological impact of these findings remains to be established, we propose a detrimental role for OxPL during bacterial infections. Beside infections, OxPL are found predominantly during chronic inflammation such as atherosclerosis. The here described observations of impaired endocytosis in the presence of OxPL could explain the very recent finding of diverse bacterial products within a single plaque specimen from patients with coronary heart disease (48). The possibility exists that OxPL, present in atherosclerotic plaques, prevent the effective elimination of bacteria that are encountered during asymptomatic phases of bacteremia (such as after dental procedures or translocation from the intestines) thus leading to the accumulation of intracellular bacterial products that in turn might contribute to ongoing inflammation.

It should be noted that although OxPAPC and CD14 deficiency negatively influenced the outcome of *E. coli* peritonitis by different mechanisms, OxPAPC did not further impair host defense in $CD14^{-/-}$ mice. Importantly, however, OxPAPC and CD14 deficiency both impacted on PMNs and macrophages, and although OxPAPC profoundly diminished the capacity of $CD14^{-/-}$ PMNs and macrophages to phagocytose *E.coli in vitro*, apparently this immune suppressing effect did not further impact on the outcome of $CD14^{-/-}$ mice *in vivo* due to the fact that these animals had very few PMNs and macrophages in their peritoneal cavity and as a consequence already had a severely hampered cellular immune response.

In conclusion, we here demonstrate that OxPAPC reduce host defense against abdominal sepsis caused by *E. coli* most likely by inhibiting the phagocytosing capacity of cells involved in innate immunity by a CD14 independent mechanism. While OxPL might be able to prevent overwhelming inflammation in settings of sterile inflammatory disorders, our results suggest that OxPL generated at sites of inflammation impair the innate immune response to bacterial infections.

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CHAPTER 11

Pulmonary LPS-binding protein inhibits the LPS-induced lung inflammation *in vivo*

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Abstract

Lipopolysaccharide binding protein (LBP) facilitates the interaction of the Gram-negative cell wall component LPS with CD14, thereby enhancing the immune response to LPS. Although lung epithelial cells have been reported to produce LBP *in vitro*, knowledge of the *in vivo* role of pulmonary LBP is limited. Therefore, in the present study we sought to determine the function of pulmonary LBP in lung inflammation induced by intranasal administration of LPS *in vivo*. Using LBP deficient ($LBP^{-/-}$) and normal wild-type mice we show that the contribution of LBP to pulmonary LPS responsiveness depended entirely on the LPS dose. While the inflammatory response to low dose (1ng) LPS was attenuated in $LBP^{-/-}$ mice, neutrophil influx and cytokine/chemokine concentrations in the brochoalveolar compartment were enhanced in $LBP^{-/-}$ mice treated with higher (>10ng) LPS doses. This finding was specific for LBP, since the exogenous administration of LBP to $LBP^{-/-}$ mice reversed this phenotype and reduced the local inflammatory response to higher LPS-doses. Our results indicate that pulmonary LBP acts as an important modulator of the LPS response in the respiratory tract *in vivo*. This newly identified function of pulmonary LBP might prove beneficial by enabling a protective immune response to low LPS doses while preventing an overwhelming, potentially harmful immune response to higher doses of LPS.

Introduction

LPS is the major constituent of the outer cell wall of Gram-negative bacteria and the predominant inducer of inflammatory responses to these bacteria (1, 2). The recognition of LPS by cells of the innate immune system provides an important first step to identify invading pathogens and to initiate a protective immune response. However, overwhelming inflammation in response to LPS can be harmful and may cause septic shock with organ failure (1, 2). Therefore, a balanced immune response to pathogens is crucial.

The cellular LPS receptor complex comprises the three molecules CD14, Toll-like receptor (TLR) 4 and MD-2 (1). While TLR4 signals the presence of LPS to the cellular interior, resulting in the production of proinflammatory cytokines and chemokines, the presence of MD-2 - that is associated with the extracellular domain of TLR4 - is crucial for the binding of LPS (3-5). The interaction between LPS and MD-2 is strongly facilitated in the presence of CD14 (5). However, the earliest events following the release of LPS require the transfer of LPS to its receptor complex. LPS binding protein (LBP) acts as a lipid-transfer protein that captures LPS out of aggregates formed by LPS monomers due to its amphiphilic properties and transfers it to CD14 (6-8). The spontaneous diffusion of LPS monomers to the cellular binding site is very slow and transfer by LBP enhances the immune response to LPS up to 1000-fold *in vitro* (9, 10). Beside its clear role in facilitating the presentation of LPS to its cellular receptor complex, LBP has also been demonstrated to be involved in the neutralization of LPS via transfer of LPS to lipoproteins such as HDL, LDL and VLDL (11, 12). This neutralizing capacity of LBP seems to play a greater role in clinical conditions like sepsis, which are associated with high serum LBP levels (12). Indeed, addition of high LBP levels or serum from septic patients containing high LBP concentrations has been demonstrated to attenuate the inflammatory response of macrophages and monocytes to LPS *in vitro* (13, 14).

While LBP is constitutively produced by hepatocytes under normal conditions, IL-1β and IL-6 synergize in inducing strongly enhanced LBP synthesis during an acute phase reaction (15). Beside increased LBP serum levels upon systemic infection, elevated LBP levels were also found in bronchoalveolar lavage fluid (BALF) during infectious and allergic lung inflammation (9, 16-18). Moreover, recent evidence indicates that LBP production is not restricted to hepatocytes and that both lung and intestinal epithelial cells represent additional sources (19-21). Synthesis of biologically active LBP by human and murine respiratory epithelial cells was observed upon stimulation with IL-1β, TNF-α and IL-6 (19). In addition, the inflammatory response of rabbit alveolar macrophages is amplified in the presence of LBP *in vitro* (9). Together, these observations indicate a direct and local role for LBP in LPS responsiveness in the respiratory tract and in host defense against Gram-negative pneumonia. Indeed, we and others recently demonstrated that LBP gene deficient ($LBP^{-/-}$) mice are highly susceptible to pneumonia caused by *Klebsiella pneumoniae* (22, 23) suggesting that endogenous LBP contributes to an adequate recognition of Gram-negative bacteria in the

lung. In the present study we sought to determine the role of pulmonary LBP in acute lung inflammation induced by LPS.

Materials and Methods

Mice

Pathogen-free 10-12 wk-old female C57BL/6 wild-type mice were purchased from Harlan Sprague-Dawley (Horst, The Netherlands). $LBP^{-/-}$ mice were generated as described previously (10), backcrossed to C57BL/6 background 11 times and bred in the animal facility of the Academic Medical Center in Amsterdam. Age and sex matched mice were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments.

Induction of Lung Inflammation

Mice were anesthetized by inhalation of isoflurane (Abbott Laboratories, Kent, U.K.) and LPS (from *Escherichia (E.) coli* O55:B5, Sigma, St. Louis, MO) diluted in 50µl sterile saline, was instilled intranasally (i.n.). Control mice received sterile saline. In some experiments mice received purified human LBP (80 ng; HyCult Biotechnology, Uden, The Netherlands) i.n. simultaneously with LPS. After 6h or 22h mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands) and sacrificed by bleeding out the vena cava inferior. Blood was collected in EDTA containing tubes and plasma was stored at –20C until further usage.

Bronchoalveolar lavag

The trachea was exposed through a midline incision and canulated with a sterile 22-gauge Abbocath-T catheter (Abott, Sligo, Ireland). Bilateral bronchoalveolar lavage (BAL) was performed by instilling two 0.5 ml aliquots of sterile saline. Approximately 0.9-1 ml BALF was retrieved per mouse. Total cell numbers were counted from each sample using a hemocytometer (Türck chamber), BALF differential cell counts were done on cytospin preparations stained with Giemsa. BALF supernatant was stored at -20°C for cytokine and LBP measurements.

Histology

Lungs for histology were harvested at 6 or 22h after infection, fixed in 10% formaline and embedded in paraffin. 4 µm sections were stained with hematoxylin and eosin (H&E), and analyzed by a pathologist who was blinded for groups. To quantify lung inflammation and damage, the entire lung surface was semi-quantitatively scored as described previously (24). In brief, the following parameters were analyzed: interstitial inflammation, intra-alveolar inflammation, edema, endothelialitis, bronchitis, pleuritis and thrombi formation. Each

parameter was graded on a scale of 0 to 3, with 0: absent, 1: mild, 2: moderate and 3: severe. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 21.

Assays

Murine LBP was measured using a commercially available ELISA (HyCult Biotechnology, Uden, The Netherlands) according to the manufacturer's instructions; the detection limit was 0.4 ng/ml. TNF-α and KC were measured using specific ELISAs (R&D Systems, Minneapolis, MN) according to the manufacturers` instructions. The detection limits were 31 pg/ml for TNF-α and 12 pg/ml for KC. Protein levels in BALF were measured using the BCA protein kit according to the manufacturer's instructions (Pierce, Rockford, IL).

LPS responsiveness of alveolar macrophages

The murine alveolar macrophage cell line MH-S was purchased from American Type Culture Collection (ATCC, Rockville, MD) and grown in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 2mM L-glutamine, 5mM Hepes buffer, 10% FCS (unless otherwise indicated), penicillin, streptomycin and β-mercaptoethanol. *In vitro* stimulation was carried out in 96-well plates (Greiner, Alphen a/d Rijn, the Netherlands) at a density of $1x10^6$ cells/ml. Following over-night culture in serum-free medium at 37°C in 5% CO_2, adherent cells were washed twice in serum-free medium then stimulated over-night with 0.1ng/ml to 1μg/ml LPS (*E.coli* O55:B5, Sigma) in the presence or absence of 0.1ng/ml to 10μg/ml hLBP (HyCult Biotechnology) in serum-free medium. Some experiments were performed in the presence of scavenger receptor A (SR-A) blocking antibodies (clone: 2F8, Serotec, Kidlington, United Kingdom) at a concentration of 10μg/ml.

Statistical analysis

Serial data were analyzed by one-way ANOVA, differences between two groups were calculated by Mann-Whitney *U* test. Correlations were calculated by Pearson test. Values are expressed as mean ± SEM. A p-value ≤ 0.05 was considered statistically significant.

Results

LBP is detectable in BALF of healthy mice and rapidly increases in acute lung inflammation

To investigate whether pulmonary LBP is detectable in normal BALF and whether acute lung inflammation induces local release of this protein, we measured LBP in BALF of wild type mice before and 6h after i.n. administration of 10μg LPS or NaCl. Low levels of alveolar LBP were readily detectable in normal mice and LPS induced a significant rise in LBP concentrations (Table 1). Plasma LBP levels, which are known to be 100-fold higher, were unaffected by pulmonary inflammation (Table 1) and no LBP was detectable in $LBP^{-/-}$ mice.

Hence, i.n. administration of LPS induced a rapid increase in alveolar LBP levels, supporting the notion of locally produced LBP *in vivo*.

LBP concentration	***BALF (ng/ml)***	***Plasma (μg/ml)***
Control	11.7 ± 3.9	5.3 ± 0.9
6h post NaCl i.n.	16.8 ± 5.8^{b}	3.5 ± 0.5
6h post 10μg LPS i.n.	47.5 ± 4.7^{a,b}	3.5 ± 0.9

Table 1: ***LBP concentrations in BALF and plasma.*** Wild type mice (n=6-8) were inoculated with saline or 10μg LPS i.n. and BAL was performed after 6h. LBP concentrations were measured by ELISA in BALF and plasma. Samples of untreated mice served as controls (n=6). Data are mean ± SEM, ^{a} indicates $p<0.05$ versus NaCl treated mice, ^{b} $p<0.05$ versus untreated controls.

Lung inflammation induced by high dose LPS is enhanced in the absence of LBP

To determine the specific contribution of pulmonary LBP to acute lung inflammation, we i.n. inoculated wild type and LBP$^{-/-}$ mice with 10μg LPS or saline. LPS induced a strong cell influx that predominantly was caused by polymorphonuclear cells (PMNs) ($p<0.05$ LPS treated wild type versus saline treated wild type mice). Surprisingly, the comparison of wild type and LBP$^{-/-}$ mice revealed significantly more PMNs as well as higher TNF-α and KC concentrations in BALF of LBP$^{-/-}$ mice (Fig. 1). Hence, these data suggested that pulmonary LBP inhibits lung inflammation induced by 10μg LPS *in vivo*.

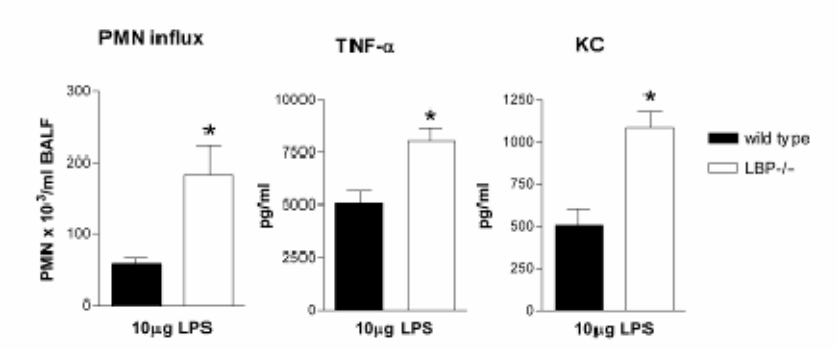

Fig. 1: LBP$^{-/-}$ mice demonstrate enhanced lung inflammation upon i.n. administration of 10 μg LPS. PMN counts, TNF-α and KC concentrations in BALF of wild type (filled bars) and LBP$^{-/-}$ mice (open bars) 6h after i.n. administration of 10μg LPS (n=6 per strain). Data are mean ± SEM, * indicates $p<0.05$ versus wild type mice.

The influence of LBP on LPS-induced pulmonary inflammation depends on the dose of LPS

This surprising finding of a more pronounced pulmonary inflammation in LBP$^{-/-}$ mice following LPS challenge led us to investigate whether this observation was attributable to the LPS dose. We therefore repeated these *in vivo* studies with increasing, though lower LPS doses. Wild type and LBP$^{-/-}$ mice were inoculated with 1, 10 or 100ng LPS i.n. 6h before BAL was performed and cell influx and TNF-α and KC responses were evaluated. In mice

that received the lowest LPS dose (1ng) a LBP-dependent increase of pulmonary inflammation was found: 1ng LPS induced a significantly higher PMN influx and slightly higher BALF TNF-α levels in wild type than LBP$^{-/-}$ mice (Fig. 2 and 3).

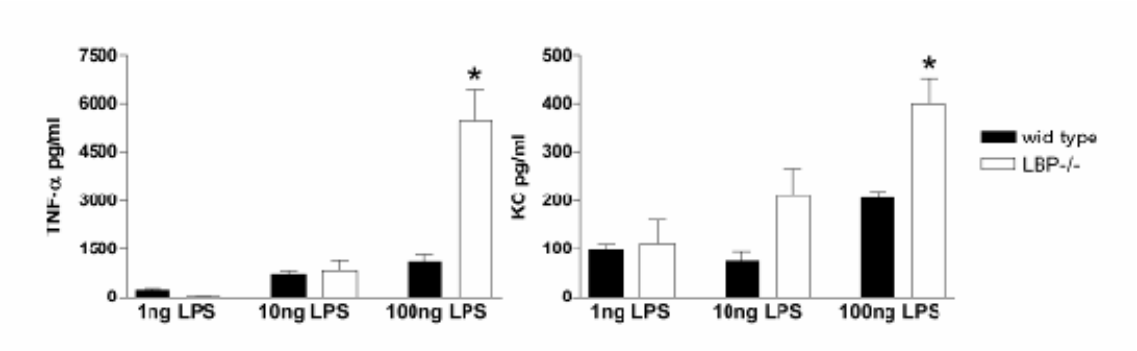

Fig. 2: Pulmonary LPS responsiveness of LBP$^{-/-}$ mice depends on the LPS dose administered. Increasing LPS doses (1, 10, or 100ng) were administered to wild type (filled bars) and LBP$^{-/-}$ (open bars) mice. After 6h TNF-α and KC concentrations were assessed in BALF. Data are mean ± SEM of 5-6 mice per group for each LPS dose. * indicates $p<0.05$ versus wild type mice.

In contrast, PMN influx as well as TNF-α and KC release in LBP$^{-/-}$ mice exceeded values observed in wild type mice when treated with 100ng LPS (Fig. 2 and 3). Results of mice that received 10ng LPS were indistinguishable between wild type and LBP$^{-/-}$ animals (Fig. 2 and 3). Together, the acute pulmonary inflammation induced by LPS doses as low as 1ng was augmented in the presence of LBP whereas cell influx and cytokine/chemokine production following higher LPS doses (≥ 10ng LPS) were independent from or even inhibited by the presence of LBP.

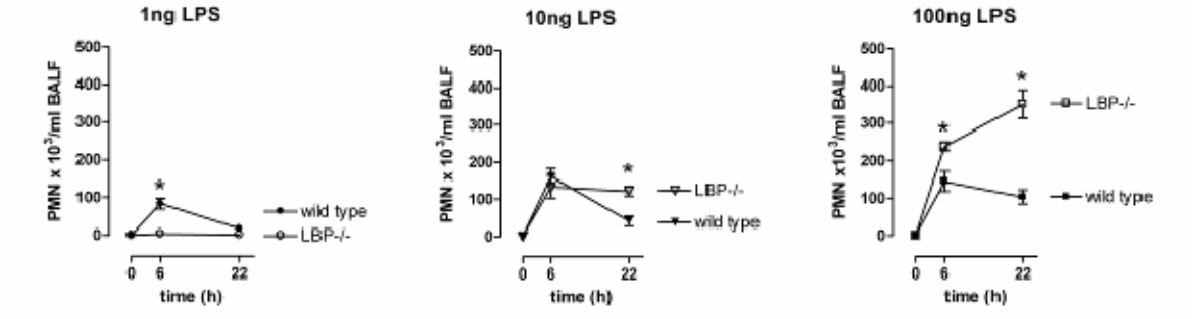

Fig. 3: Prolonged LPS-induced PMN-influx in LBP$^{-/-}$ mice. PMN influx obtained in BALF of wild type (filled symbols) and LBP$^{-/-}$ mice (open symbols) at 0h, 6h and 22h after i.n. administration of 1, 10 or 100ng LPS (n=5-6 per strain). Data are mean ± SEM, * indicates $p<0.05$ versus wild type mice.

To evaluate these findings over a longer period of time, we repeated the *in vivo* experiments and assessed pulmonary inflammation in response to different LPS doses (1, 10, and 100ng) after 22h. At this late time-point, cytokines or chemokines could not be detected anymore in most of the BALF samples. A small amount of TNF-a (65±12 pg/ml) was detected in BALF of LBP$^{-/-}$ mice challenged with 100ng LPS. As shown Fig. 3, all wild type mice showed a trend towards resolution of the inflammation in terms of a reduction in alveolar PMN counts (as compared to t=6h) at this later time-point. In contrast, alveolar PMN counts continued to

be elevated (10ng LPS) or increased even further (100ng LPS) in $LBP^{-/-}$ mice. To obtain further proof for enhanced lung inflammation in $LBP^{-/-}$ mice after i.n. administration of 100 ng LPS, histological analysis of lungs slides obtained 6h and 22h post-challenge were performed. Using the semiquantitative scoring system described in the M&M section, we could confirm our findings of enhanced and prolonged pulmonary inflammation in $LBP^{-/-}$ mice that received 100ng LPS i.n.. As shown on representative slides in Fig. 4, the extent of lung inflammation was more severe in LBP-/- mice when compared to their wild type counterparts (lung inflammation score at t=6h: wild type: 7.4± 1.2, $LBP^{-/-}$: 11.7±0.8; $p<0.05$ and at 22h: wild type: 3.0±0.6 and $LBP^{-/-}$: 7.5± 0.8; $p<0.05$). This experiment confirms our previous finding of an exaggerated pulmonary inflammation in the absence of LBP and illustrates furthermore that LBP is involved in the timely resolution of pulmonary inflammation.

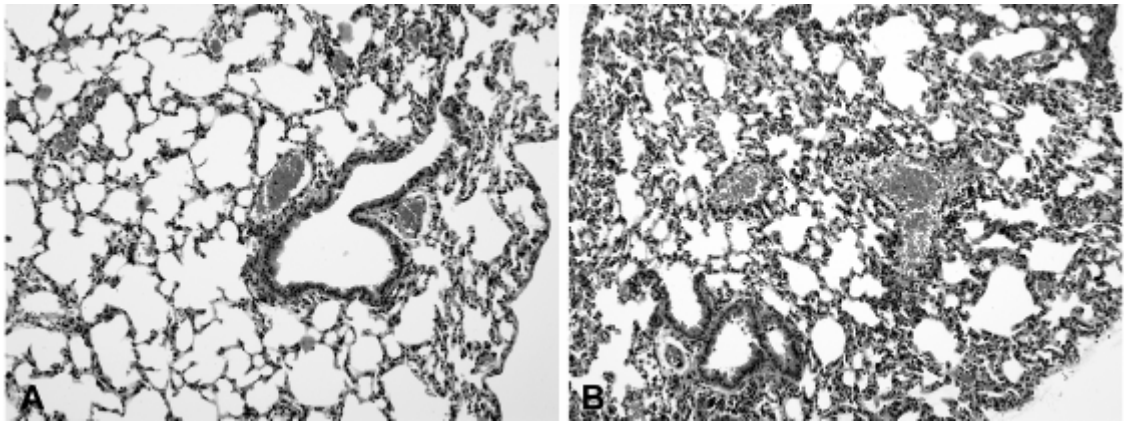

Fig. 4: More pronounced lung inflammation in $LBP^{-/-}$ mice. Wild type (A) and $LBP^{-/-}$ (B) mice (n=6 per strain) were inoculated with 100ng LPS i.n. and representative histological images of lungs at t=22h are depicted. H&E staining, magnification x10.

Pulmonary LBP concentrations rise in a LPS dose-dependent manner

In Table 1, we show elevated LBP protein concentrations within the alveolar compartment following LPS challenge. To study whether this increase in LBP concentrations correlates to the dose of LPS administered, we measured LBP levels in BALF of mice treated with increasing LPS doses (1, 10, or 100ng). While in wild type mice 1ng LPS did not induce any change in LBP levels, a dose-dependent rise in alveolar LBP could be revealed in response to higher LPS doses ($\geq$ 10ng LPS) (Fig. 5A). To exclude the possibility that this increase in alveolar LBP levels is a result of extravasated serum proteins, we compared LBP levels with total BALF protein concentrations in BALF of wild type mice (Fig. 5B): while LBP concentrations increased in a LPS-dose dependent manner (Pearson correlation LPS dose versus LBP concentration: $r=0.79$, $p=0.003$), protein levels remained essentially unaltered and no correlation was found between LBP and total protein concentrations in BALF of wild type mice (Pearson correlation LBP concentration versus total protein concentrations: $r=0.39$, $p=0.14$). As expected, LBP could not be detected in BALF or plasma of $LBP^{-/-}$ mice whereas

in these animals total alveolar protein concentrations increased in response to LPS and correlated with PMN influx (Pearson correlation total protein concentrations versus PMN count: r=0.79, p=0.0003) and TNF-α levels (Pearson correlation total protein concentrations versus TNF-α levels: r=0.82, p=0.0001). Together, alveolar LBP (but not total protein) concentrations increased in a LPS-dose dependent manner in wild type mice. The increase in alveolar protein concentrations most likely reflects the extent of capillary leakage found during acute lung injury and exclusively correlated with alveolar inflammation parameters as reflected by the high levels measured in LBP$^{-/-}$ mice challenged with 100ng LPS.

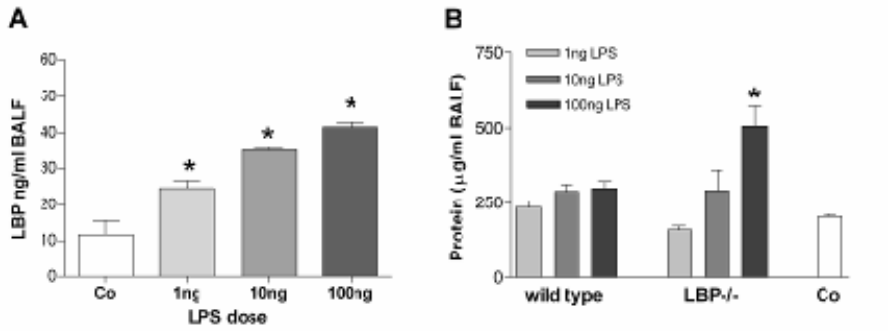

Fig. 5: Increased BALF LBP concentrations upon administration of LPS. **A:** LPS dose-dependent rise in BALF LBP concentrations 6h after administration of increasing LPS doses or saline to wild type mice. **B:** Total protein concentrations in BALF obtained 6h after LPS or saline administration to wild type or LBP$^{-/-}$ animals. Data are mean ± SEM of 5-6 mice per LPS dose; * indicates $p<0.05$ compared to saline (Co).

The increased lung inflammation in LBP$^{-/-}$ mice induced by high dose LPS is reversed by exogenous LBP

In order to investigate whether the augmented lung inflammation in LBP$^{-/-}$ mice following administration of ≥10ng LPS is specifically attributable to the lack of LBP, we administered human LBP in a dose of 80ng together with 100ng LPS to both wild type and LBP$^{-/-}$ mice and assessed the inflammatory response. Exogenous LBP did not influence LPS responsiveness in the lungs of wild type mice (Fig. 6).

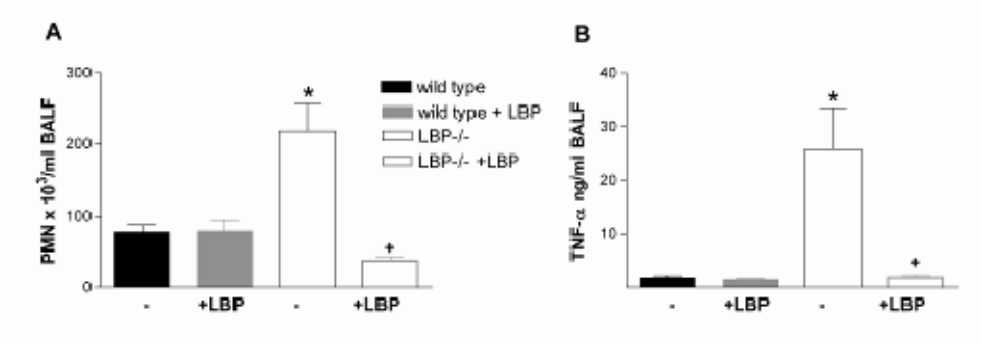

Fig. 6: Exogenous LBP inhibits pulmonary inflammation in LBP$^{-/-}$ mice. Wild type and LBP$^{-/-}$ mice (n=5-6 per group) received 100ng LPS with or without 80ng LBP i.n. and PMN counts (A) and TNF-α concentrations (B) were assessed in BALF after 6h. * indicates $p<0.05$ versus wild type mice, $^{+}$ indicates $p<0.05$ versus LBP$^{-/-}$ mice treated with LPS only. Data are mean ± SEM.

In LBP$^{-/-}$ mice, however, the administration of LBP in addition to LPS completely reversed the amplified pulmonary inflammation observed in LBP$^{-/-}$ mice treated with LPS only. Thus, the co-administration of LBP virtually transformed LBP$^{-/-}$ mice to a wild type phenotype with regard to both PMN influx and alveolar TNF-α concentrations, confirming the specificity of the findings.

The LPS-responsiveness of alveolar macrophages in vitro depends on LBP

Because we consider the enhanced PMN recruitment a consequence of exaggerated proinflammatory stimuli we attempted to elucidate the mechanisms involved. In a next step we therefore examined whether our findings could be reproduced *in vitro* and whether alveolar macrophages are responsible for our *in vivo* observations. For this purpose we stimulated murine alveolar macrophages (MH-S cells) with increasing doses of LPS and LBP in the absence of serum. By doing so we found a clear LBP and LPS dose dependent response with respect to TNF-α production (Fig. 7A). Because it has been reported that 'supranormal' LBP concentrations can exert inhibitory actions, we also performed stimulations using LBP concentrations up to 10µg/ml (Fig. 7B). Even 10µg/ml LBP further increased TNF-α levels released by alveolar macrophages *in vitro*.

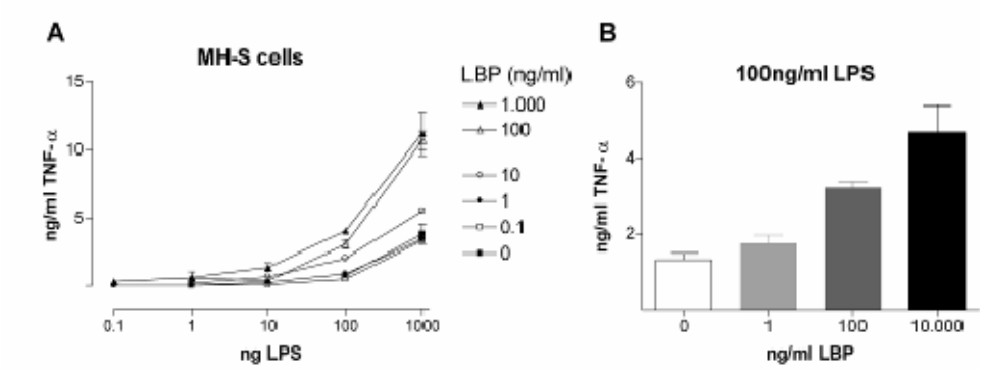

Fig. 7: LBP enhances TNF-α production by alveolar macrophages *in vitro*. MH-S cells were seeded at 1x10^6/ml and stimulated with indicated amounts of LPS in the absence or presence of increasing LBP concentrations for 18h at 37C. Supernatants were assayed for TNF-α by ELISA. Data are mean ± SEM of triplicates.

Another major receptor interacting with LPS-responsiveness and expressed on alveolar macrophages is SR-A. In an attempt to investigate whether LBP also transfers LPS to SR-A and thereby causes a dampened inflammatory response, we stimulated MH-S cells with LPS and increasing doses of LBP in the presence or absence of a blocking antibody against SR-A. As depicted in Fig.8, blockage of SR-A increases the inflammatory response irrespective of LBP. Together, no inhibitory properties of LBP were observed *in vitro*, indicating either that this observation is not reproducible *in vitro* or that alveolar macrophages are not the primary target cells for the inhibitory properties of LBP seen *in vivo*.

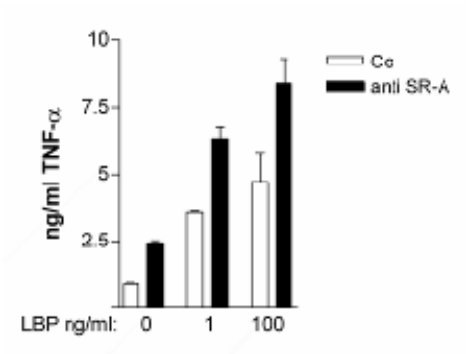

Fig. 8: LBP does not interact with SR-A associated LPS neutralization *in vitro*. MH-S cells were seeded at $1x10^6$/ml and stimulated with100ng/ml LPS in the absence or presence of indicated LBP concentrations and/or anti-SR-A blocking antibody for 18h at 37C. Supernatants were assayed for TNF-□by ELISA. Data are mean ± SEM of triplicates.

Discussion

LBP is an important acute phase protein that has attracted much attention as an enhancer of cellular LPS responsiveness via its unique property to transfer LPS to the CD14/TLR4 receptor complex on immune cells (8) thereby facilitating the inflammatory response to LPS and Gram-negative bacteria *in vivo* (23, 25, 26). Additionally, there is evidence suggesting that very high LBP concentrations, such as found in plasma samples of septic patients, inhibit the pro-inflammatory effects of LPS (13). Conceivably, the function of LBP depends on diverse aspects, such as concentration, cell types investigated and – possibly – localization of the stimulus *in vivo*. The finding of LBP production by respiratory epithelial cells *in vitro* (19) led us to evaluate the specific *in vivo* contribution of pulmonary LBP to the inflammatory response induced by LPS. Firstly, we here establish that LPS elicits an increase in alveolar LBP *in vivo* in a dose-dependent manner, supporting the hypothesis of local, pulmonary LBP production. Secondly, using $LBP^{-/-}$ mice, we were able to demonstrate for the first time that pulmonary LBP – at a naturally occurring low concentration - specifically inhibits and abridges the LPS-induced acute lung inflammation *in vivo*. These inhibitory properties of pulmonary LBP depended entirely on the LPS-dose administered – with a threshold set at 10ng LPS.

While our studies were in progress, Brass *et al.* reported on the role of endogenous LBP in lung inflammation induced by repeated LPS inhalation during periods up to 4 weeks (27). The authors found reduced cell influx and lower cytokine levels in BALF of $LBP^{-/-}$ mice when compared to wild type mice, indicating that LBP was required for full LPS responsiveness in their model. Of note, the LPS dose administered, albeit repeatedly, was relatively low. Although the precise LPS dose inhaled by an individual mouse is difficult to determine, the approximate LPS concentration in the exposure chambers was determined to be 5µg LPS per m^3 air (27). We also found LBP to promote lung inflammation when a low LPS-dose (<10ng) was administered. Of crucial importance, higher LPS doses showed quite the opposite, i.e. a diminished inflammatory response in the presence of LBP.

How can this observation be explained? LBP is a transfer protein that binds anionic structures like LPS and shuttles them to respective receptors or other proteins. Since LBP itself is not directly involved in cellular signaling processes, pulmonary LBP likely transfers LPS to other LPS-binding structures (e.g. receptors, lipid-binding molecules, lipoproteins) that neutralize

LPS activity. Alternatively, LBP might actively prevent the transfer of LPS to the CD14/TLR4/MD2 receptor that mediates pro-inflammatory pathways.

Regarding the latter possibility the CD14/TLR4/MD2 receptor complex prominently features as "the" LPS-signaling structure. As suggested by a recent report, moderate to high LBP levels inhibit the LPS transfer from CD14 to the TLR4/MD-2 signaling receptor *in vitro* (28). Thompson *et al.* showed that LBP removed cell-bound LPS from mCD14 and MD-2 resulting in an attenuated inflammatory response (28). However, the LBP dose required for this to take place was 3μg/ml, which is comparable to plasma levels, but much higher than the concentrations we found in BALF (although LBP levels in the alveolar lining fluid likely are higher considering the dilution caused by the BAL procedure). We studied the interaction of LPS with alveolar macrophages *in vitro* and found – in accordance with a previous report – a dose-dependent enhancement of the inflammatory response in the presence of LBP (9). Like Thompson *et al.* (28), we added LPS a few minutes before LBP but we were unable to find any LBP-related reduction of the inflammatory response even in the presence of LBP doses as high as 10μg/ml. This *in vitro* observation is in contrast to the results we found in mice. By stimulating isolated alveolar macrophages of $LBP^{-/-}$ and wild type mice with LPS *in vitro* we observed no difference in their capacity to produce TNF-α, ruling out the possibility of an impaired or exaggerated cytokine secretion of $LBP^{-/-}$ macrophages (data not shown). Already in 1995 Gegner *et al.* described the potential dual role of LBP and mCD14 in LPS-signal enhancement and LPS neutralization (29). They concluded that LBP is also part of the LPS clearance mechanism and that this pathway bifurcates after binding to mCD14. A more recent publication delineated that TLR4 is responsible for LPS-signaling while LPS-neutralization is mediated by other receptors *in vitro* (30). Using murine blood monocytes and endothelial cells, Dunzendorfer *et al.* demonstrated that LBP contributes substantially to the neutralization of LPS via transfer to CD14 as well as to other anionic structure binding receptors, presumably scavenger receptors (30). Scavenger receptors (SR) are expressed on macrophages and able to bind anionic ligands including LPS. It is known that $SR\text{-}A^{-/-}$ mice are hypersensitive to LPS, which might be explained by the fact that SR-A can degrade LPS without concomitant release of proinflammatory cytokines *in vitro* (31). However, when blocking SR-A with an antibody, we could not delineate a role for LBP in the enhanced inflammatory response seen with anti-SR-A treated cells *in vitro* (Fig. 8).

Lung epithelial cells have been disclosed to importantly contribute to LPS-induced pulmonary inflammation *in vivo* via NF-κB activating pathways (32, 33). Of great interest, lung epithelial cells have also been demonstrated to have the ability to internalize LPS without activation of NF-κB (34). Hamann *et al.* revealed that the internalization and neutralization of LPS by respiratory epithelial cells depended on the presence of LBP (and partly mCD14) *in vitro* (34). In an attempt to investigate the potential role for LBP we measured the internalization of FITC-labeled LPS by respiratory epithelial cells in the presence or absence of LBP. Although we found LPS rapidly internalized by respiratory epithelial cells, we could not identify a role of LBP herein (data not shown). Furthermore, Hamann *et al.* described that moderate LBP

concentrations enhanced the uptake and signaling of LPS by alveolar macrophages, whereas high LBP concentrations (≥1μg/ml) diminished the inflammatory response *in vitro* (34). Stimulations of alveolar macrophages in the presence of up to 10μg/ml LBP did not enable us to reproduce this observation *in vitro*. However, the major difference between our studies and those by Hamann *et al.* is the LPS chemotype cells were stimulated with. While Hamann *et al*. focused on rough LPS mutants we made use of smooth LPS. The vast importance of distinct LPS chemotypes has just been highlighted by a recent article disclosing that the TLR4-MD-2 complex is able to differentiate between these LPS chemotypes (35). While CD14 is required for MyD88-independent signaling by either LPS chemotype, rough LPS (but not smooth LPS) can bind and signal via the MyD88-dependent pathway in the absence of CD14 (35). Together, Hamann *et al.* utilized rough LPS that elicits also CD14-independent pathways, while our data were generated with CD14-dependent smooth LPS. Our data clearly could not confirm the hypothesis of a LBP mediated neutralization of smooth LPS by respiratory epithelial cells or alveolar macrophages *in vitro.*

The presence of surfactant proteins (SP) is another unique and specific feature of the pulmonary compartment. SP-A, like LBP, is able to bind anionic structures including rough LPS and to inhibit the inflammatory response of alveolar macrophages to rough or smooth LPS *in vitro* (36, 37). Furthermore, SP-$A^{-/-}$ mice challenged with intratracheal LPS display increased TNF-α and macrophage inflammatory protein (MIP)-2 BALF-concentrations when compared to wild-type controls (38). The potential role of LBP in the SP-A related anti-inflammatory mechanisms is controversial. While Stamme *et al.* explained the anti-inflammatory properties of SP-A via its capacity to inhibit the binding of rough LPS to LBP, these data could not be confirmed by Alcorn *et al.* (36, 37). However, *in vivo* data thus far only demonstrated the SP-A related decrease in cytokine/chemokine levels whereas PMN influx was not affected (38). Nevertheless, the possibility exists that the anti-inflammatory properties of SP-A are masked in mice lacking LBP, which results in higher pulmonary cytokine/chemokine concentrations that indirectly attract more PMNs.

The airways are a site of constant exposure to inhaled pathogens or LPS and it certainly is of crucial importance to fight these pathogens to prevent pneumonia. Conversely, the continuous daily exposure to minute amounts of inhaled LPS might warrant mechanisms that attenuate an excessive inflammatory response that would be detrimental to the host. Our *in vivo* findings indicate that LBP could be considered an important regulator of the pulmonary immune response: LBP enables the host to respond to small amounts of LPS with a self-limited inflammatory response while contributing to the neutralization of higher LPS doses and thus preventing the harmful consequences of exaggerated lung inflammation. Although premature at this point, therapeutic implications of these findings may prove beneficial in the future.

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CHAPTER 12

Lipoteichoic acid induced lung inflammation depends on Toll-like Receptor (TLR)2 and the concerted action of TLR4 and the Platelet Activating Factor Receptor

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Submitted

Abstract

Lipoteichoic acid (LTA) is a major outer cell wall component of Gram-positive bacteria that has been implicated as an important factor in the inflammatory response following bacterial infection. *In vitro* data indicate roles for Toll-like receptor (TLR) 2, platelet-activating factor receptor (PAF-R), CD14 and LPS-binding protein (LBP) in cellular responsiveness to LTA whereas the mechanisms contributing to LTA effects *in vivo* have never been investigated.
Utilizing mice deficient for LBP, CD14, TLR2, TLR4 or PAF-R we now examined the role of these molecules in pulmonary inflammation induced by highly purified LTA *in vivo*. While pulmonary LBP increased dose-dependently following administration of LTA, the inflammatory response was unaltered in $LBP^{-/-}$ mice. TLR2 proved to be indispensable for the initiation of an inflammatory response, as PMN-influx, TNF-α, KC and MIP-2 release were abolished in $TLR2^{-/-}$ mice. Minor effects such as moderately decreased TNF-α and MIP-2 levels were observed in the absence of CD14, indicating a role for CD14 as a co-receptor. Quite surprisingly, the absence of TLR4 greatly diminished pulmonary inflammation and the same phenotype was observed in $PAF\text{-}R^{-/-}$ animals. In contrast to all other mice studied, only $TLR4^{-/-}$ and PAF-R-/- mice displayed significantly elevated IL-10 pulmonary concentrations.
These data suggest that TLR2 is the single most important receptor signaling the presence of LTA within the lungs *in vivo*, whereas TLR4 and PAF-R may influence lung inflammation induced by LTA either by sensing LTA directly or through recognition and signaling of endogenous mediators induced by the interaction between LTA and TLR2.

Introduction

Gram-positive infections are a worldwide threat and major cause of mortality. Infections of the respiratory tract are frequently caused by Gram-positive bacteria and pneumonia is the 7th leading cause of mortality in the United States (1). While much has been learned about Gram-negative infections and the importance of LPS therein, less is known about the host response to Gram-positive pathogens.

Lipoteichoic acid (LTA) is a major constituent of the outer cell wall of Gram-positive bacteria and the predominant mediator of inflammatory responses to these microorganisms (2, 3). LTA shares many pathogenic properties with LPS and is able to induce the production of a variety of pro-inflammatory cytokines and chemokines by cells of the innate immune system (4-7). From *in vitro* studies it is known that the cellular recognition and signaling receptor for LTA is Toll-like receptor (TLR)2 (8-13). However, there is continuing controversy regarding the possibility of TLR4 being a receptor for LTA (14, 15). While most investigators who excluded TLR4 as a signaling receptor for LTA studied cells from C3H/HeJ mice (i.e. mice that harbor a mutated TLR4), transfected cell lines or applied neutralizing anti-TLR4 antibodies, Takeuchi *et al.* examined peritoneal macrophages from TLR4$^{-/-}$ mice and thereby found a diminished TNF-α and IL-6 response following stimulation with purified LTA from *Staphylococcus aureus* (*S. aureus*) (8, 11, 12, 14-17). A more recent article even disclosed TLR-independent (but CD14-dependent) responses by human polymorphonuclear cells (PMN) that were incubated with LTA (18). Importantly, the roles of TLR2 and TLR4 in the lung inflammatory response to LTA *in vivo* have not been investigated thus far.

Besides TLR2 and TLR4, several other molecules have been implicated in cellular responsiveness to LTA, in particular CD14, LBP and the receptor for platelet activating factor (PAF-R). TLRs such as TLR2 co-localize with CD14 and LTA in lipid rafts of epithelial cells before trafficking to the Golgi apparatus (10) and CD14 dependent responses to diverse LTA preparations from different bacteria have been studied in various cell types *in vitro* (5, 11, 12, 15, 17-19). Most of these investigators described some degree of CD14-dependency when studying endpoints like cytokine/chemokine release or the rate at which PMNs undergo apoptosis. Of note, CD14 expression within the pulmonary compartment is not restricted to macrophages since CD14 has been detected on respiratory epithelial cells (20). Moreover, soluble CD14 can be recovered from alveolar lavage fluid (21). LBP is well known as an acute phase protein that transfers LPS monomers from aggregates to the CD14/TLR4 receptor complex, thereby enhancing the immune response to LPS up to 1000-fold *in vitro* (22-24). LBP is also produced by respiratory epithelial cells *in vitro* and LPS administration results in a dose dependent increase in bronchoalveolar lavage fluid (BALF) LBP *in vivo* (21, 25, 26). However, LBP does not unequivocally augment inflammation as illustrated by our recent finding that pulmonary LBP inhibits the inflammatory response during LPS-induced lung inflammation *in vivo* (26). Less is known about the potential role of LBP during LTA-induced inflammation. While few studies illustrated the requirement for LBP (11, 27), another report showed LBP independent responses to LTA (19). Another important receptor for LTA,

specifically on respiratory epithelial cells, has been identified to be PAF-R (28). Lemjabbar et al. disclosed that the interaction of LTA with PAF-R induced the induction of NFκB and mucin production within the lungs while epithelial TLR2, in contrast to macrophage TLR2, did not play a role (28). More recent investigations revealed PAF-R as an important factor in the LTA-induced nitric oxide production *in vitro* (29).
Pulmonary infections due to *S. aureus* are a serious threat with LTA being a major immunogenic determinant. Although LBP is produced locally and CD14, TLR2, 4 and PAF-R are expressed on alveolar macrophages as well as on respiratory epithelial cells (20, 25 , 26, 28, 30, 31), knowledge of the precise function of these molecules during pulmonary inflammation induced by Gram-positive cell wall components *in vivo* is highly limited Therefore, in the present study we sought to systematically determine the importance of pulmonary LBP, CD14, TLR2 and TLR4 as well as PAF-R in acute lung inflammation induced by highly purified LTA *in vivo*.

Materials and Methods

Mice

Pathogen-free 8-12 wk-old C57BL/6 wild-type mice were purchased from Harlan Sprague-Dawley (Horst, The Netherlands). $LBP^{-/-}$ mice were generated as described previously and backcrossed to a C57BL/6 background 11 times (24). $CD14^{-/-}$ C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) (32). $TLR2^{-/-}$ (14) and $TLR4^{-/-}$ (33) mice were generated as described and backcrossed 6 times to a C57BL/6 background. $PAFR^{-/-}$ mice were generated as described (34) and backcrossed 7 times to a C57BL/6 background. All mice were bred in the animal facility of the Academic Medical Center in Amsterdam. Age matched female mice were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments.

Induction of Lung Inflammation

Acute lung inflammation was induced as described previously (6, 26, 35). Briefly, mice were anesthetized by inhalation of isoflurane (Abbott Laboratories, Kent, U.K.) and highly purified LTA (from *S. aureus*, (16), LPS content below 5 pg/mg LTA as assessed by Limulus amebocyte lysate assay), diluted in 50µl sterile saline, was instilled intranasally. Control mice received sterile saline. After 6h mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Meidrecht, the Netherlands) and sacrificed by bleeding out the vena cava inferior. Blood was collected in EDTA containing tubes and plasma was stored at –20°C until further usage.

Bronchoalveolar lavage

The trachea was exposed through a midline incision and canulated with a sterile 22-gauge Abbocath-T catheter (Abott, Sligo, Ireland). Bilateral bronchoalveolar lavage (BAL) was performed by instilling two 0.5 ml aliquots of sterile saline. Approximately 0.9 ml of BALF was retrieved per mouse. Total cell numbers were counted from each sample using a hemocytometer (Türck chamber), BALF differential cell counts were done on cytospin preparations stained with Giemsa. BALF supernatant was stored at -20°C for cytokine and LBP measurements.

Assays

Murine LBP was measured using a commercially available ELISA (HyCult Biotechnology, Uden, The Netherlands) according to the manufacturer's instructions; the detection limit was 0.4 ng/ml. TNF-α, keratinocyte-derived chemokine (KC), macrophage-inflammatory protein (MIP)-2 (all R&D Systems, Minneapolis, MN) and IL-10 (Bender MedSystems, Vienna, Austria) were measured using specific ELISAs according to the manufacturers` instructions. The detection limits were 31 pg/ml for TNF-α, 12 pg/ml for KC, 31 pg/ml for MIP-2 and 39 pg/ml for IL-10.

Statistical analysis

Differences between groups were calculated by Mann-Whitney *U* test. Values are expressed as mean ± SEM. A p-value ≤ 0.05 was considered statistically significant.

Results

Alveolar LBP concentrations rise upon LTA administration but do not affect pulmonary inflammation *in vivo*.

We earlier showed that LTA is a potent inducer of chemotactic stimuli (4). Prior to studying the role of pulmonary LBP in LTA-induced lung inflammation *in vivo*, we first investigated whether LTA administration influences pulmonary LBP concentrations. As shown in Table 1, low levels of alveolar LBP were detectable in saline-treated mice while LTA induced a significant rise in LBP concentrations. Plasma LBP levels were unaffected by pulmonary LTA administration (Table 1) and no LBP was detectable in $LBP^{-/-}$ mice. Hence, intranasal administration of LTA induced a rapid increase in pulmonary LBP.

LBP concentrations	*BALF (ng/ml)*	*Plasma (μg/ml)*
Control	11.7 ± 3.9	5.3 ± 0.9
6h post NaCl i.n.	16.8 ± 5.8^{+}	3.5 ± 0.5
6h post 100 μg LTA i.n.	55.7 ± 6.1*$^{+}$	4.1 ± 0.3

Table 1.LBP concentrations in BALF and plasma Wild type mice (n=6-8) were inoculated with saline or 100μg LTA i.n. and BAL was performed after 6h. LBP concentrations were measured with ELISA in BALF and plasma. Samples of untreated mice served as controls (n=6). Depicted are mean ± SEM, * indicates $p<0.05$ versus NaCl treated mice, $^{+}$ $p<0.05$ versus control.

Arguing that the local increase in pulmonary LBP might contribute to LTA-induced lung inflammation, we repeated these experiments using wild type and LBP$^{-/-}$ mice and administered 100μg LTA intranasally and enumerated infiltrating PMNs 6 hours thereafter. LTA induced a strong influx of cells to the alveolar compartment and differential counts disclosed a predominance of PMNs, although macrophages were also attracted (Fig. 1).

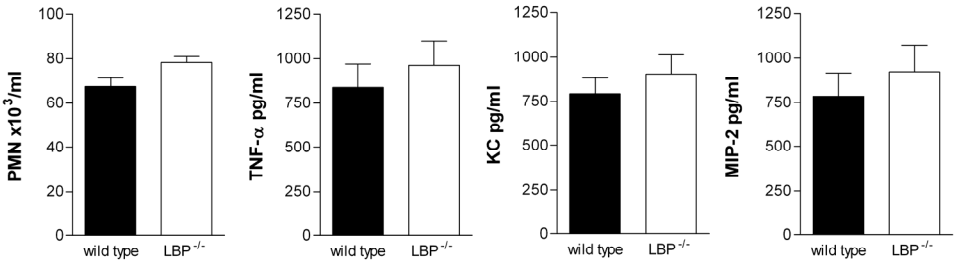

Fig. 1: LTA induces acute pulmonary TNF-α and KC production independent of LBP. PMN counts, TNF-α, KC and MIP-2 concentrations obtained from BALF (8 mice per strain) 6h after intranasal administration of 100μg LTA. Filled bars indicate wild type mice, open bars LBP$^{-/-}$ mice. Data are mean±SEM.

However, the LTA induced acute cell influx was not impaired in the absence of LBP. In addition, an impressive increase in TNF-α, KC and MIP-2 concentrations in BALF could be measured upon LTA challenge, which again did not depend on the presence of LBP (Fig. 1). Hence, these data indicate that the LTA induced acute pulmonary inflammation does not depend on the presence of LBP *in vivo*.

CD14 has a moderate effect on LTA-induced lung inflammation.

Next we were interested whether the reported importance of CD14 in the inflammatory response to LTA can be reproduced *in vivo* (5, 11, 12, 15, 17-19). Wild type and CD14$^{-/-}$ mice were inoculated intranasally with LTA and the inflammatory response was evaluated. Although pulmonary TNF-α and MIP-2 concentrations were reduced in CD14$^{-/-}$ animals, KC levels and PMN influx were not altered in these mice (Fig. 2). Therefore, CD14 has only a moderate effect on pulmonary inflammation induced by LTA.

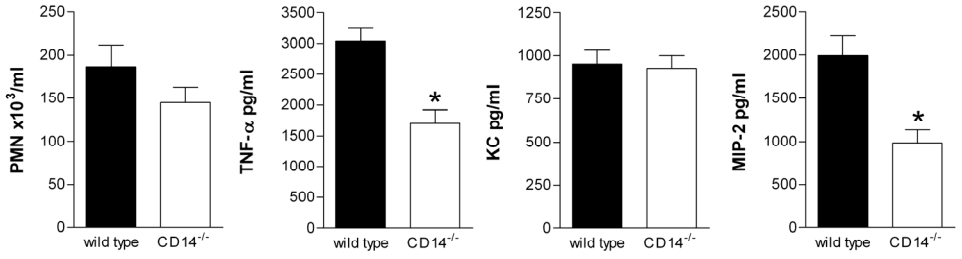

Fig. 2: LTA induced pulmonary inflammation partially depends on CD14. PMN counts, TNF-α, KC and MIP-2 concentrations obtained from BALF (n=6 per strain) 6h after intranasal administration of 100µg LTA. Filled bars indicate wild type mice, open bars $CD14^{-/-}$ mice. Data are mean±SEM; * indicates $p<0.05$ versus wild type mice.

TLR2 and TLR4 contribute to LTA-induced lung inflammation *in vivo*.

TLR2 has been repeatedly shown to be the most important receptor mediating the response to LTA *in vitro* (8-13). In accordance, we found mice lacking TLR2 to be non-responsive to intranasal LTA. While in wild type mice LTA induced brisk responses, the number of PMN as well as local TNF-α, KC or MIP-2 release was abolished in $TLR2^{-/-}$ mice (Fig. 3).

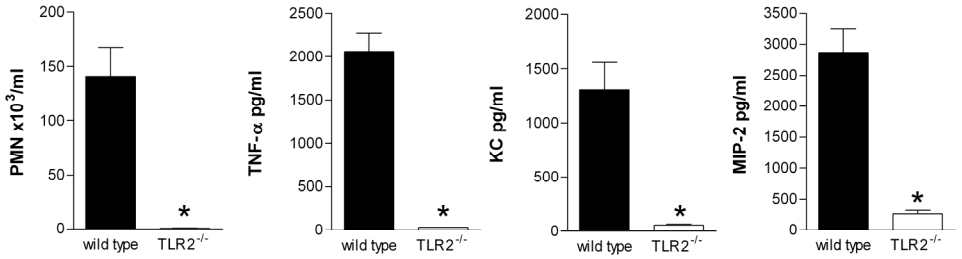

Fig. 3: LTA induced pulmonary inflammation entirely depends on TLR2. Mice (n=6 per strain) were inoculated intranasally with 100µg LTA and PMN counts, TNF-α, KC and MIP-2 concentrations were evaluated after 6h. Filled bars indicate wild type mice, open bars $TLR2^{-/-}$ mice. Data are mean±SEM; * $p<0.05$ versus wild type mice.

Although the role of TLR4 in cellular responsiveness to LTA has been debated (8, 11, 12, 14, 15, 17), we, much to our surprise, observed a significantly attenuated pulmonary inflammation in response to LTA in $TLR4^{-/-}$ mice. Indeed, alveolar PMN counts as well as TNF, KC and MIP-2 concentrations were clearly reduced in $TLR4^{-/-}$ mice as compared to wild-type animals (Fig. 4).

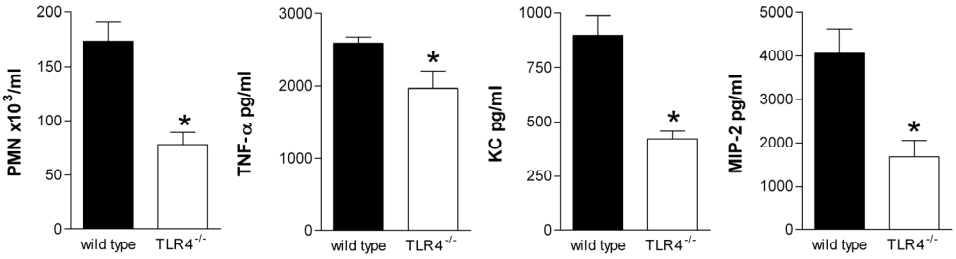

Fig. 4: TLR4 has an impact on LTA induced pulmonary inflammation. PMN counts, TNF-α, KC and MIP-2 concentrations obtained from BALF (n=6 per strain) 6h after intranasal administration of 100µg LTA. Filled bars indicate wild type mice, open bars TLR4$^{-/-}$ mice. Data are mean±SEM; * $p<0.05$ versus wild type mice.

PAF-R and TLR4 have similar effects on pulmonary LTA-responsiveness.

PAF-R has been recognized as the most important epithelial LTA-recognition receptor within the pulmonary compartment *in vitro* (28). In order to evaluate this finding *in vivo* and compare results with TLR4, we inoculated PAF-R$^{-/-}$, TLR4$^{-/-}$ and wild-type mice with LTA and evaluated the inflammatory response (Fig. 5). These experiments confirmed our findings regarding the role of TLR4 in LTA-induced lung inflammation presented in Fig. 4. PAF-R and TLR4 had a comparable impact on pulmonary inflammation, indicated by strongly diminished cell-influx and local TNF, KC and MIP-2 concentrations in mice lacking either receptor (Fig. 5). Hence, both TLR4 and PAFR importantly contribute to pulmonary inflammation induced by LTA *in vivo*.

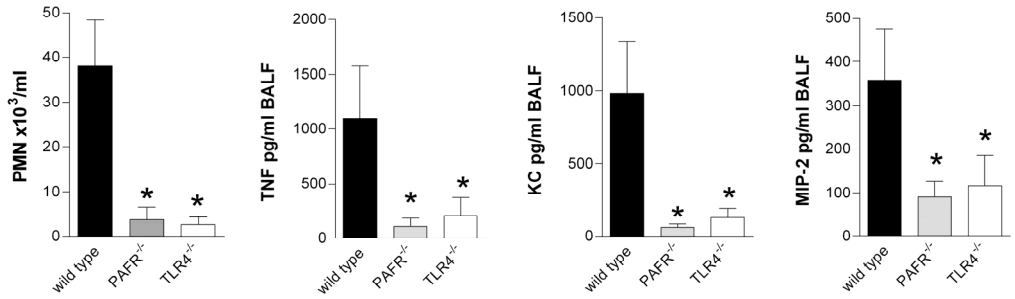

Fig. 5: TLR4 and PAFR equally contribute to LTA induced pulmonary inflammation. Indicated mice (n=5-6 per strain) were treated intranasally with 100µg LTA and PMN counts, TNF-α, KC and MIP-2 concentrations were assessed after 6h. Filled bars indicate wild type mice, open bars TLR4$^{-/-}$ mice, shaded bars PAF-R$^{-/-}$ mice. Data are mean±SEM; * $p<0.05$ versus wild type mice.

PAF-R and TLR4 influence the anti-inflammatory response.

After having investigated the requirement for LBP, CD14, TLR2, 4 and PAF-R for an appropriate pro-inflammatory response we asked whether these receptors also affect the anti-inflammatory response. Neither CD14 nor TLR2 deficiency resulted in altered IL-10 concentrations (data not shown). Of great interest however, PAFR$^{-/-}$ and TLR4$^{-/-}$ mice displayed significantly higher alveolar IL-10 levels when compared to wild-type mice.

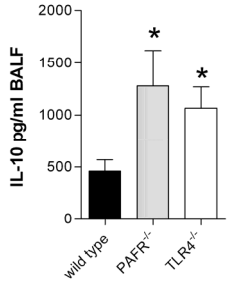

Fig. 6: Enhanced anti-inflammatory IL-10 response in TLR4$^{-/-}$ and PAF-R$^{-/-}$ mice. IL-10 concentrations were measured in BALF of indicated mice (n=5-6 per strain) 6h after i.n. administration of 100µg LTA. Filled bars indicate wild type mice, open bars TLR4$^{-/-}$ mice, shaded bars PAF-R$^{-/-}$ mice. Date are mean±SEM; * indicates $p<0.05$ versus wild type mice.

Discussion

Pneumonia is associated with a profound inflammatory response within the pulmonary compartment. Inflammation induced by Gram-positive pathogens is predominantly elicited by bacterial cell wall components such as LTA and peptidoglycan (3, 6). While many investigators studied the requirement of pattern recognition receptors that sense the presence of LTA *in vitro*, little is known about these pathways *in vivo*. We here for the first time investigated the recognition machinery for LTA during pulmonary inflammation *in vivo* using mice lacking LBP, CD14, TLR2, TLR4 or PAF-R. In accordance with earlier *in vitro* studies we revealed in particular TLR2 and to a lesser extent PAF-R to contribute to pulmonary inflammation caused by LTA *in vivo*. Quite surprisingly, we discerned an important role for TLR4 in LTA-induced lung inflammation *in vivo*, while CD14 played a moderate role and LBP was not required.

Pulmonary host defense against invading pathogens invariably involves the activation of macrophages and epithelial cells, the release of proinflammatory cytokines and chemokines and the recruitment of PMNs. Our data underline the crucial importance of TLR2 during LTA-induced lung inflammation *in vivo* since the inflammatory response was abolished in the absence of TLR2. However, we observed that despite the presence of TLR2, other surface receptors such as PAF-R and TLR4 also contributed – albeit to a lesser degree – to the pulmonary immune response. Because the absence of these receptors did not abolish but rather diminish pulmonary inflammation, we hypothesize two potential scenarios: first, LTA might be an actual ligand for TLR4 and/or PAF-R; second, the LTA-TLR2 induced inflammation instigates the generation of endogenous mediators that serve as ligands for PAF-R and/or TLR4 and thus synergize with LTA in the inflammatory response. Potential endogenous mediators that are in particular generated during pulmonary inflammation, such as PAF, fragmented hyaluronan, oxidation products, biglycans or heat shock proteins have been identified as PAF-R and/or TLR4 ligands (36-42).

The finding that TLR4 played a role during LTA-induced lung inflammation *in vivo* certainly warranted us to exclude the possibility of LPS contamination. We believe that LPS contamination cannot explain our findings because the LPS content of the LTA preparation was very low (<5pg/mg LTA), which in theory results in intranasal inoculation of maximal 0.5pg LPS; this amount is not sufficient to induce a detectable inflammatory response in the

mouse lung (26, 43). In addition, contaminating LPS would have elicited an inflammatory response in TLR2$^{-/-}$ mice, which clearly did not occur. To our knowledge, this is the first study investigating the function of TLR4 in LTA-induced inflammation *in vivo*. Earlier reports that studied and excluded the role of TLR4 *in vitro* used either transfected cell-lines, neutralizing antibodies or cells from C3H/HeJ mice (8, 11, 12, 15-17). In fact, Takeuchi et al. were the only ones that utilized cells from the same mouse strain described here and found TLR4$^{-/-}$ macrophages to be hyporesponsive to LTA (14). Hence, either LTA is a ligand of TLR4 but resulting effects only become visible when specifically using TLR4$^{-/-}$ animals, or the *in vivo* effects described here are related to the multifaceted interplay between different cell types and mediators within the lungs that cannot be revealed using *in vitro* techniques.

PAF-R has been reported to recognize LTA. Lemjabbar et al. demonstrated that respiratory epithelial cells signal the presence of LTA via PAF-R and that this process occurs, in contrast to macrophages, irrespective of TLR2 (28). However, the precise participation of PAF-R in the inflammatory response to LTA is difficult to discern. While LTA has been proposed as a direct ligand for PAF-R by some investigators, other reports indicate that LTA may (possibly via TLR2) induce the release of PAF that in turn acts via PAF-R (28, 29). Our studies do not allow distinguishing between these two pathways and PAF concentrations are hard to determine in diluted samples such as BALF due to the low sensitivity of available assays and the fact that PAF largely exists in a cell-associated form (29, 44). However, our data clearly indicate an important role for PAF-R during LTA-induced acute lung injury, irrespective of the mode of activation.

Of great interest, we observed increased IL-10 levels in BALF from both TLR4 and PAF-R gene-deficient mice challenged with LTA. It is known that under normal circumstances anti-inflammatory pathways are activated within the pulmonary compartment, most likely to prevent perpetual inflammation due to inhalation of minute amounts of e.g. polluted air in daily life (45). Bronchial epithelial cells have been identified as the source of constitutive pulmonary IL-10 in humans and mice (45, 46). Once the lung is exposed to potentially harmful substances, such as bacteria, the initiation of an inflammatory response is crucial for the elimination of the infection and to prevent further damage to the host (47). Enhanced IL-10 levels have been repeatedly shown to worsen the host defense during bacterial pneumonia *in vivo* (47-50). LTA-triggered activation via TLR2 induces an inflammatory response, while TLR4 and/or PAF-mediated signaling might also counteract the constitutive anti-inflammatory milieu. Counteracting anti-inflammatory pathways might be a direct result of LTA-induced TLR4/PAF involvement or an indirect consequence of above mentioned endogenous mediators that are generated in the course of TLR2-triggered inflammation. The lack of inflammation and consecutive absence of endogenously generated mediators would explain our observation of unaltered IL-10 levels in TLR2$^{-/-}$ mice. Another explanation, not excluding the one suggested above, could be that the presumptive immunosuppressive effects mediated by TLR2 (51), may become more visible in the absence of receptors mediating inflammatory responses (such as TLR4 and PAF-R).

Together, our data illustrate the principal importance of pulmonary TLR2 as the signaling receptor for LTA *in vivo*. We in addition discovered that PAF-R and TLR4 importantly contribute to the pulmonary inflammation induced by LTA *in vivo*. Although LTA might be a direct ligand for either TLR4 or PAF-R, the possibility exists that pulmonary TLR4 and/or PAF-R signal the presence of TLR2-triggered endogenous, proinflammatory mediators *in vivo*.

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CHAPTER 13

CD14 facilitates invasive respiratory tract infection by *Streptococcus pneumoniae*

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Submitted

Abstract

Rationale: 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.

Objectives: To study the role of CD14 in Gram-positive bacterial pneumonia.

Methods: CD14 knockout (KO) and normal wild-type (WT) mice were intranasally infected with *Streptococcus (S.) pneumoniae*.

Measurements and main results: 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.

Conclusion: 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/50µl *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 (Prism , Amsterdam, NL) . 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.

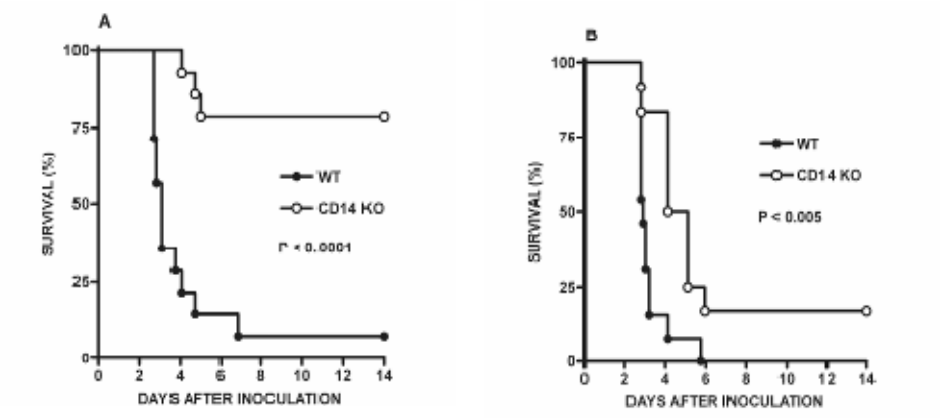

Fig. 1: CD14 KO mice are protected against pneumococcal pneumonia. Survival after intranasal infection with $1x10^4$ CFU (A) or 5x104 CFU (B) *S. pneumoniae*. Mortality was assessed four times daily for 14 days (N = 13-14 per group in each experiment). WT mice, closed symbols. CD14 KO mice, open symbols. * $P < 0.0001$ versus WT mice. † $P < 0.005$ versus WT mice.

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). Together these data suggested that CD14 contributes to the invasion of *S. pneumoniae* from the alveolar space into lung tissue and the circulation.

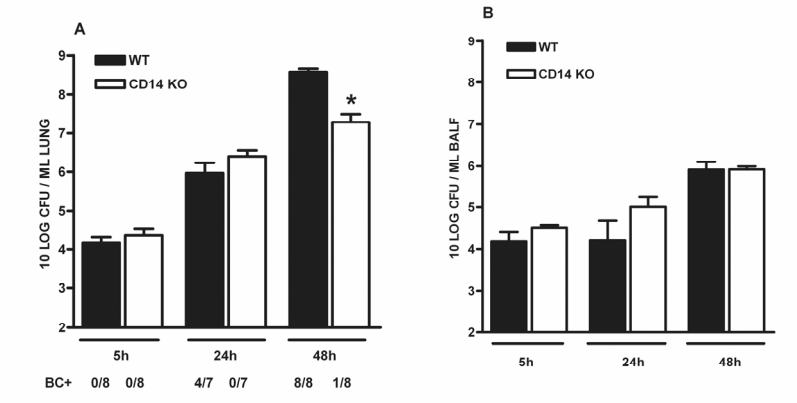

Fig. 2: CD14 KO mice demonstrate reduced invasiveness and dissemination of the infection. Bacterial loads in lungs (A) and bronchoalveolar lavage fluid (B) at 5, 24 and 48 hours after infection with 5 x 10^4 CFU *S. pneumoniae*. BC+ indicate the number of positive blood cultures. WT mice, closed bars. CD14 KO mice, open bars. 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 (data not shown), 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 (Fig. 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; at this time point the total protein concentration in BALF of WT mice was 743 ± 123 µg/ml versus 362 ± 47 µg/ml in CD14 KO mice ($P < 0.05$).

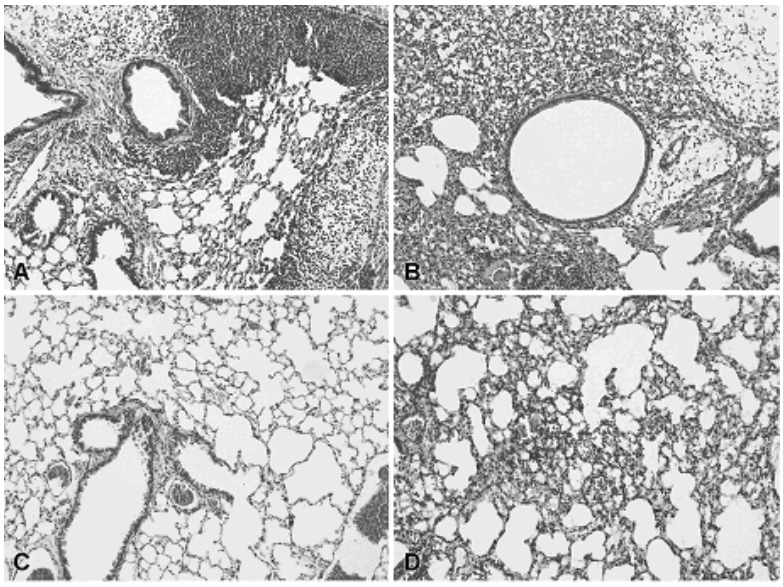

Fig. 3: CD14 KO mice display reduced lung inflammation. Representative lung slides of WT (panels A and B) and CD14 KO (panels C and D) mice 24 hours (panels A and C) and 48 hours (panels B and D) after infection with 5 x 10^4 CFU *S. pneumoniae*. CD14 KO mice displayed less lung inflammation at both time points as determined by the semi-quantitative histology scores described in the Methods section (24 h: 7 ± 2 in WT and 3 ± 1 in CD14 KO mice, $P < 0.05$; 48 h: 13 ± 2 and 5 ± 2 respectively, $P < 0.005$; data are mean ± SEM of 8 mice per strain at each time point). 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). Forty-eight hours after infection, neutrophil counts were equal in BALF of both mouse strains (Fig. 4A).

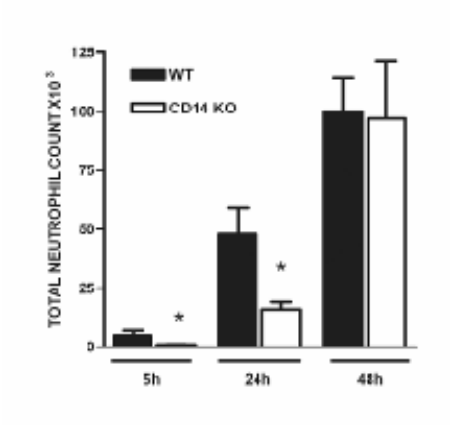

Fig. 4: CD14 KO mice demonstrate a reduced neutrophil influx into bronchoalveolar lavage fluid. Neutrophil numbers in BALF at 5, 24 and 48 hours after infection with 5 x 10^4 CFU *S. pneumoniae*. Data are means ± SEM (N = 7-8 mice per group at each time point). * $P < 0.05$ versus WT mice.

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. 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 (data not shown).

pg/ml	Wt	CD14 KO
TNF-α	421 ± 82	528 ± 229
IL-1β	12596 ± 865	4275 ± 2872*
IL-6	2972 ± 542	479 ± 192‡
IL-10	2038 ± 423	566 ± 17‡
MCP-1	7498 ± 955	1568 ± 459†
MIP-2	19131 ± 4928	7591 ± 3166*
KC	5922 ± 748	2352 ± 459‡

Table 1: CD14 KO mice have reduced cytokine and chemokine levels in lung homogenates 48 hours after infection Mice were intranasally infected with 5x10^4 CFU *S. pneumoniae* and lung homogenates were prepared 48 hours later. Data are means ± SEM (N=8 per group). * P <0.05 † P<0.001 ‡ P<0.005.

At 48 hours CD14 KO mice demonstrated reduced concentrations of all mediators except TNF-α (all $P < 0.05$, Table I). TNF-α, MCP-1 and IL-6 levels in blood of CD14 KO mice were significantly lower compared to WT mice 48 hours after inoculation (all $P < 0.005$; data not shown).

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. 5A). 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. 5B).

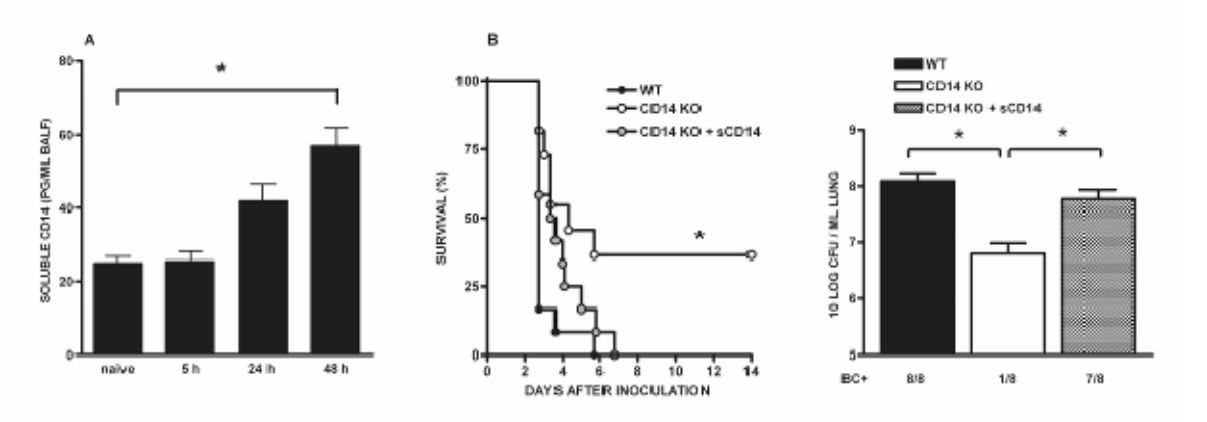

Fig. 5: Treatment with recombinant soluble CD14 results in invasive infection in CD14 KO mice. (A) Soluble CD14 concentrations in BALF of WT mice infected with 5 x 10^4 CFU *S. pneumoniae*. Data are means ± SEM (N = 7-8 mice per group at each time point) * $P < 0.05$ compared to naïve mice (B) Survival of WT and CD14 KO mice which received either saline or recombinant mouse sCD14 intranasally (1 µg; 0, 24 and 48 hours). WT mice, closed symbols. CD14 KO mice, open symbols. CD14 KO mice + sCD14, grey symbols. * 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. WT mice, closed bars. CD14 KO mice, open bars. CD14 KO mice treated with recombinant mouse sCD14 (1 µg; 0 and 24 hours), hatched bar. Data are means ± SEM (N = 7-8 mice per group at each time point) * $P < 0.05$ CD14 KO mice vs WT mice and vs CD14 KO mice + sCD14.

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. 5C). 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 (Fig. 6). In line, the lung and plasma concentrations of cytokines and chemokines were increased by sCD14 administration to CD14 KO mice (data not shown). Together these data indicate that the presence of sCD14 in the bronchoalveolar compartment of CD14 KO mice can fully compensate for CD14 gene deficiency.

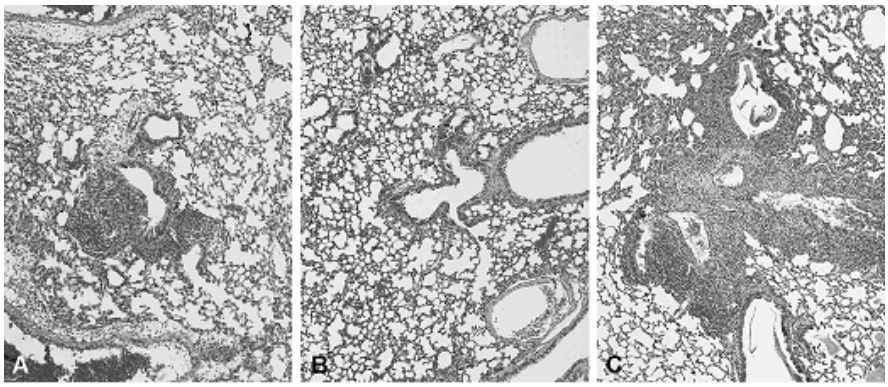

Fig. 6: Treatment with recombinant soluble CD14 enhances lung inflammation in CD14 KO mice. Representative lung slides of WT (panel A) and CD14 KO treated with saline (panel B) or sCD14 (panel C). Mice were sacrificed 48 hours after infection with 5 x 10^4 CFU *S. pneumoniae*. CD14 KO mice displayed less lung inflammation as determined by the semi-quantitative histology scores described in the Methods section, which was reversed by sCD14 administration (WT: 15 ± 2, CD14 KO: 4 ± 2, CD14 KO + sCD14: 17 ± 2, $P < 0.05$ for the difference between CD14 KO mice and the other two groups; data are mean ± SEM of 7-8 mice per group). H&E staining: magnification x 4.

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). Such knowledge is not readily available for Gram-positive infections. 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*, the most commonly isolated pathogen in patients with community-acquired pneumonia. 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 intravenous or intraperitoneal injection of *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, as reflected by increased neutrophil numbers in cerebrospinal fluid and elevated TNF-α and MIP-2 levels in brain homogenates. 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, a different route of administration 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, bacteria are delivered into the lower airways where they meet the natural components of the pulmonary innate immune system including an intact alveolar-capillary barrier. As such, we provide clear evidence that either cell-associated or sCD14 present in the bronchoalveolar compartment facilitates the invasion of pneumococci into lung tissue and blood. Indeed, whereas the number of *S. pneumoniae* CFU remained similar in BALF of CD14 KO and WT mice throughout, 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. It remains to be established whether CD14 can play a similar role in passing other anatomical barriers, including the blood-brain barrier such as during meningitis.

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 was the only cytokine that was not affected by CD14 deficiency; all other mediators measured, 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). At present it is not clear why CD14 deficiency influenced cytokine levels differently in *S. aureus* sepsis and *S. pneumoniae* meningitis than in *S. pneumoniae* pneumonia, although – as described above - the first two models differ in several ways from the model used here.

Fundamental research has elucidated an important mechanism by which the pneumococcus interacts with cells lining the respiratory tract to cause tissue invasion. 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) CD14 is involved in the presentation (of components) of *S. pneumoniae* to the PAFR so that the phosphoryl – PAFR mediated invasion is facilitated. At present, however, such a mechanism remains speculative. On the other hand, an interaction between CD14 and TLRs is unlikely to explain our observations: we recently established that TLR2 does not contribute to host defense against pneumococcal pneumonia whereas TLR4 has been found to play a protective role (30, 31, 39). Moreover, mice deficient for the common TLR adaptor protein MyD88 displayed a strongly reduced resistance against nasopharyngeal infection with *S. pneumoniae* (40).

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*. Considering that CD14 contributes to an effective protective immune response during Gram-negative respiratory tract infection and considering that TLR signaling contributes to host defense during pneumococcal pneumonia, 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 14

Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense

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Abstract

Toll-like receptors (TLR) are crucial pattern recognition receptors in innate immunity. The importance of TLR2 in host defense against Gram-positive bacteria has been suggested by the fact that this receptor recognizes major Gram-positive cell-wall components, such as peptidoglycan and lipoteichoic acid. To determine the role of TLR2 in pulmonary Gram-positive infection, we first established that TLR2 is indispensable for alveolar macrophage responsiveness toward *Streptococcus pneumoniae*. Nonetheless TLR2 gene deficient mice intranasally inoculated with *S. pneumoniae* at doses varying from nonlethal (with complete clearance of the infection) to lethal displayed only a modestly reduced inflammatory response in their lungs and an unaltered antibacterial defense when compared with normal wild type mice. These data suggest that TLR2 plays a limited role in the innate immune response to pneumococcal pneumonia and that additional pattern recognition receptors likely are involved in host defense against this common respiratory pathogen.

Introduction

Streptococcus pneumoniae is the leading causative pathogen in community-acquired pneumonia and a major cause of morbidity and mortality in humans (1-3). Pneumococci account for up to 36% of adult community-acquired pneumonia in the Unites States. An estimated 570.000 cases of pneumococcal pneumonia occur annually in the United States, including 175.000 hospitalized cases (4). The rise in antibiotic resistance of this pathogen urges further efforts to understand the host response mechanisms involved in pneumococcal pneumonia (5, 6).

The first line of defense against invading bacteria is provided by the innate immune system, which recognizes pathogen associated molecular patterns (PAMPs), conserved microbial patterns shared by large groups of pathogens, but not found in higher eukaryotes (7-9). Over the last few years it has become evident that both the recognition and the subsequent response to pathogens is mainly transferred by members of the Toll-like receptor (TLR) family (for review see: (8, 10, 11)). Ten TLRs have been described so far, among which TLR2 and TLR4 are the best-investigated family members. TLR4, the LPS receptor, is important for the recognition of Gram-negative bacteria, while TLR2 has been designated the major receptor for Gram-positive bacteria by virtue of its capacity to recognize major cell wall constituents of Gram-positive microorganisms, such as peptidoglycan (PGN), lipoteichoic acid (LTA) and lipoproteins (12-16). TLR2, together with TLR1 and TLR6, have been demonstrated to migrate to phagosomes within phagocytic cells, where they might sample the contents and signal the presence of an invader (17, 18). TLR2 has to cooperate and heterodimerize with other TLRs like TLR6 or TLR1 to activate TNF-α production in macrophages (18). Moreover, evidence exists that TLR2 is directly involved in bacterial killing by monocytes and macrophages (19).

The role of TLRs in the innate recognition of *S. pneumoniae* has been the subject of several recent investigations. In a model of a Chinese hamster ovary (CHO) reporter fibroblast cell line, heat-killed *S. pneumoniae* were found to partially stimulate CHO cells in the absence of TLR expression (13). The responsiveness of CHO cells markedly increased after TLR2 expression, suggesting that *S. pneumoniae* stimulates both a TLR2-dependent and a TLR2-independent pathway (13). In the human embryonic kidney cell line HEK 293, transfection with either TLR2 or TLR4 resulted in cellular activation by *S. pneumoniae* (20). However, peritoneal macrophages harvested from mice lacking functional TLR2 and/or TLR4 responded normally to *S. pneumoniae*, suggesting that for activation of these cells neither TLR2 nor TLR4 is required (20). Two investigations examined the role of TLR2 in host defense against *S. pneumoniae in vivo*, both making use of a meningitis model in which pneumococci were injected directly into the central nervous system (CNS) (20, 21). Both studies reported an increased disease severity in TLR2 deficient ($TLR2^{-/-}$) mice together with a moderately elevated bacterial outgrowth in the CNS when compared with normal wild type (wt) mice. Very recently Malley *et al.* reported an important role for TLR4 in the innate immune response to *S. pneumoniae* in the nasopharynx (22). Indeed, C3H/HeJ mice, which

display a mutant nonfunctional TLR4, were found to be more susceptible to invasive disease after colonization of the nasopharynx with pneumococci (22).
Knowledge of the role of TLR2 in host defense against respiratory tract infections is highly limited. Therefore, in the present study we sought to determine the role of TLR2 in the innate immune response to pneumococcal pneumonia.

Materials and Methods

Mice

Pathogen-free 10-12 wk-old male C57BL/6 wild-type (wt) mice were purchased from Harlan Sprague-Dawley (Horst, The Netherlands). $TLR2^{-/-}$ mice were generated as described previously (12) and backcrossed to C57BL/6 background 6 times and bred in the animal facility of the Academic Medical Center in Amsterdam. Age and sex matched mice were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments.

Induction of pneumonia

Pneumonia was induced as described previously (23-25). Briefly, *S. pneumoniae* serotype 3 obtained from American Type Culture Collection (ATCC 6303, Rockville, MD) were grown for 6 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. Bacteria were then resuspended in sterile isotonic saline at a concentration of $2x10^3$ to $5x10^5$ CFUs/50µl, as determined by plating serial 10-fold dilutions on sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, The Netherlands) and 50 µl (containing $2x10^3$ to $5x10^5$ CFU, depending on the experiment) was inoculated intranasally.

Evaluation of TLR2 mRNA levels by RT-PCR

Total RNA was extracted from lungs of wt mice 24h after inoculation with *S. pneumoniae* or saline using chloroform extraction and isopropanol precipitation. After treatment with RQ1 RNase-Free DNase (Promega, Madison, WI), total RNA was reverse transcribed using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Paisley, U.K.) according to the supplier's recommendations. cDNA samples were standardized based on the content of β-actin cDNA as housekeeping gene. β-actin cDNA was evaluated by performance of a β-actin PCR on multiple dilutions of each cDNA sample. The amount of amplified product was estimated by densitometry of ethidium bromide-stained 1.2% agarose gels using a CCD camera and Imagemaster VDS software (Pharmacia, Uppsala, Sweden). Primers for TLR2 were 5'-TCT GGG CAG TCT TGA ACA TTT-3' (sense primer) and 5'-AGA GTC AGG TGA TGG ATG TCG-3' (antisense primer), yielding a 321-bp fragment; primers for murine β-actin were 5'-TAA AAC GCA GCT CAG TAA CAG TCG G-

3' (sense primer) and 5'-TGC AAT CCT GTG GCA TCC ATG AAA C-3' (antisense primer). Using appropriate dilutions of the cDNA, PCR was performed as described previously (26). Levels of TLR2 mRNA expression were evaluated by densitometric image analysis and relative TLR2 mRNA levels were calculated by comparison of band intensities of the TLR2 RT-PCR products with standard curves prepared by PCR amplifications on dilution series of a highly concentrated murine lung cDNA.

Preparation of alveolar macrophages

Alveolar macrophages (AM) were harvested from TLR2$^{-/-}$ and wt mice by bronchoalveolar lavage (BAL) (n=8 per strain). The trachea was exposed through a midline incision and canulated with a sterile 22-gauge Abbocath-T catheter (Abott, Sligo, Ireland). BAL was performed by instilling two 0.5 ml aliquots of sterile saline. Total cell numbers were counted from each sample using a hemocytometer. Cells of two mice were pooled, washed and resuspended in RPMI 1640 containing 1mM pyruvate, 2mM L-glutamine, penicillin, streptomycin and 10% FCS in a final concentration of 1x10^{5}cells/ml. Cells were then cultured in 96-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) for 2h and washed with RPMI 1640 to remove non-adherent cells. Adherent monolayer cells were stimulated with LPS (from *E.coli* O55:B5, 1 μg/ml, Sigma (St.Louis, MO)), LTA (from *S. aureus*, 10μg/ml, kind gift of Dr. T. Hartung, Univ. Konstanz, Germany, (27)), heat-killed *S. pneumoniae* (5x10^{7}CFU/ml, ATCC 6303) or RPMI 1640 for 16h. Supernatants were collected and stored at –70°C until assayed for TNF-α and KC.

Determination of bacterial outgrowth

Six or 48h after infection, mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Meidrecht, the Netherlands) and sacrificed by bleeding out the vena cava inferior. Blood was collected in EDTA containing tubes. Whole lungs were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs were determined from serial dilutions of lung homogenates and blood, plated on blood agar plates and incubated at 37°C for 16 h before colonies were counted.

Preparation of lung tissue for cytokine measurements

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.

Histology

Lungs for histology were harvested at 6, 48 or 72h after infection, fixed in 4% formaline and embedded in paraffin. 4 μm sections were stained with H&E, 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: interstitial inflammation, intra-alveolar inflammation, edema, endothelialitis, bronchitis, pleuritis and thrombi formation. Each parameter was graded on a scale of 0 to 3, with 0: absent, 1: mild, 2: moderate and 3: severe. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 21. Granulocyte staining was done as described previously (23, 28). Briefly, slides were deparaffinized and endogenous peroxidase activity was quenched by a solution of methanol/0.03% H_2O_2 (Merck, Darmstadt, Germany). After digestion with a solution of pepsine 0.25% (Sigma, St Louis, MO) in 0.01M HCl, the sections were incubated in 10% normal goat serum (Dako, Glostrup, Denmark) and then exposed to FITC-labelled anti-mouse Ly-6G mAb (BDPharmingen, San Diego, CA). Slides were incubated with a rabbit anti-FITC antibody (Dako) followed by a further incubation with a biotinylated swine anti-rabbit antibody (Dako), rinsed again, incubated in a streptavidin-ABC solution (Dako) and developed using 1% H_2O_2 and 3.3'-diaminobenzidin-tetra-hydrochloride (Sigma) in Tris-HCl. The sections were mounted in glycerin gelatin without counter staining and analyzed. All antibodies were used in concentrations recommended by the manufacturers.

Immunohistochemistry for TNF-α

Lungs from wt and TLR2$^{-/-}$ mice (n=8 per group) were harvested 48h after induction of pneumonia. Pulmonary lung suspensions were obtained by crushing lungs through a 40μm cell strainer (BDPharmingen), cells were washed, resuspended in PBS and cytospins were prepared. Endogenous peroxidase activity was quenched by a solution of 0.3% H_2O_2 in PBS 0.1%NaN_3 (Merck). After a blocking step with 10% normal goat serum (Dako), slides were incubated with rabbit-anti-mouse TNF-α antibodies (Bioscource, Camarillo, CA) in the presence of 1% saponin (Sigma) followed by exposure to streptavidin goat-anti-rabbit antibody (ImmunoLogic, Duiven, the Netherlands) and developed using 1% H_2O_2 and 3.3'-diaminobenzidin-tetra-hydrochloride (Sigma) in Tris-HCl. Antibodies were used in concentrations recommended by the manufacturers.

Cytokines

Cytokines and chemokines (TNF-α, IL-1β, IL-6, IL-10, KC, MIP-2) were measured using specific ELISAs (R&D Systems, Minneapolis, MN) according to the manufacturers' instructions. The detection limits were 31 pg/ml for TNF-α and IL-10, 8 pg/ml for IL-1β, 16 pg/ml for IL-6, 12 pg/ml for KC and 94 pg/ml for MIP-2.

Statistical analysis

Differences between groups were analyzed using Mann-Whitney *U* test. For survival analyses, Kaplan-Meier analysis followed by log rank test was performed. Results of the *in vitro* stimulation were calculated with one-way analysis of variance followed by Bonferroni's multiple comparison tests. Values are expressed as mean ± SEM. A p-value ≤ 0.05 was considered statistically significant.

Results

Lung TLR2 mRNA expression is up-regulated during pneumococcal pneumonia

The pulmonary TLR2 expression was determined in lungs from healthy control mice (receiving saline) and animals challenged with *S. pneumoniae* to obtain constitutive and infection-induced TLR2 mRNA levels. As depicted in Fig. 1, TLR2 mRNA was constitutively expressed in healthy lung tissue and markedly enhanced following pulmonary infection with pneumococci.

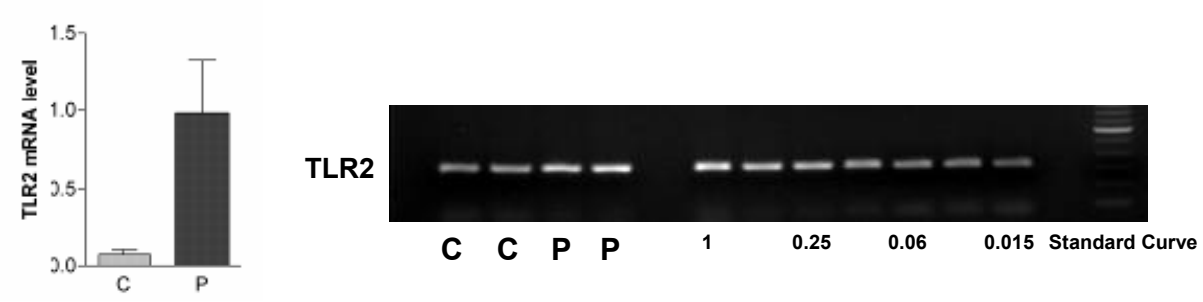

Fig. 1: Lung TLR2 mRNA expression after infection with *S. pneumoniae.* Pulmonary TLR2 mRNA expression was determined by specific RT-PCR amplification of lung cDNA samples of wt mice inoculated i.n. with saline (control, C) or *S. pneumoniae* (pneumonia, P). *S. pneumoniae* induced an increased expression of TLR2 compared to saline treated controls. Results shown are representative of 3 independent RT-PCR of lung cDNA samples; cDNA was standardized for β-actin content. Semi-quantitative data were generated by densitometric evaluation of RT-PCR products, which were compared with a standard curve obtained by amplification of a serial dilution of highly concentrated cDNA.

TLR2 is essential for responsiveness of AM to heat-killed *S. pneumoniae in vitro*.

To obtain a first insight into the function of TLR2 in the pulmonary host response to *S. pneumoniae* we determined the responsiveness of AM to heat-killed pneumococci and, as controls, LPS (signaling in a TLR2 independent way) and LTA (signaling in a TLR2 dependent way) (12, 15, 27).

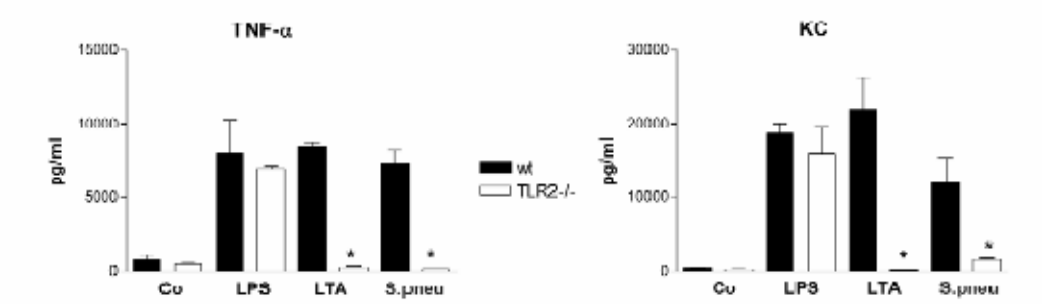

Fig. 2: TLR2 is required for responsiveness of alveolar macrophages to *S. pneumoniae.* Freshly isolated alveolar macrophages of wt and TLR2$^{-/-}$ mice (n = 8 per group: cells from 2 mice were pooled yielding 4 samples per group) were incubated with RPMI (control), LPS (1μg/ml), LTA (10μg/ml) or heat-killed *S. pneumoniae* (equivalent of 5x10^7 CFU/ml) for 16h before TNF-α and KC production was measured (* $p<0.05$ TLR2$^{-/-}$ versus wt).

Freshly isolated AM from TLR2$^{-/-}$ mice failed to release TNF-α or KC upon stimulation with heat-killed *S. pneumoniae* whereas wt AM released high amounts of TNF-α and KC ($p<0.05$, Fig. 2). As expected, LPS stimulated TLR2$^{-/-}$ and wt AM to release similar amounts of TNF-α and KC, whereas LTA induced TNF-α and KC secretion in cultures of wt but not of TLR2$^{-/-}$ AM (Fig.2). Hence, the responsiveness of AM to *S. pneumoniae* depended on the presence of TLR2.

TLR2 plays a role in the lung inflammatory response to *S. pneumoniae in vivo*

Having established that TLR2 mediates *S. pneumoniae* induced TNF-α and KC release by AM *in vitro*, we next determined whether the pulmonary cytokine/chemokine response to respiratory tract infection with pneumococci was altered in the absence of TLR2 *in vivo*. For this, the concentrations of TNF-α, IL-1β, IL-6, IL-10, KC and MIP-2 were assessed in lung homogenates obtained from TLR2$^{-/-}$ and wt mice 48h after infection with a low (10^4 CFU), intermediate (approximate LD_{60}; 10^5 CFU) or high (lethal; $5x10^5$ CFU) *S. pneumoniae* dose. We chose to measure these mediators 48 h postinfection, since we previously established that this time-point is suitable for this purpose in this model (23, 24, 29). Mediator levels did not differ between TLR2$^{-/-}$ and wt mice (data not shown), except for KC and MIP-2 concentrations after infection with 10^5 *S. pneumoniae* CFU, which were lower in TLR2$^{-/-}$ mice (1.6±0.5 and 8.3±2.1 ng/ml, respectively) than in wt mice (3.1±0.5 and 17.9±4.7 ng/ml respectively; both $p\leq0.05$ for the difference between groups).

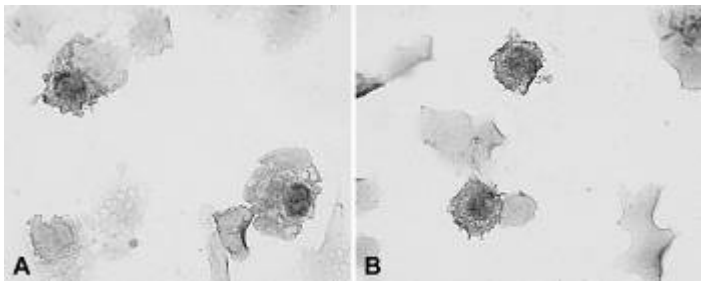

Fig. 3: TNF-α is produced by macrophages *in vivo*. Representative immunohistochemical stainings for TNF-α on lung cell suspensions of wt (A) and TLR2$^{-/-}$ mice (B) 48h after infection with $5x10^3$ CFU *S. pneumoniae* (n=7 per group) showing macrophages with similar staining intensities. Epithelial cells and granulocytes did not display positive staining.

Since the *in vivo* finding of unaltered TNF-α concentrations in the lungs of wt and TLR2$^{-/-}$ mice contrasted with *in vitro* studies using alveolar macrophages (Fig. 2), we decided to investigate the cellular source of TNF-α *in vivo* and performed immunohistochemical stainings on lung cell suspensions obtained 48h after induction of pneumonia. Pulmonary macrophages proved to be the main cellular source of TNF-α in wt and TLR2$^{-/-}$ mice *in vivo* (Fig. 3). To further evaluate the role of TLR2 in lung inflammation induced by *S. pneumoniae in vivo*, lung histology slides, obtained 48 h after infection with either 10^4 or 10^5 CFU *S. pneumoniae*, were scored as described in the Methods section. At both inoculum doses, TLR2$^{-/-}$ mice displayed significantly less inflammation, edema and pleuritis when compared

to wt mice (Fig. 4, Table 1). In accordance, granulocyte staining of lung slides from TLR2$^{-/-}$ mice revealed less granulocytes than lung tissue obtained from wt mice (inserts in Fig 4).

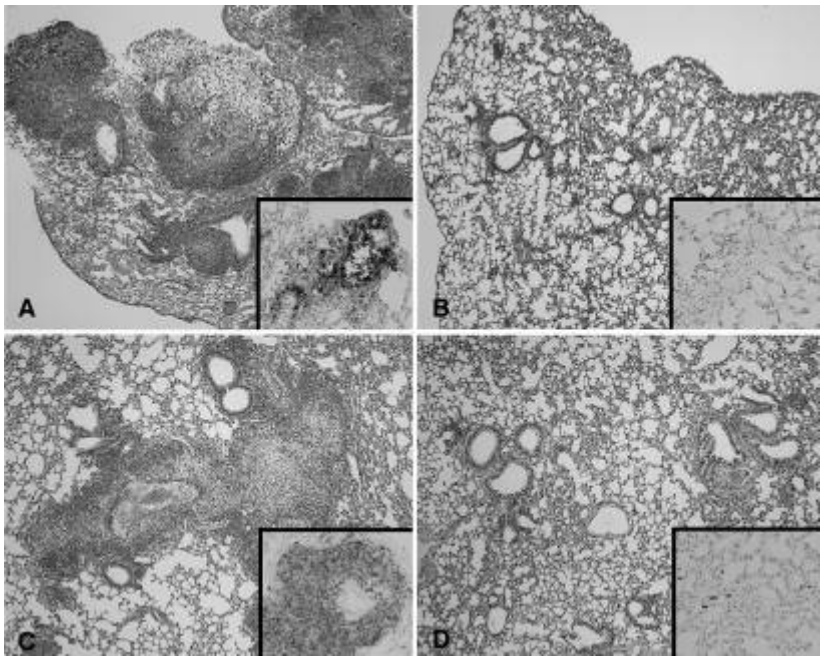

Fig. 4: Reduced lung inflammation in TLR2$^{-/-}$ mice 48h after infection. Representative lung histology of wt (A and C) and TLR2$^{-/-}$ (B and D) mice 48 h after infection with 10^4 CFU (A and B) or 10^5 CFU (C and D) *S. pneumoniae* showing significantly more inflammation, pleuritis, edema, bronchitis and endothelialitis in wt mice compared to TLR2$^{-/-}$ animals. The inserts are representative pictures of immunostaining for granulocytes, confirming the dense granulocytic infiltration in wt mice. The histological lung sections are representative for at least 5 mice per group. H&E staining: magnification x 4; inset (Ly-6-staining): magnification x 20.

S. pneumoniae:	***10^4 CFU***		***10^5 CFU***	
	Wt	**TLR2$^{-/-}$**	**Wt**	**TLR2$^{-/-}$**
Inflammation Score	10.9±1.6	6.4±1.6 *	17.0±0.5	9.6±2.8 *

Table 1: Less severe pulmonary inflammation in TLR2$^{-/-}$ as compared to wt mice. Inflammation scores (as described in the Methods section) in wt and TLR2$^{-/-}$ mice 48h after infection with 10^5 or 10^4 CFU *S. pneumoniae*, respectively. Data are presented as mean ± SEM of n= 7-8 mice per strain; * $p<0.05$ versus wt mice.

TLR2 does not contribute to bacterial clearance

The clearance of bacteria from the respiratory tract during pneumococcal pneumonia strongly depends on the efficacy to mount a local inflammatory response (30, 31). To evaluate whether the lack of TLR2 interferes with bacterial clearance, we determined bacterial loads in lungs 48 hours after infection with $5x10^3$, 10^4, 10^5 or $5x10^5$ CFU *S. pneumoniae*. At all four bacterial doses, TLR2$^{-/-}$ mice tended to have less *S. pneumoniae* CFU in their lungs than wt mice, although the differences between the two mouse strains did not reach significance (Fig. 5). Likewise, the proportion of bacteremic mice tended to be lower in TLR2$^{-/-}$ mice, irrespective of the size of inoculum (data not shown).

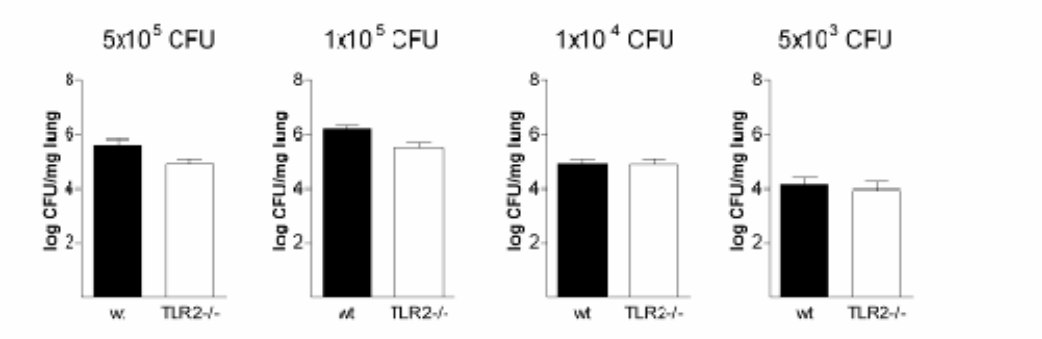

Fig. 5: TLR2$^{-/-}$ mice display an unaltered bacterial clearance. Wt and TLR2$^{-/-}$ mice were inoculated with 5x10^5, 10^5, 10^4 or 5x10^3 CFU *S. pneumoniae* and bacterial outgrowth in lungs were obtained 48h later. Data represent mean ± SEM of n=7-8 mice per strain, 48h after infection.

Moreover, survival did not differ between TLR2$^{-/-}$ and wt mice after infection with a high (10^5 CFU) or low (5x10^3 CFU) bacterial inoculum (Fig. 6), whereas both mouse strains completely cleared pneumococci from their lungs after infection with a bacterial dose of 2x10^3 CFU after 72h. Hence, these data suggest that TLR2 does not contribute to local antibacterial defense or containment of the infection during pneumococcal pneumonia.

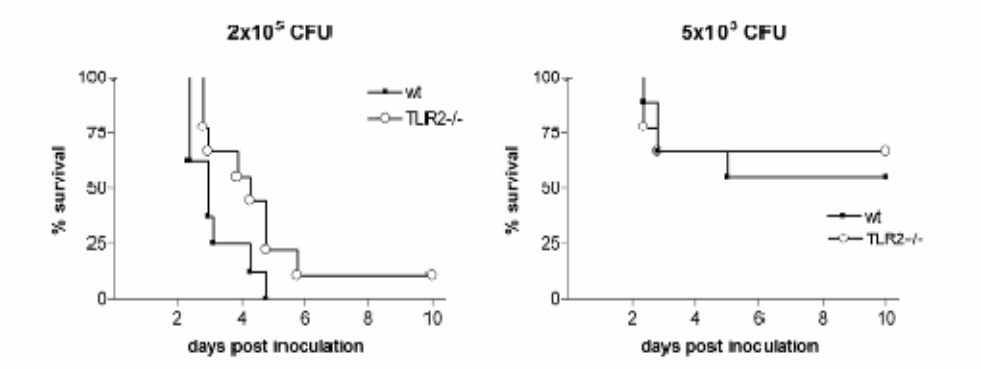

Fig. 6: Unaltered survival in TLR2$^{-/-}$ mice . Wt and TLR2$^{-/-}$ mice (n=8-9) were i.n. inoculated with 2x10^5 (A) or 5x10^3 (B) *S. pneumoniae* and survival was monitored.

Role of TLR2 early after infection

Postulating that TLR2 might play a more important role in the early initiation phase of the host response to pneumococcal pneumonia, we infected mice with 10^5 CFU *S. pneumoniae* and sacrificed them 6h later. IL-1β, IL-6 and KC levels were significantly lower in TLR2$^{-/-}$ than in wt mice at this early time-point, whereas TNF-α, IL-10 and MIP-2 levels did not differ between the two mouse strains (Table 2). The extent of lung inflammation was found slightly lower in TLR2$^{-/-}$ mice at this time-point (data not shown). No difference in bacterial loads in lungs of TLR2$^{-/-}$ and wt mice was observed (2.4±1.5x10^4 CFU/ml and 5.4±1.9x10^4 CFU/ml, respectively, n.s.) and blood cultures were all negative.

pg/ml	*Wt*	*TLR2$^{-/-}$*
TNF-α	2631 ± 297	2374 ± 173
IL-1β	1787 ± 424	549 ± 88 *
IL-6	153 ± 36	78 ± 4 *
IL-10	462 ± 57	464 ± 35
KC	3132 ± 504	1880 ± 87 *
MIP-2	4328 ± 632	4421 ± 417

Table 2: Reduced lung concentrations of IL-1β, IL-6 and KC in TLR2$^{-/-}$ mice 6 h after infection with *S. pneumoniae*. Cytokines and Chemokines measured in lung homogenates 6h after infection with 10^5 CFU *S. pneumoniae*. Data are means ± SEM, n=9 per strain; * $p<0.05$ versus wt mice.

Discussion

S. pneumoniae is the most frequently isolated pathogen in community-acquired pneumonia and responsible for an estimated ten million deaths annually, making pneumococcal pneumonia a major health threat worldwide (32, 33). Previous observations have pointed to TLR2 as the key pattern recognition receptor in the immune response to Gram-positive bacteria (12-14, 34). In this report we sought to obtain insight into the contribution of TLR2 in Gram-positive pneumonia and investigated the requirement for TLR2 during both *in vitro* and *in vivo* infection with *S. pneumoniae*. We used AM in *in vitro* studies and showed that TLR2 is required for responsiveness to *S. pneumoniae*. However, although TLR2 played a role in the induction of an inflammatory response in the lung during pneumococcal pneumonia, TLR2$^{-/-}$ mice displayed an uncompromised antibacterial defense against pneumococci in the respiratory tract using infectious inocula ranging from non-lethal to lethal. Together these data indicate that TLR2 is not indispensable in the innate immune response to *S. pneumoniae* pneumonia.

There is abundant evidence from *in vitro* observations that TLR2 is the predominant receptor signaling the presence of cell wall components of Gram-positive bacteria, such as PGN or LTA (13-15, 35, 36). However, data that confirm these observations *in vivo* are still scarce. Both LTA and PGN alone are capable of inducing acute lung inflammation *in vivo*, indicating that these PAMPs likely contribute to the local inflammatory response during Gram-positive pneumonia (28). In this report we demonstrated that AM require TLR2 to mount an inflammatory response to LTA. Similarly, TLR2$^{-/-}$ AM did not respond to *S. pneumoniae in vitro*. Considering that AM are major players in the induction and regulation of lung inflammation during respiratory tract infections (30), we expected TLR2$^{-/-}$ mice to mount a reduced inflammatory response to pneumococcal pneumonia, and as a consequence thereof an impaired bacterial clearance. We indeed observed less inflammation in lungs of TLR2$^{-/-}$ mice, albeit the differences with wt mice were modest. Importantly, antibacterial defense was indistinguishable in TLR2$^{-/-}$ and wt mice Given the multitude of factors involved in the innate immune response against pneumococcal pneumonia, such as natural antibodies and

complement, these mediators are likely candidates to compensate for the lack of TLR2 (37). This might also explain why *in vivo* $TLR2^{-/-}$ macrophages stained positive for TNF-α during pneumonia, whereas incubation of isolated $TLR2^{-/-}$ macrophages with *S. pneumoniae in vitro* did not result in TNF-α production. Thus, these results suggest that TLR2 plays a modest role in the immune response to *S. pneumoniae* in the respiratory tract, which does not influence the overall antibacterial defense during pneumococcal pneumonia.

Two recent reports investigated the role of TLR2 in pneumococcal meningitis *in vivo* and demonstrated an aggravated course in mice lacking TLR2 using a clinical severity score (20, 21). One study provided survival data showing a 100% fatality rate in both $TLR2^{-/-}$ and wt mice (21). In both investigations, in which *S. pneumoniae* was injected intracisternally, differences between $TLR2^{-/-}$ and wt mice in bacterial clearance were modest at best. Indeed, in the study by Echchannaoui *et al.* bacterial counts in blood and cerebrospinal fluid were similar in both mouse strains (21). Using luciferase-tagged *S. pneumoniae* these investigators reported higher fluorescence intensity in brains of $TLR2^{-/-}$ mice than in wt brains. In the study by Koedel *et al.* $TLR2^{-/-}$ mice displayed more pneumococci in their cerebellum and blood, but not in their spleen (20). The recruitment of neutrophils to the CNS and blood cytokine concentrations were unaltered (20, 21). In contrast to this CNS infection model we tried to reproduce the natural route of pulmonary infection and infected mice i.n. with *S. pneumoniae*. By using this approach we found no major difference between wt and $TLR2^{-/-}$ mice in their pulmonary host response to pneumococci. In *S.aureus* sepsis, Takeuchi *et al.* reported higher susceptibility and impaired bacterial clearance in $TLR2^{-/-}$ mice when high bacterial doses were administered, whereas no clear difference between wt and $TLR2^{-/-}$ animals could be found when mice were treated with lower bacterial doses (34). We investigated the role of TLR2 in host defense against *S. pneumoniae* using high and low infectious doses and did not observe differences between $TLR2^{-/-}$ and wt mice. All together, the observations of moderately impaired host defense in pneumococcal meningitis (20, 21) and our results of an unaltered host defense of $TLR2^{-/-}$ mice in pneumococcal pneumonia, point towards the simultaneous involvement of different recognition receptors in the innate immune response to *S. pneumoniae*. TLR2 is apparently one participating receptor, but *in vivo* findings thus far could not reveal an unequivocal role for TLR2 as the prime receptor in this Gram-positive infection. Moreover, the absence of MyD88 rendered mice more susceptible to *S. aureus* sepsis than the lack of TLR2 alone, suggesting the involvement of additional MyD88-dependent mechanisms (34).

The immune response to *S. pneumoniae* continues to be a point of interest. *In vitro* experiments using CHO cells could not demonstrate an equally strong dependency on TLR2 as it was found for *S. aureus*, since a partial, though less pronounced, immune response to whole *S. pneumoniae* could be observed in the absence of TLR2 (13). Evidence is increasing that the innate immune response to pathogens does not solely depend on the composition of the outer cell wall of bacteria but also involves factors produced and released by bacteria. Moreover, that these bacterial factors can also signal in a TLR-dependent way has been shown for group B streptococci and Yersinia (38-40). Of great interest, a recent report

demonstrated that pneumolysin, known as a major toxic component of *S. pneumoniae*, signals via TLR4 (22). Pneumolysin gets released upon lysis of pneumococci during multiplication of bacteria and has many biological activities like induction of cytokines and cytotoxicity (41-43). Malley *et al.* used a model of i.n. inoculation in unanesthetized mice, in which the infecting organism is confined to the upper respiratory tract, not progressing to the lung (22). These investigators monitored nasopharyngeal colonization using luminescent pneumococci and found a much higher bacterial nasopharyngeal burden (as determined by photon emission) in TLR4 mutant than in wt mice. While Malley *et al.* focused on the role of TLR4 during nasopharyngeal colonization with *S. pneumoniae* (22), our laboratory recently studied the role of this receptor in the pneumonia model also used in the current investigation (44). In *S. pneumoniae* pneumonia, TLR4 appeared to play a modest protective role, as indicated by slightly higher bacterial loads in lungs of TLR4 mutant mice than in wt mice (44). Interestingly, in the model by Malley *et al.* TLR4 mutant mice demonstrated an increased mortality when infected with wt but not with pneumolysin deficient pneumococci, indicating that the interaction between pneumolysin and TLR4 during colonization of the nasopharynx is important for protection against invasive pneumococcal disease (22). This report also showed that the host response to pneumolysin was synergistically potentiated by costimulation with TLR2-dependent cell-wall components of *S. pneumoniae*. The lack of costimulatory signals in the absence of TLR2 likely contributed to the diminished pulmonary inflammation we observed in $TLR2^{-/-}$ mice. Of note, the immune response to *S. pneumoniae* in the lungs likely includes other receptors and/or pathways. Thus, the roles of for example TLR6 and TLR1, which can form heterodimers with TLR2 for optimal detection of some Gram-positive PAMPs (18, 45), and TLR9, which recognizes bacterial DNA (46), in *S. pneumoniae* pneumonia remain to be established.

In conclusion, we here show that TLR2 does not importantly contribute to host defense in pneumococcal pneumonia. By ruling out the absolute requirement for TLR2 in host defense against *S. pneumoniae in vivo*, our data contribute valuable information to the paradigm of complex interactions between multiple pattern recognition receptors in host defense against a single pathogen *in vivo*.

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CHAPTER 15

The role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice

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Abstract

To determine the role of Toll-like receptor (TLR) 4 in the immune response to pneumonia, C3H/HeJ mice (which display a mutant non-functional TLR4), and C3H/HeN wild type mice were intranasally infected with either *Streptococcus pneumoniae* or *Klebsiella pneumoniae*, common Gram-positive and Gram-negative respiratory pathogens respectively. In pneumococcal pneumonia, TLR4 mutant mice showed a reduced survival only after infection with low bacterial doses, which was associated with a higher bacterial burden in their lungs 48 hours post infection. In *Klebsiella* pneumonia, TLR4 mutant mice demonstrated a shortened survival after infection with either a low or a high bacterial dose, together with an enhanced bacterial outgrowth in their lungs. These data suggest that TLR4 contributes to a protective immune response in both pneumococcal and *Klebsiella* pneumonia, and that its role is more important in respiratory tract infection caused by the latter - Gram-negative - pathogen.

Introduction

Pneumonia is a common and serious illness that is a major cause of morbidity and mortality in humans. *Streptococcus pneumoniae* and *Klebsiella pneumoniae* are frequently isolated causative pathogens of pneumonia (1, 7, 8, 34, 53). Because of the high incidence of pneumonia and the increasing resistance of several bacterial strains to antimicrobial agents (8-10), it is vital to gain more insight into the pathogenesis of pneumonia.

The innate immune system is important for the elimination of bacteria from the pulmonary compartment. One of the first steps in activating host defense mechanisms is recognition of pathogens by phagocytic cells. Phagocytes recognize highly conserved motifs (pathogen-associated molecular patterns; PAMPs) shared by large groups of microorganisms, leading to intracellular signaling and ultimately resulting in the production of cytokines and chemokines and activation of the adaptive immune system (45)**.** One of the best-known PAMPs is endotoxin (lipopolysaccharide, LPS), part of the outer membrane of Gram-negative bacteria and responsible for activating innate host defense mechanisms in Gram-negative infections (52). Gram-positive bacteria do not contain LPS in their cell wall, but express other PAMPs such as lipoteichoic acid (LTA), peptidoglycan (PGN) and lipoproteins.

Recognition of and responses to PAMPs are controlled by several pattern recognition receptors (PRRs). CD14 has been widely accepted as a PRR for a variety of bacterial cell wall components among which LPS (52), LTA (13, 25) and PGN (15). However, CD14 does not contain a cytoplasmic domain and therefore cannot transduce activating signals across the cell membrane. Since a few years, Toll-like receptors (TLRs) are emerging as the key regulators of innate immune responses to infection in mammals (for review, see refs (6, 30, 45)). By now, 10 different members of the TLR family have been identified and for most of them one or more PAMPs have been described (2, 45). TLR4, in complex with CD14 and MD-2, a secreted cell bound protein (32, 40, 43), has been shown to mediate LPS responsiveness, implying that TLR4 is the pattern recognition receptor for LPS (12, 14, 21, 22, 43, 47). In contrast, cell wall components of Gram-positive bacteria (PGN, LTA) induce inflammatory responses predominantly through TLR2 (27, 31, 42, 46, 54). However, in vitro studies done by Takeuchi et al. (46) show that LTA can also signal via TLR4**.** Recent investigations studied the role of TLR4 in host defense against respiratory tract infection by Gram-negative bacteria in vivo, revealing that this receptor contributes to a protective innate immune response against *Haemophilus influenzae* (50) and *Pasteurella pneumotropica* (11, 19), but not against *Legionella pneumophila* (28). In the present study, we conducted experiments in which Gram-positive (*Streptococcus pneumoniae*) and Gram-negative (*Klebsiella pneumoniae*) pneumonia was induced in C3H/HeJ mice, which have non-functional TLR4, and normal wild type (WT) C3H/HeN mice. With these experiments, we sought to determine the role of TLR4 in host defense mechanisms in Gram-positive and Gram-negative pneumonia in mice.

Materials and Methods

Animals

Pathogen-free 8-10 week old, sex-matched, C3H/HeJ (TLR4 mutant) and C3H/HeN (WT) mice were purchased from Charles River (Someren, the Netherlands). C3H/HeJ mice have been demonstrated to have a mis-sense mutation in the third exon of TLR4 resulting in a Pro712→His substitution, yielding a nonfunctional TLR4 (22, 35, 36) .All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, the Netherlands).

Induction of pneumonia

Pneumonia was induced as described before (26, 37, 41). *Streptococcus pneumoniae* serotype 3 (ATCC 6303; Rockville, MD) was used for Gram-positive infection. Pneumococci were cultured for 16 h at 37°C in 5% CO_2 in Todd-Hewitt broth (Difco, Detroit, MI). This suspension was diluted 1:100 in fresh medium and grown for 5 h to midlogaritmic phase. *Klebsiella pneumoniae* serotype 2 (ATCC 43816; Rockville, MD) was used for Gram-negative infection. *Klebsiella* bacteria were cultured for 16 h at 37°C in 5% CO_2 in Tryptic Soy broth (Difco, Detroit, MI). This suspension was diluted 1:100 in fresh medium and grown for 3 h to midlogaritmic phase. *S. pneumoniae* and *K. pneumoniae* were harvested by centrifugation at 1500 x *g* for 15 min and washed twice in sterile 0.9% saline. Bacteria were resuspended in saline at different concentrations (see results), as determined by plating 10-fold dilutions of the suspensions on blood agar plates. After preparation of the bacterial inocula, mice were lightly anesthesized by inhalation of isoflurane (Upjohn, Ede, the Netherlands) and 50 μl of the bacterial suspension was inoculated intranasally. Control mice received 50 μl saline.

Determination of bacterial outgrowth

At 6, 24 and 48 h after infection, mice were anesthesized by FFM (fentanyl citrate 0.079 mg/ml, fluanisone 2.5 mg/ml, midazolam 1.25 mg/ml in H_2O; of this mixture 7.0 ml/kg intraperitoneally), and sacrificed by bleeding out the vena cava inferior. Blood was collected in EDTA containing tubes. Whole lungs were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Serial 10-fold dilutions were made in sterile saline and 10 μl volumes were plated on blood agar plates. In addition, 20 μl volumes of blood were plated. Plates were incubated at 37°C in 5% CO_2, and Colony Forming Units (CFU) were counted after 16 h.

Cell counts in the lungs

In separate experiments, whole lungs were harvested at 6, 24 and 48 h after induction of infection. Lungs were crushed and filtered through a 40 μm cell strainer (Becton Dickinson, Franklin Lakes, NJ); and pulmonary cells were suspended in RPMI (Bio Whittaker, Verviers,

Belgium). Erythrocytes were lysed with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 100 mM EDTA, pH 7.4); the remaining cells were resuspended in RPMI medium. Total cell numbers in each sample were counted using a hemacytometer (Emergo). Differential counts (macrophages, granulocytes, lymphocytes) in the cell suspensions were assessed using cytospin preparations stained with a modified Giemsa stain (Diff-Quick, Baxter, McGraw Park, IL).

Cytokine and chemokine measurements in lung tissue

For cytokine measurements, lung homogenates were diluted 1:2 in lysis buffer (150 mM NaCl, 15 mM Tris, 1mM $MgCl.H_2O$, 1 mM $CaCl_2$, 1% Triton X-100, 100 μg/ml Pepstatin A, Leupeptin and Aprotinin, pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g for 15 min after which the supernatants were stored at -20°C until further use. Cytokine and chemokine levels in lung homogenates were measured by ELISA according to the manufacturer's instructions: TNF, IL-6, MIP-2 and KC assays were all obtained from R&D (Minneapolis, MN).

Histologic examination

Lungs for histologic examination were harvested at 6 and 24 h (*K. pneumoniae* pneumonia) and at 24 and 48 h (*S. pneumoniae* pneumonia) after inoculation, fixed in 10% formaline and embedded in paraffin. Four μm sections were stained with haematoxylin and eosin, and analyzed by a pathologist who was blinded for groups.

Statistical analysis

All data are expressed as mean ± SEM. Differences between groups were analyzed by Mann-Whitney U test. Survival studies were analyzed using Kaplan-Meier. $P < 0.05$ was considered to represent a statistically significant difference.

Results

Survival

In Gram-positive pneumonia induced by *S. pneumoniae*, survival did not consistently differ between TLR4 mutant and WT mice. At low infectious doses, 4 and 6 x 10^3 CFU, survival was reduced in TLR4 mutant mice, significantly so after inoculation with 6 x 10^3 CFU ($P < 0.05$). However, a higher bacterial dose, 6 x 10^4 CFU, caused a similarly high mortality with a similar time course in TLR4 mutant and WT mice (Figure 1). In contrast, in Gram-negative pneumonia induced by *K. pneumoniae*, survival was consistently and significantly shortened in TLR4 mutant mice, both after infection with a low (50 CFU) and a high (600 CFU) bacterial dose (Figure 2). Hence, these data suggest that TLR4 may play a modest protective role against mortality during pneumococcal pneumonia induced by relatively low bacterial inocula, whereas TLR4 has a more important role in the protective immune response to *K.*

pneumoniae pneumonia. Subsequent experiments were done with 10^4 CFU *S. pneumoniae* and 200 CFU *K. pneumoniae.*

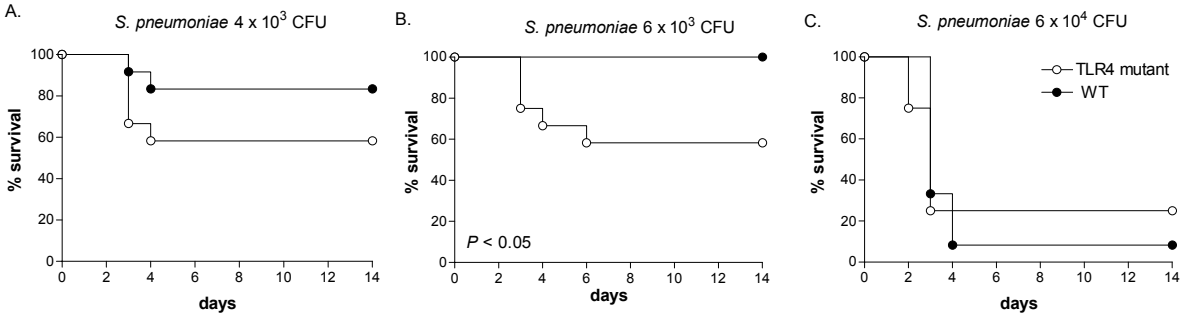

Fig. 1: Survival of TLR4 mutant and WT mice after intranasal inoculation with (A) 4 x 10^3 CFU, (B) 6 x 10^3 CFU or (C) 6 x 10^4 CFU *S. pneumoniae.* Twelve mice per group were studied. Survival in TLR4 mutant mice was significantly decreased compared to WT mice when inoculated with 6.5 x 10^3 CFU ($P < 0.05$).

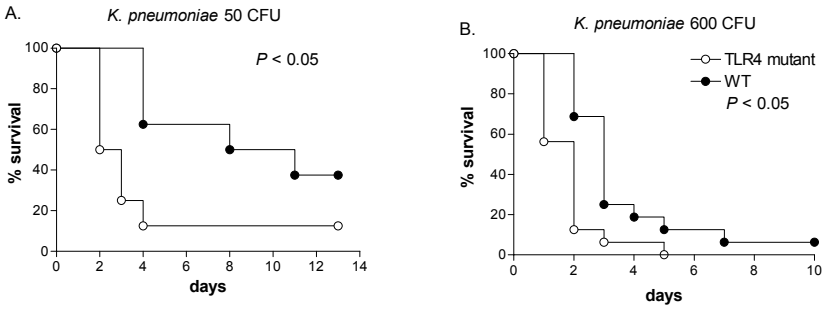

Fig. 2: Survival of TLR4 mutant and WT mice after intranasal inoculation with (A) 50 or (B) 600 CFU *K. pneumoniae.* 8-16 mice per group were studied. Survival in TLR4 mutant mice was significantly decreased compared to WT mice in both experiments ($P < 0.05$).

Bacterial outgrowth

To obtain more insight into the role of TLR4 in early host defense against Gram-positive and Gram-negative pneumonia, bacterial outgrowth in the lungs and blood of TLR4 mutant and WT mice was compared. After infection with 10^4 CFU *S. pneumoniae,* bacteria were counted at 24 and 48 hours. Although, at 24 h, the number of CFU was similar in both mouse strains, at 48 h post infection TLR4 mutant mice had significantly more bacteria in their lungs than WT mice ($P < 0.05$; Figure 3A). The percentage of positive blood cultures in TLR4 mutant and WT mice was similar (24 h: 0% of TLR4 mutant and 12.5% of WT mice; 48 h: 50% of TLR4 mutant and 37.5% of WT mice). In Gram-negative pneumonia, the bacterial load in the lungs was assessed 6 h and 24 h after inoculation with 200 CFU *K. pneumoniae*. These time points were chosen earlier than in the pneumococcal model considering the early mortality of TLR4 mutant mice during *Klebsiella* pneumonia. At both time points, lungs of TLR4 mutant mice contained more bacteria, although significance was reached only at 24 h ($P < 0.05$;

Figure 3B). The percentages of positive blood cultures were equal in both mouse strains**,** 0 % at 6 h and 12.5 % at 24 h.

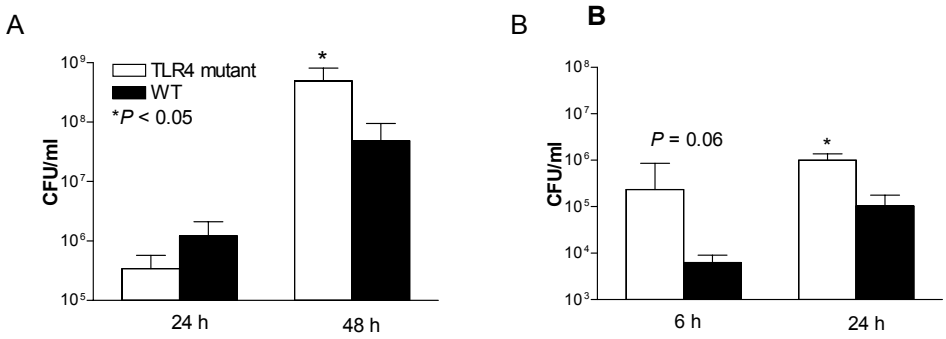

Fig. 3: Bacterial outgrowth in lungs in TLR4 mutant and WT mice (A) at 24 and 48 h after intranasal inoculation with 10^4 CFU *S. pneumoniae* and (B) at 6 and 24 h after intranasal inoculation with 200 CFU *K. pneumoniae.* Data are mean ± SEM of 8 mice. * $P < 0.05$ vs. WT mice.

Granulocyte influx in the lungs

The influx of granulocytes to the site of inflammation early in infection is an important characteristic of innate host defense mechanisms (55). We therefore determined leukocyte counts and differentials in the lungs at 6 and 24 h after induction of infection. Induction of both Gram-positive and Gram-negative pneumonia caused an increase of granulocyte numbers in TLR4 mutant and WT mouse lungs compared to saline controls (data not shown). In Gram-positive pneumonia, TLR4 mutant mouse lungs contained similar numbers of granulocytes compared to WT mouse lungs at 24 h (Table I). Similarly, in pneumonia induced by *K. pneumoniae,* no difference in granulocyte influx in lungs of TLR4 mutant and WT mice was seen after 6 and 24 h (Table I).

		Cells $\times 10^4$	Granulocytes %	Macrophages %	Lymphocytes %
S. pneumoniae					
TLR4 mutant	24 h	45.6 ± 6.2	31.1 ± 3.7	61.3 ± 3.7	7.6 ± 1.7
WT		46.2 ± 6.4	34.0 ± 4.9	57.3 ± 5.0	8.6 ± 1.1
K. pneumoniae					
TLR4 mutant	6 h	63.5 ± 8.7	30.2 ± 3.1	44.1 ± 3.1	25.6 ± 2.1*
WT		77.6 ± 5.4	37.2 ± 5.1	55.0 ± 5.8	7.8 ± 1.1
TLR4 mutant	24 h	82.3 ± 11.1	42.5 ± 5.6	35.4 ± 4.8	22.1 ± 2.5*
WT		90.5 ± 13.7	41.6 ± 3.7	23.3 ± 3.3	35.1 ± 1.5

Table I. Cellular composition of lungs during pneumonia. Total leukocyte counts and differential counts (as percentage) in lungs of TLR4 mutant and WT mice infected with S. pneumoniae or K. pneumoniae, 6 and/or 24 h after infection. Data are mean ± SEM of 6 mice per group at each time-point. *P < 0.05 vs. WT at the same time-point.

Cytokine and chemokine response to pneumonia

Local production of cytokines and chemokines plays a role in the protective immune response to respiratory tract infection (44, 55). Therefore, we determined the influence of TLR4 deficiency on pulmonary cytokine concentration during Gram-positive and Gram-negative pneumonia. Cytokine (TNFα, IL-6) and chemokine (MIP-2, KC) levels measured in lung homogenates 24 and 48 h after induction of pneumococcal pneumonia (Table II), and 6 and 24 h after infection with *K. pneumoniae* (Table III), did not differ between TLR4 mutant and WT mice .

	24 h post-infection		*48 h post-infection*	
ng/g lung	**TLR4 mutant**	**WT**	**TLR4 mutant**	**WT**
TNF	4.8 ± 0.8	3.1 ± 0.3	8.1 ± 2.6	4.6 ± 2.1
IL-6	9.6 ± 5.1	9.7 ± 4.6	66.7 ± 27.1	40.1 ± 29.9
KC	35.8 ± 1.1	32.3 ± 0.7	110.6 ± 33.5	71.2 ± 30.8
MIP-2	5.1 ± 1.7	6.2 ± 1.7	20.5 ± 7.1	10.0 ± 5.4

Table II. Cytokine and chemokine concentrations in the lung during *S. pneumoniae* pneumonia. Data are mean ± SEM of 8 mice per group.

	6 h post-infection		*24 h post-infection*	
ng/g lung	**TLR4 mutant**	**WT**	**TLR4 mutant**	**WT**
TNF	2.7 ± 0.1	2.7 ± 0.1	1.9 ± 0.1	2.2 ± 0.3
IL-6	4.6 ± 1.4	2.8 ± 0.2	3.3 ± 0.4	3.3 ± 0.3
KC	38.6 ± 1.0	38.5 ± 2.5	32.3 ± 0.7	34.2 ± 0.6
MIP-2	33.8 ± 2.7	32.1 ± 4.5	33.3 ± 2.2	36.9 ± 2.3

Table III. **Cytokine and chemokine concentrations in the lung during *K. pneumoniae* pneumonia.**Data are mean ± SEM of 8 mice per group.

Histopathology

After infection with *S. pneumoniae*, the lungs of WT mice showed a mild interstitial inflammation composed of monocytes and lymphocytes (Figure 4A). The inflammation was slightly more pronounced in TLR4 mutant mice with more perivascular infiltrates (Figure 4B). In *K. pneumoniae* infection, lungs from WT mice had thickened alveolar septae due to mild interstitial inflammation with infiltration of lymphocytes and monocytes (Figure 4C). In TLR4 mutant mice, the interstitial inflammation was also slightly more pronounced (Figure 4D).

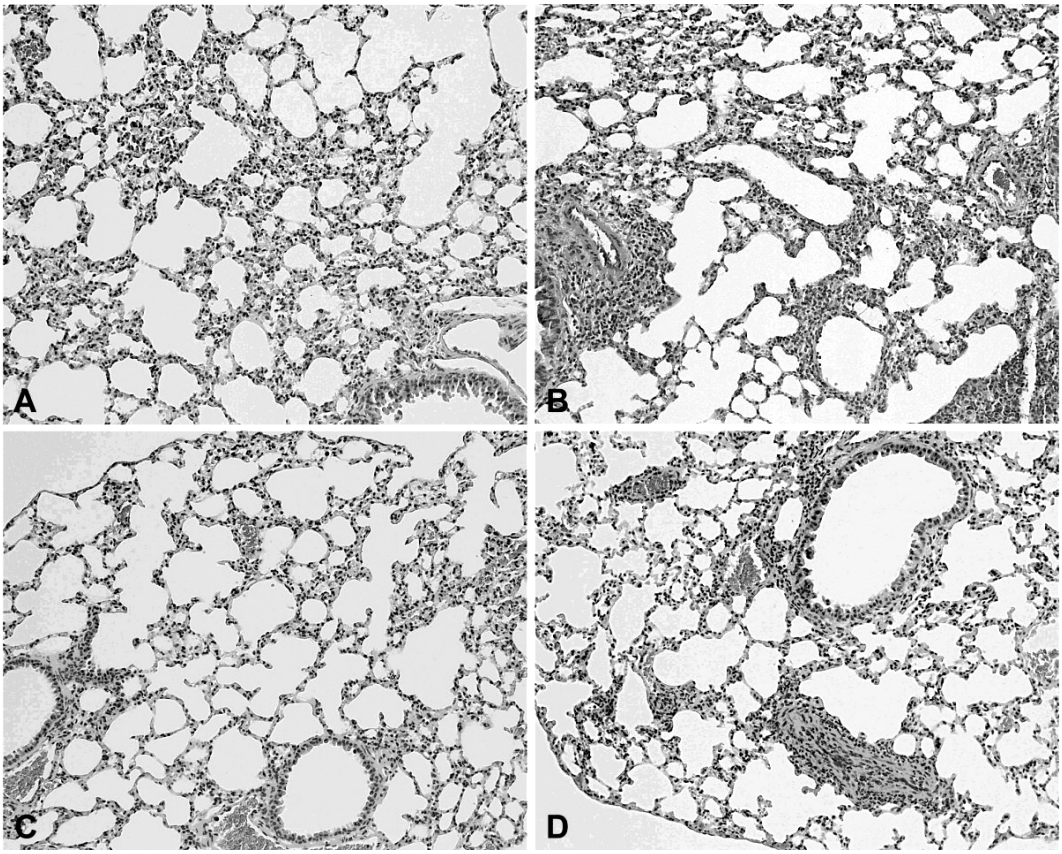

Fig. 4: Representative lung histology of WT (A and C) and TLR4 mutant mice (B and D) 24 hours after *S. pneumoniae* (A, B) and after *K. pneumoniae* (C, D) inoculation. Data are representative of 5 mice per group, H&E staining, magnification × 10.

Discussion

TLR4 has been implicated to play an essential role in host defense against Gram-negative bacteria by virtue of its capacity to signal LPS-induced inflammatory responses (12, 14, 21, 22, 43, 47). In contrast, components of Gram-positive bacteria have been demonstrated to signal predominantly via TLR2 (42, 54), although TLR4 may also play a role herein (27, 29, 31, 42, 46, 54). To determine the relevance of TLR4 in inducing an innate host response to pulmonary infection, we induced pneumonia caused by two common respiratory pathogens (Gram-positive, *S. pneumoniae*, and Gram-negative, *K. pneumoniae*) in TLR4 mutant and WT mice. The outcome of *S. pneumoniae* pneumonia was modestly influenced by TLR4 deficiency, as reflected by a reduced survival of TLR4 mutant mice after inoculation with a relatively low infectious dose, and an increased bacterial outgrowth in lungs. In *K. pneumoniae* pneumonia, the protective role of TLR4 was clearer, i.e. TLR4 mutant mice displayed an impaired host response compared to WT mice as illustrated by a consistently and significantly shortened survival and an increased number of bacteria in their lungs.

Several studies have attributed an important role to TLR2 in activating cells upon stimulation with components of *S. pneumoniae.* In CD14-expressing Chinese Hamster Ovary (CHO)

cells, heat killed *S. pneumoniae* stimulated NF-κB-translocation, a response that was greatly enhanced in cells co-expressing TLR2 and CD14, suggesting that the pneumococcus activates both TLR2 dependent and a TLR2 independent signaling pathways. Activation of the TLR2 pathway by *S. pneumoniae* can be attributed to several PAMPs expressed within the pneumococcal cell wall, including PGN and LTA (27, 31, 39, 42, 54). The role of TLR2 in pneumococcal infection in vivo was investigated in a model of meningitis, in which *S. pneumoniae* was injected intracerebrally in TLR2 deficient (TLR2-/-) and WT mice (16, 24). Both studies showed an increased susceptibility of TLR2-/- compared to WT mice in the early phase of infection as expressed by increased bacterial counts in the brain (16, 24) and blood (24), while one study also reported a reduced survival time in TLR2-/- mice (16). However, in preliminary experiments our laboratory did not observe major differences in antibacterial defense and survival between TLR2-/- and WT mice in pneumococcal pneumonia (S. Knapp, S. Florquin, O Takeuchi, S. Akira and T. van der Poll, Abstr. 42nd Intersc. Conf. Antimicrob. Agents Chemother., abstr B-693, 2002). Interestingly, in the previous meningitis studies TLR2-/- mice displayed a virtually unchanged inflammatory response to *S. pneumoniae* (16, 24). Together, these data suggest that although TLR2 may play a role in the innate immune response to pneumococcal infection in some organs (like the brain), other PRRs likely are involved.

While our studies were in progress, Malley et al. reported a role for TLR4 in host defense against nasopharyngeal colonization by *S. pneumoniae* (29). In that study, TLR4 mutant and WT mice were intranasally inoculated with *S. pneumoniae* without the use of anesthesia, which resulted in nasopharyngeal colonization rather than in lower respiratory tract infection such as in our present investigation (29). Using a bioluminescent pneumococcal strain, these authors demonstrated a much higher bacterial nasopharyngeal burden (as determined by photon emission) in TLR4 mutant mice in the first 3 days after intranasal infection, which was associated with the subsequent development of bacteremia and increased lethality. In a series of elegant experiments it was further shown that pneumolysin, a pore-forming cytolysin toxin secreted by pneumococci (38), interacts with TLR4 and that pneumolysin deficient pneumococci are unable to induce invasive disease after nasopharyngeal colonization in either TLR4 mutant or WT mice (29). Together these data suggest that in the nasopharynx the interaction between pneumolysin and TLR4 is critically involved in the innate immune response to *S. pneumoniae*. Our experiments, using a pneumolysin producing *S. pneumoniae*, indicate that once pneumococci have reached the lower respiratory tract, TLR4 plays a limited role in pulmonary defense against infection. Although we found that TLR4 mutant mice had an increased mortality at low infectious doses and, after 48 h, increased bacterial counts in the lungs compared to WT mice, survival rates were similar at a higher inoculum. Malley et al. used only one bacterial dose, which resulted in a 12.5% lethality rate in WT mice without evidence of lower respiratory tract infection (29). In that study, the absolute difference in mortality rates between TLR4 mutant and WT mice was similar to the difference between these mouse strains observed in our current study using low infectious doses causing 17% lethality in WT mice. Together, these findings point to distinct roles for TLR4 in the upper

and lower respiratory tract in innate immunity against pneumococcal infection. Notably, another report showed no difference in outcome between TLR4 mutant and WT mice in a peritoneal infection model induced by pneumococci (3).

It should be noted that in the two survival studies using low *S. pneumoniae* doses of 4 and 6 x 10^3 CFU mortality was 2/12 and 0/12 respectively in wild type mice (i.e. only mortality after the lowest dose). In this respect it is important to realize that these studies were done several months apart using different shipments of mice, and that some variation is not uncommon when studying the outcome of live infections in this setting. These data suggest that these bacterial challenges are at or just over the edge of what can be handled by the normal innate immune system. Importantly, however, all comparisons between wild type and TLR4 mutant mice were done in experiments in which all mice were inoculated at the same time with exactly the same inoculum. Hence, comparisons between the two mouse strains were always done in an adequate and valid way. Thus based on the slightly reduced antibacterial defense and the modestly reduced survival of TLR4 mutant mice in our pneumococcal pneumonia model using low doses, together with the recent findings of Malley et al. discussed above (29), we feel that the conclusion that TLR4 plays a modest role in host defense against respiratory tract infection by *S. pneumoniae* is justified.

C3H/HeJ mice have long been known to be hypo-responsive to LPS. A mutation in the *Tlr4* gene proved responsible for this hypo-responsiveness (35, 36). In vitro studies also identified TLR4 as an essential receptor for LPS (12, 14, 21, 22, 43, 47). In addition to TLR4, recognition of LPS requires other molecules such as LPS-binding protein, CD14 and MD-2 (23, 43). Since LPS is an important antigen in Gram-negative bacteria, capable of inducing a strong immune response, it was expected that the lack of functional TLR4 would render mice susceptible to Gram-negative infections (5). Indeed, TLR4 seems to be important in host defense against some Gram-negative bacteria, as shown by an impaired defense of TLR4 mutant mice during urinary tract infection with *E. coli* (18), intraperitoneal infection with *Neisseria* (51), *Klebsiella* (49) and *Salmonella* species (4). However, host defense was not impaired in TLR4 mutant mice with *E. coli* peritonitis (17, 20). Our results obtained in the *Klebsiella* model corroborate earlier studies that reported a protective role of TLR4 in Gram-negative respiratory tract infection caused by *Haemophilus influenzae* (50) or *Pasteurella pneumotropica* (11, 19). Of note, in a previous study our laboratory documented an unimpaired host defense in TLR4 mutant mice infected with *Legionella pneumophila*, which may have been caused by the unique structure of *Legionella* LPS that also fails to interact with CD14 (28, 33).

TLR4 mutant mice displayed an unaltered inflammatory response to pneumococcal and *Klebsiella* pneumonia. It remains to be established which PRRs play a role in the induction of lung inflammation during infection by the pathogens used here. In this respect the recent description of receptor clusters recognizing LPS is of considerable interest, i.e. accumulating evidence suggests that following LPS stimulation a signaling complex of receptors is formed which comprises heat shock protein (HSP)70, HSP90, CXC chemokine receptor 4 (CXCR4) and growth differentiation factor 5 (48). Although it is absolutely clear that TLR4 is important

for LPS signaling, it is tempting to speculate that receptor clusters like described above can in part compensate for a lack of TLR4 during Gram-negative infection in vivo.
We conclude that TLR4 is involved in innate immunity during pneumonia caused by either *S. pneumoniae* or *K. pneumoniae*. The role of TLR4 in pneumococcal pneumonia is relatively limited, providing protection only after infection of the lower respiratory tract with low bacterial doses causing little if any mortality in WT mice. In *Klebsiella* pneumonia, TLR4 is a more important part of an effective immune response in the lungs.

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CHAPTER 16

Differential roles of CD14 and Toll-like Receptors 4 and 2 in murine *Acinetobacter* pneumonia

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Abstract

Rationale: *Acinetobacter baumannii* is an opportunistic bacterial pathogen that is increasingly associated with Gram-negative nosocomial pneumonia, but the molecular mechanisms that play a role in innate defenses during *A. baumannii* infection have not been elucidated.

Objective: To gain first insight into the role of CD14, Toll-like receptor 4 and 2 in host response to *A. baumannii* pneumonia.

Methods: Respective gene-deficient mice were intranasally infected with *A. baumannii* and bacterial outgrowth, lung inflammation and pulmonary cytokine/chemokine responses were determined. To study the importance of lipopolysaccharide in the inflammatory response, mice were also challenged with *A. baumannii* lipopolysaccharide.

Measurements and Main Results: Bacterial counts were increased in CD14 and Toll-like receptor 4 gene-deficient mice and only these animals developed bacteremia. The pulmonary cytokine/chemokine response was impaired in Toll-like receptor 4 knock-out mice and the onset of lung inflammation was delayed. In contrast, Toll-like receptor 2 deficient animals displayed an earlier cell-influx into lungs combined with increased macrophage inflammatory protein-2 and monocyte chemoattractant protein-1 concentrations, which was associated with accelerated elimination of bacteria from the pulmonary compartment. Neither CD14 nor Toll-like receptor 4 gene-deficient mice responded to intranasal administration of lipopolysaccharide, whereas Toll-like receptor 2 knock-out mice were indistinguishable from wild-type animals.

Conclusions: Our results suggest that CD14 and Toll-like receptor 4 play a key role in innate sensing of *A. baumannii* via the LPS moiety, resulting in effective elimination of the bacteria from the lung, whereas Toll-like receptor 2 signaling seems to counteract the robustness of innate responses during acute *A.baumannii* pneumonia.

Introduction

Members of the genus *Acinetobacter* have recently gained increased recognition as bacterial pathogens that have the potential to cause severe infections in critically ill patients in intensive care units (1, 2). One of the species within the genus, *A. baumannii*, has gained particular notoriety as one of the leading causes of opportunistic nosocomial infections worldwide (3-7). The predominant site of *A. baumannii* infection is the pulmonary compartment and 15-25% of ventilator-associated pneumonias (VAP) are attributable to this pathogen (8, 9). The crude mortality of VAP due to *A. baumannii* has been shown to be as high as 75% (8, 10). There is additional evidence that *A. baumannii* induces community-acquired pneumonia, predominately among young alcoholics in tropical climates (11). Pneumonia induced by *A. baumannii* is frequently associated with a sudden and severe onset that in most cases requires mechanical ventilation and systemic complications including septic shock have been repeatedly described (11). The high rate of antibiotic resistance and widespread colonization of skin, mucosal membranes and medical equipment makes *A. baumannii* a pathogen of high importance and concern (12-18). Given the increased clinical importance of *A. baumannii* and the lack of knowledge regarding host defense mechanisms against this opportunistic pathogen, we developed a murine model of *A. baumannii* pneumonia to address this.

The first line of defense against invading bacteria is provided by the innate immune system, which recognizes pathogen-associated molecular patterns, conserved microbial patterns shared by large groups of pathogens, but not found in higher eukaryotes (19-21). In recent years it has become evident that both the recognition and the subsequent response to pathogens are mainly transferred by members of the Toll-like receptor (TLR) family (for review see: (22-25)). Of the 11 described TLRs, TLR4 and TLR2 are the key receptors signaling the presence of bacteria. TLR4 signaling is triggered by the interaction with lipopolysaccharide (LPS), the major cell-wall component of Gram-negative bacteria (26). CD14, a glycosylphosphatidylinositol-anchored molecule, is an important player in the LPS-signaling process, by enhancing LPS binding to MD-2 (27). This process, in turn, enables LPS binding to TLR4. In the absence of either CD14 or TLR4 the LPS-induced inflammatory responses are greatly reduced (28, 29). TLR2, in contrast to TLR4, has received attention primarily as an important pattern recognition receptor for Gram-positive bacteria, although it might also contribute to the host innate immune defense against Gram-negative pathogens (30-34). TLR2 recognizes peptidoglycan and lipoproteins, which are major constituents of the cell-wall of Gram-positive bacteria but, to a lesser degree, are also present in Gram-negative microorganisms. Here, we provide first insight into the role of these important signaling receptors in *A. baumannii* pneumonia.

Materials and Methods

Mice

Pathogen-free 7-9 wk-old C57/BL6 mice were obtained from Harlan Sprague-Dawley (Horst, the Netherlands), CD14 gene deficient ($CD14^{-/-}$) mice from Jackson Laboratories (Bar Harbor, ME) (35). $TLR4^{-/-}$ and $TLR2^{-/-}$ mice were generated as described (29, 32). All mice were bred in the animal facility of the Academic Medical Center in Amsterdam and backcrossed 6 times to C57/BL6 background. Age and sex matched C57/BL6 wild type mice were used as controls. The institutional Animal Care and Use Committee approved all experiments.

Induction of pneumonia

A. baumannii (strain RUH 2037, allocated to the European clone I, (36)) was isolated during an *Acinetobacter* outbreak in 1986 from sputum of a patient suffering from pneumonia. Detailed information regarding the bacterial strain is provided in the online data supplement. Bacteria were grown to midlogarithmic phase at 37°C using Luria Bertani broth (Difco, Detroit, MI), washed and resuspended in sterile isotonic saline (10^6 to 10^8 CFU/50µl). Mice were anesthetized by inhalation of isoflurane (Upjohn, Ede, The Netherlands) and 50 µl were inoculated intranasally (i.n.). At indicated timepoints mice were sacrificed and bacterial counts were determined as described (34, 37). In some experiments a broncho-alveolar lavage was performed, total cell numbers were counted using a coulter counter (Beckmann Coulter, Fullerton, CA) and differential cell counts were done on cytospin preparations stained with Giemsa.

Cytokine/chemokine and myeloperoxidase measurements

Lungs were homogenized as described (34, 37, 38). Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10 and monocyte chemoattractant protein-1 (MCP-1) were measured using the cytometric bead array (BD Bioscience, St. Jose, CA). IL-1β, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2 were measured using ELISAs (R&D Systems, Minneapolis, MN) as was myeloperoxidase (MPO) (HyCult Biotechnology, Uden, the Netherlands).

Histologic examination

Lungs were harvested at indicated timepoints, fixed in 10% formaline and embedded in paraffin. Four µm sections were stained with hematoxylin and eosin (H&E), and analyzed by a pathologist blinded for groups. To score lung inflammation and damage, the entire lung surface was analyzed regarding the presence of: interstitial inflammation, edema, endothelialitis, bronchitis and pleuritis as described (34, 38): Granulocyte staining was done as described previously (34, 37).

LPS pneumonitis

LPS (100 ng) purified from *A. baumannii* strain 24 (RUH 872, allocated to the European clone I (39, 40) additional information on this LPS preparation is provided in the online data supplement) was administered (in 50 µl saline) i.n. to mice that were anesthetized by inhalation of isoflurane (Upjohn). Six hours later, a broncho-alveolar lavage was performed, total cell numbers were counted using a hemocytometer (Türk chamber) and differential cell counts were done on cytospin preparations stained with Giemsa (41).

Statistical analysis

Differences between groups were calculated by Mann-Whitney *U* test or one-way analysis of variance where appropriate using GraphPad software (San Diego, CA). Values are expressed as mean ± SEM. A p-value < 0.05 was considered statistically significant.

Results

A. baumannii pneumonia model

To enable the investigation of host defense mechanisms in *A. baumannii* pneumonia *in vivo* we first developed a suitable mouse model. A well-documented strain of *A. baumannii* isolated from a clinical case of *Acinetobacter* pneumonia was selected for this purpose and mice (n=5 per group) were infected i.n. with inocula ranging from 10^6 to 10^8 CFU. Mice were sacrificed after 1, 4, 24, 48 or 72h to follow the inflammatory response over time. As depicted in Fig. 1, lung bacterial counts temporarily increased until 4h post-infection, and gradually declined thereafter (Fig. 1). However, bacteria were still detectable in lung tissue 72h after inoculation. Likewise, pulmonary TNF-α production reached peak levels at t=4h and quickly decreased thereafter, while the number of infiltrating polymorphonuclear cells (PMNs) (determined as MPO-concentration) remained at a constant level between 4 and 48h post-infection (Fig. 1).

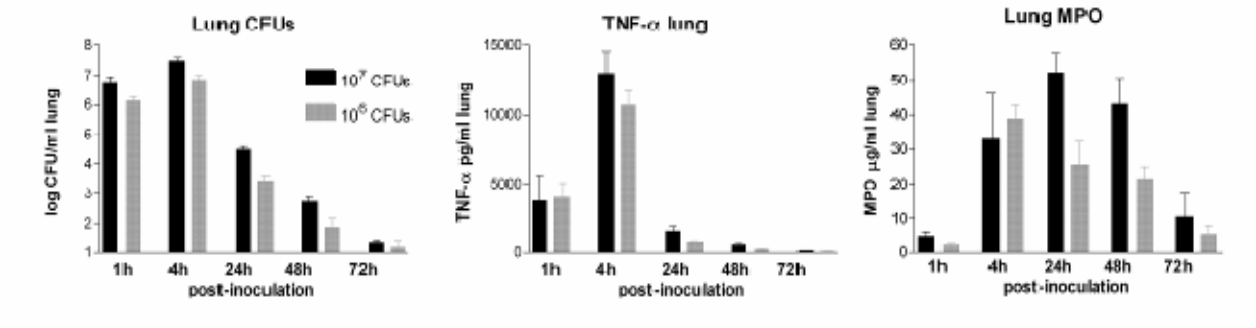

Fig. 1: *A. baumannii* pneumonia model. Mice (n=5 per time-point) were infected i.n. with *A. baumannii* (10^6 or 10^7 CFU) and lung bacterial counts (A), TNF-α (B) and MPO (C) were determined in lung homogenates at given time-points. Results are expressed as mean±SEM.

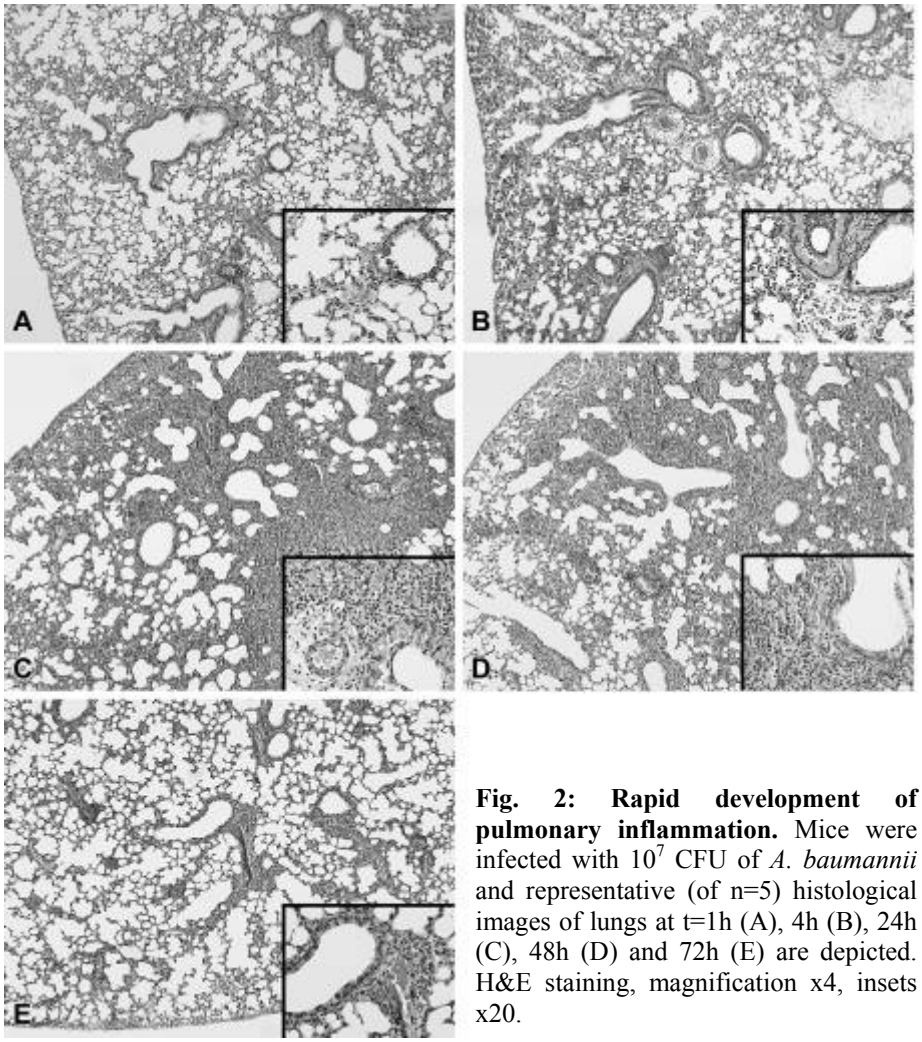

Fig. 2: Rapid development of pulmonary inflammation. Mice were infected with 10^7 CFU of *A. baumannii* and representative (of n=5) histological images of lungs at t=1h (A), 4h (B), 24h (C), 48h (D) and 72h (E) are depicted. H&E staining, magnification x4, insets x20.

Histological examination of lung tissue illustrated the early onset of pneumonia as reflected by dense pulmonary infiltrates (Fig. 2). Of the mice challenged with 10^8 CFU of *A. baumannii*, two died after 24h. Based on these pilot experiments, subsequent studies were performed with a bacterial inoculum of 10^7 CFU and mice were sacrificed at 4h (peak of cytokine response and bacterial counts) and 24h (peak of PMN influx and onset of bacterial clearance) following challenge.

CD14 and TLR4 contribute to clearance of A. baumannii.

Having established a murine model of *A. baumannii* pneumonia we next studied host innate defense pathways possibly involved in the observed inflammatory responses. CD14 and TLRs are important pattern recognition receptors that contribute to the initiation of an adequate inflammatory response during infections and, hence, to an effective host immune defense. Because CD14 and TLR4 are known to recognize LPS from Gram-negative bacteria, we first investigated their respective roles during *A. baumannii* pneumonia *in vivo*. Wild-type and gene-deficient mice were inoculated with 10^7 CFU of *A. baumannii* and sacrificed after 4 and

24h. At 4h post-inoculation, significantly higher lung bacterial counts were found in CD14$^{-/-}$ and TLR4$^{-/-}$ mice as compared to wild-type animals (Fig. 3). At 24h, wild-type mice had lower bacterial counts than either group of gene-deficient mice, though this difference was only statistically significant for TLR4$^{-/-}$ animals (Fig. 3). To investigate systemic bacterial dissemination, blood samples were cultured for the presence of *A. baumannii*. Whereas blood cultures were sterile at 4h in all mice, approximately 50% of mice lacking either CD14 or TLR4 had positive blood cultures at t=24h (6/10 and 4/9 mice, respectively) as compared to wild-type animals (0/8 mice).

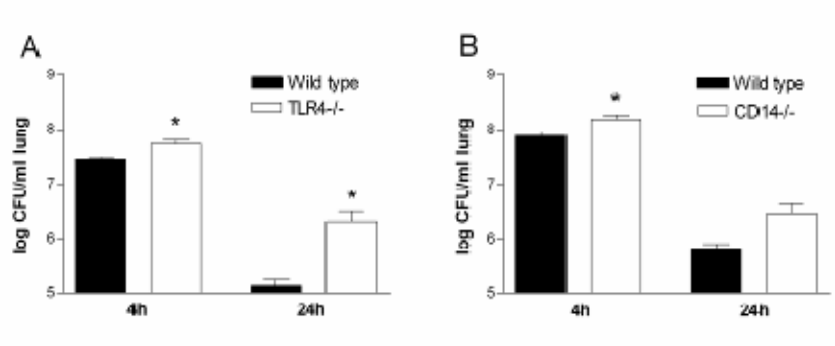

Fig. 3: Reduced bacterial clearance in TLR4$^{-/-}$ and CD14$^{-/-}$ mice. Wild-type and (A) TLR4$^{-/-}$ or (B) CD14$^{-/-}$ mice (n=7-10 per group at each time-point) were inoculated with 10^7 CFU of *A. baumannii* and lung bacterial counts were determined after 4 and 24 h. Results are expressed as mean±SEM; * indicates $p<0.05$ vs. wild-type.

We next examined whether TLR2 might also play a role in host innate defense against *A. baumannii*. TLR2$^{-/-}$ and wild-type mice were infected and lung bacterial counts enumerated. No difference was found at t=4h after infection but, somewhat surprisingly, a significantly lower number of bacteria was observed in lungs of TLR2$^{-/-}$ mice 24h after induction of pneumonia (Fig. 4). The observed difference in bacterial clearance between wild-type mice and mice lacking TLR2 was still observed at 44h post-inoculation (Fig. 4).

Taken together, the above results suggested that CD14 and TLR4 contribute to lung bacterial clearance and prevent systemic dissemination of *A. baumannii*, whereas TLR2 signaling counteracts elimination of the bacteria from the lungs.

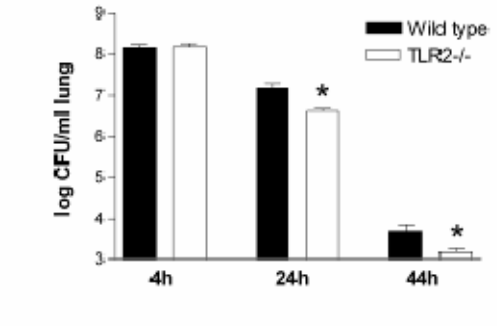

Fig. 4: Accelerated bacterial clearance in TLR2$^{-/-}$ mice. Wild-type and TLR2$^{-/-}$ mice (n=8 per group at each time-point) were inoculated with *A. baumannii* (10^7 CFU) and lung bacterial counts were determined after 4, 24 and 44h. Results are expressed as mean±SEM, * indicates $p<0.05$ vs. wild-type.

Humoral & Cellular factors

To gain further insight into the impaired bacterial clearance in mice lacking CD14 or TLR4, we next investigated the pulmonary cytokine and chemokine responses to *A. baumannii*. Early after infection (4h) TLR4$^{-/-}$ mice displayed a reduced ability to produce IL-6, TNF-α, KC and MIP-2 as compared to wild-type animals ($p<0.05$ in all cases), whereas IL-1β, IL-10 and MCP-1 levels were comparable to wild-type mice (Table 1). Cytokine concentrations did not differ between TLR4$^{-/-}$ and wild-type mice at 24h, whereas KC levels remained lower in the TLR4-deficient animals than in the wild types (Table 1).

	4h		*24h*	
Cytokine/chemokine	**Wild-type**	**TLR4$^{-/-}$**	**Wild-type**	**TLR4$^{-/-}$**
TNF-α	9.8±1.6	4.9±1.0*	1.1±1.8	1.4±0.3
IL-1β	8.5±2.8	11.4±2.4	3.0±0.5	3.2±0.8
IL-6	5.8±1.2	2.8±0.6*	0.9±0.1	0.7±0.1
IL-10	0.11±0.02	0.11±0.02	0.03±0.01	0.03±0.01
MCP-1	6.2±0.9	7.0±1.4	4.3±0.4	4.1±0.6
MIP-2	34.9±10.7	13.6±1.9*	2.9±0.6	1.9±0.5
KC	21.4±1.2	5.7±1.2*	5.4±0.9	1.2±0.2*

Table 1: Concentration (ng/ml) of cytokines/chemokines in wild-type and TLR4-/- mice. Wild-type and TLR4$^{-/-}$ mice (n=7-9 per strain) were i.n. inoculated with 10^7 CFU of *A. baumannii*. At indicated time points, mice were sacrificed and cytokine and chemokine levels were measured in lung homogenates. Data are mean±SEM; * $p<0.05$ vs. wild-type.

Pulmonary cytokine and chemokine concentrations in CD14$^{-/-}$ and wild-type mice were largely similar (Table E1 in supplemental online data). The onset of pulmonary inflammation, as assessed by histological examination of lungs at t=4h, was delayed in the absence of TLR4 (inflammation score at t=4h: 9.3±0.6 for wild-type and 7.1±0.6 for TLR4$^{-/-}$ mice; $p<0.05$) while no difference was observed between CD14$^{-/-}$ mice and their wild-type counterparts (inflammation score at t=4h: 10.5±0.9 in wild-type and 11.5±0.9 in CD14$^{-/-}$ mice; n.s.). At this early time point in particular TLR4$^{-/-}$ mice demonstrated a reduced influx of PMNs into BALF, whereas CD14$^{-/-}$ mice displayed moderately, albeit insignificantly, reduced PMN numbers in BALF (Table 2, $p < 0.05$ TLR4$^{-/-}$ versus wild-type mice). In line with increased bacterial counts, mice lacking CD14 had significantly higher pulmonary inflammation scores than their wild-type counterparts 24h after infection (10.0±0.6 for wild type and 12.4±0.4 for CD14$^{-/-}$ mice; $p<0.05$). Likewise, the proportion of mice with confluent pneumonia was higher in TLR4$^{-/-}$ animals (14% and 50% of wild type and TLR4$^{-/-}$ mice, respectively, displayed areas of confluent pneumonia) although no difference between the lung

inflammation score of wild type or TLR4$^{-/-}$ animals was found at t=24h (12.1±0.7 in wild type vs. 12.0±0.7 in TLR4$^{-/-}$ mice).

	Cell count/µl	*AM %*	*PMN %*	*Lymphocytes %*
Wild type	5051 ± 605	5.5 ± 0.7	93.7 ± 0.8	0.8 ± 0.2
TLR2$^{-/-}$	2616 ± 130	6.6 ± 1.2	92.4 ± 1.2	1.0 ± 0.3
TLR4$^{-/-}$	412 ± 45 *°	62.8 ± 8.2 *°	36.3 ± 8.3 *°	0.9 ± 0.2
CD14$^{-/-}$	1664 ± 231 *	12.9 ± 3.2	84.9 ± 3.2	2.1 ± 0.4

Table 2: Cell count and differentials in BALF. Wild type, TLR2$^{-/-}$, TLR4$^{-/-}$ and CD14$^{-/-}$ mice (n=6-8 per strain) were inoculated with *A. baumannii*. After 4h BALF cells were enumerated and differential counts were performed on cytospin preparations. Data are mean±SEM; * indicates $p<0.05$ vs. wild type, ° $p<0.05$ vs. TLR2$^{-/-}$ mice.

In TLR2-/- mice, the most striking finding was a significant increase in pulmonary MIP-2 and MCP-1 concentrations 4h post-infection, while TNF-α, IL-1β, IL-6 or IL-10 levels did not differ when compared to wild-type mice (Fig. 5). KC concentrations were decreased in lungs of TLR2$^{-/-}$ mice at this early time-point (see Table E2 in the online data supplement). Moreover, TLR2$^{-/-}$ mice displayed an earlier and more pronounced inflammatory cell influx into the lungs (Fig. 6 and 1E in the online data supplement). The early recruitment of PMNs into the pulmonary compartment was confirmed by Ly-6 immunohistochemical stainings and the detection of higher pulmonary MPO concentrations in TLR2$^{-/-}$ (Fig. 5 and Fig. 6 (insets)). Of note, TLR2$^{-/-}$ mice did not have an increased influx of PMNs into BALF (Table 2).

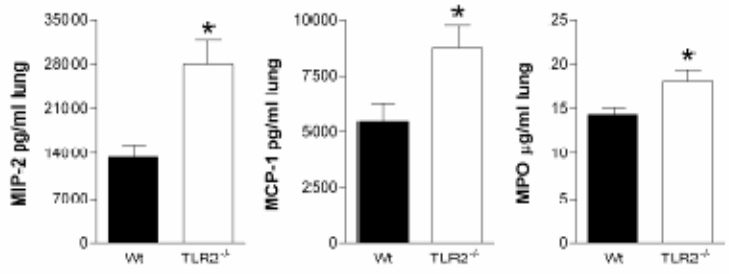

Fig. 5: Early onset of pulmonary inflammation in TLR2$^{-/-}$ animals. Wild-type (Wt) and TLR2$^{-/-}$ mice (n=8 per group) were inoculated i.n. with *A. baumannii* (10^7 CFU). After 4h, MIP-2 (A), MCP-1 (B) and MPO (C) concentrations were measured in lung homogenates. Results are the means±SEM, * indicates $p<0.05$ versus wild-type.

The role of A. baumannii LPS in pulmonary infection

So far, our studies were performed using whole bacteria. Given the involvement of LPS-signaling receptors CD14 and TLR4 in the innate response within the pulmonary compartment, we next inoculated mice i.n. with 100 ng of purified *A. baumannii* LPS of strain 24. The LPS of this strain has been shown previously to possess the same immunochemical properties as that of strain RUH 2037 (40), which was used in the studies described above.

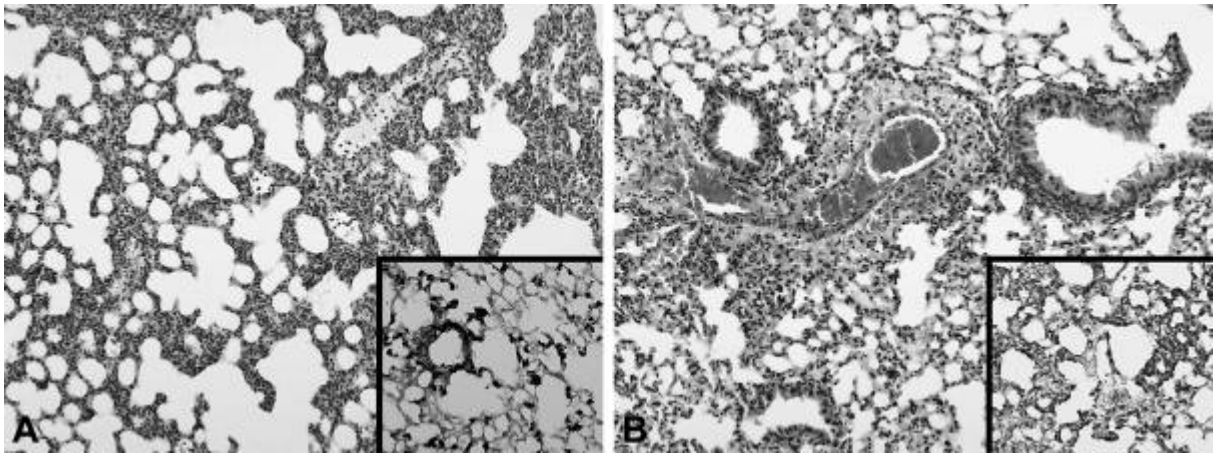

Fig. 6: Pulmonary inflammation is increased in TLR2$^{-/-}$ mice. Representative lung histology slides of wild-type (A) and TLR2$^{-/-}$ (B) mice 4 h after infection with 10^7 CFU of *A. baumannii.* The insets demonstrate immunostaining for granulocytes, confirming the dense granulocytic infiltration. Pictures are representative for at least 8 mice per group. H&E staining: magnification x 10; inset (Ly-6 staining): magnification x 20.

Broncho-alveolar PMN influx and cytokine/chemokine concentrations were assessed 6h after LPS administration. As expected, CD14 and TLR4 were crucial for the induction of the inflammatory response; mice lacking either signaling receptor did not mount a substantial PMN influx or TNF-α or IL-6 release (Fig. 7; IL-6 data not shown). These results confirm that *A. baumannii* LPS is a major immunostimulatory component that leads to a pro-inflammatory response during pneumonia with whole *A. baumannii* bacteria. Accordingly, we did not find a role for TLR2 in LPS-induced pneumonitis. Neither PMN-influx nor alveolar TNF-α or IL-6 concentrations differed between TLR2$^{-/-}$ and wild-type mice (Fig. 7; IL-6 data not shown).

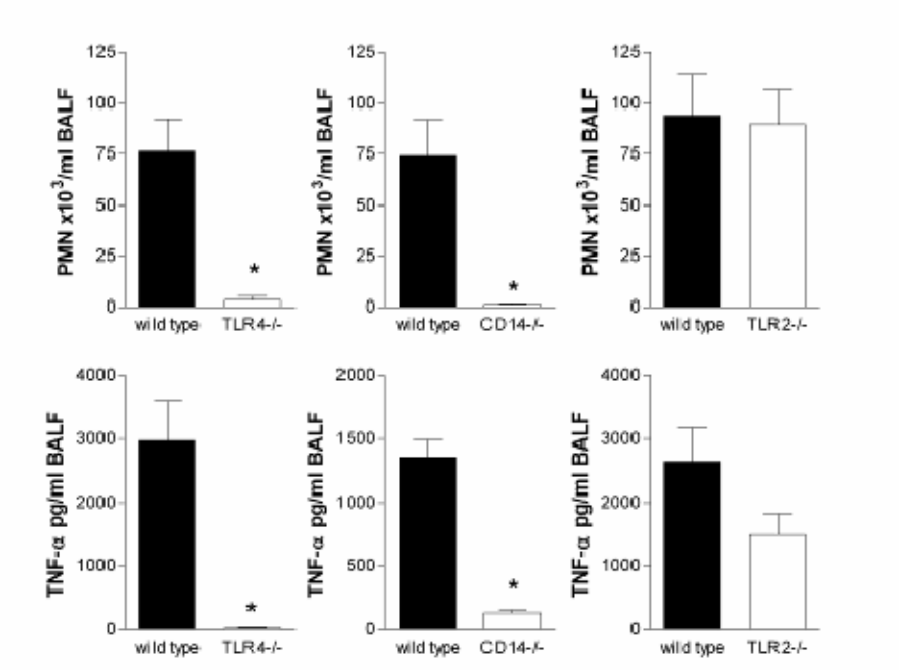

Fig. 7: LPS pneumonitis strongly depends on TLR4 and CD14. Wild type, TLR4$^{-/-}$, CD14$^{-/-}$ or TLR2$^{-/-}$ mice were inoculated i.n. with 100 ng LPS from *A. baumannii* (n=6-8 per strain) and PMN counts and TNF-α concentrations were assessed after 6h in BALF. Results are the means±SEM, * indicates $p<0.05$.

Discussion

A. baumannii pneumonia poses an increased threat to hospitalized patients, as reflected in the rising number of nosocomial pneumonia cases caused by this bacterial species and the relatively high incidence of mortality among infected individuals (4, 8). In light of the high antibiotic resistance of *A. baumannii*, knowledge about host defense mechanisms is highly warranted. We hereby introduce an acute *A. baumannii* pneumonia model that allows the *in vivo* investigation of these molecular mechanisms during infection with this bacterium. We observed that the release of pro-inflammatory cytokines mediated by CD14 and TLR4 signaling is crucial to bacterial clearance within the lungs and to the prevention of systemic bacterial spread *in vivo*. In contrast, TLR2-related pathways delayed early MIP-2 and MCP-1 release as well as pulmonary inflammation, which were accompanied by an impaired elimination of *A. baumannii* from the lungs.

A. baumannii exhibits some resemblance to *Pseudomonas aeruginosa*: both are Gram-negative non-glucose fermenters that are strongly associated with nosocomial pneumonia. While many reports have described host defense mechanisms against *P. aeruginosa*, very little is known about *A. baumannii*. We have taken first steps to fill this gap by establishing a murine model of *A. baumannii* infection and thereby encountered some similarities to the *P. aeruginosa* model we frequently study in our laboratory (42, 43). Thus, both groups of bacteria rapidly induce a robust inflammatory response within the lungs and bacteria are eventually cleared by the host unless high infection doses are administered. Until recently the role of TLRs during this type of acute pneumonia was not entirely clear and we therefore decided to investigate the respective roles of CD14, TLR4 and TLR2 during *A. baumannii* pneumonia. While this work was in progress, two other groups reported an important role for myeloid differentiation factor (MyD) 88, the main adaptor protein involved in TLR and IL-1 signaling, during *P. aeruginosa* pneumonia (44, 45). Both reports describe a severely impaired PMN influx and cytokine/chemokine response within the lungs of MyD88$^{-/-}$ mice that was associated with higher bacterial counts in this organ. Since IL-1 and IL-18 pathways (which also use MyD88 as adaptor) have been shown to play no supportive role during *P. aeruginosa* pneumonia (42, 43), Power *et al.* then focused on TLRs and investigated the contribution of TLR2 and 4 to host defense (45). Power *et al.* made use of C3H/HeJ mice that harbor a mutation in TLR4 that renders this receptor dysfunctional, whereas in the study reported here TLR4$^{-/-}$ animals backcrossed to a C57/BL6 background were used. Though in most instances the differences are minor, C3H/HeJ mice do not precisely mirror the situation found in TLR4$^{-/-}$ mice that were used here. We also performed experiments in C3H/HeJ mice (data not shown) and revealed a less prominent role for TLR4 using these mice when compared to TLR4$^{-/-}$ animals. However, just like Power *et al.* in *P. aeruginosa* pneumonia (45), we found an impaired early (4h) cytokine and PMN response in TLR4 gene-deficient mice in *A. baumannii* pneumonia. In addition, we also observed an increased bacterial load in TLR4$^{-/-}$ mice after 4h, while the difference in C3H/HeJ mice did not reach significance at this early timepoint (data not shown). Since LPS is considered the main ligand for TLR4 and

CD14, we expanded our studies and used purified LPS form *A. baumannii* to demonstrate that both TLR4 and CD14 are indeed the two crucial receptors involved in the signaling cascade during *A. baumannii* infection *in vivo*.

To investigate the receptor that counts responsible for the inflammatory response to *A. baumannii* in the absence of TLR4 or CD14, we moved on and examined the role of TLR2, the receptor for bacterial lipoproteins and peptidoglycan. Much to our surprise we found that the absence of TLR2 accelerated PMN influx into lung tissue (as assessed by MPO levels in whole lung homogenates and histology), although PMN counts in BALF of $TLR2^{-/-}$ mice did not differ from BALF PMN counts in wild-type mice early after infection. Nonetheless, TLR2 deficiency was associated with an improved bacterial clearance from this organ. When Power *et al.* investigated *P. aeruginosa* pneumonia in $TLR2^{-/-}$ mice they found a very moderately decreased PMN influx in lung tissue (as indicated by reduced MPO levels) after 4h but no impairment of the cytokine/chemokine responses (45). However, the authors did not report on lung CFU nor did they investigate later timepoints when improved bacterial clearance might have become apparent. We found an early onset of pulmonary inflammation together with increased MIP-2 and MCP-1 concentrations in $TLR2^{-/-}$ mice 4h after infection.

Alveolar macrophages have been reported to be the main source of MCP-1 during pulmonary infection (46) and LPS, via TLR4/CD14 and direct as well as indirect NFκB activation, is the major trigger for the secretion of MCP-1 (47, 48). MCP-1 has also been described in respiratory epithelial cells 24h after infection with *P. aeruginosa* (49). Moreover, two reports described the highly beneficial role of MCP-1 during Gram-negative infection: early MCP-1 administration contributed to a faster elimination of bacteria whereas late MCP-1 administration (at t=24h) reduced lung injury and improved the resolution of *P. aeruginosa* pneumonia via enhanced uptake of potentially harmful apoptotic PMNs (49, 50). Likewise, increased MIP-2 levels have been described to improve the PMN influx and phagocytosis of bacteria during Gram-negative pneumonia (51). Accordingly, we found an improved pulmonary clearance of *A. baumannii* in the presence of elevated MCP-1 and MIP-2 concentrations. The robust and early (4h) onset of pulmonary inflammation in the absence of $TLR2^{-/-}$ might therefore explain the improved bacterial clearance observed at later time points (24 and 44h).

Why and how TLR2 precisely prevents the rise in MCP-1 and MIP-2 levels remains unclear. The possibility exists that TLR2 mediates anti-inflammatory pathways that down-regulate MCP-1 production. IL-10 has been shown to reduce MIP-2 and MCP-1 secretion by activated monocytes/macrophages (48, 52, 53) but we did not find any differences in IL-10 concentrations that could explain our findings. Increased MIP-2 levels might even be a consequence of elevated MCP-1 concentrations, as illustrated by the finding of synergistically enhanced MIP-2 release in the presence of MCP-1 and LPS (54). Alternatively, the lack of TLR2 signaling in gene-deficient mice could have been associated with an upregulation of other receptors with mainly pro-inflammatory properties, such as TLR4. This phenomenon has been described in $TLR2^{-/-}$ mice infected with *P. aeruginosa* that lack pilus expression (55). Another potential explanation could be that differences in the cellular expression profile

of TLRs within the respiratory tract are associated with distinct responses. It seems quite well established that LPS induced pulmonary inflammation relies primarily on TLR4 expressing macrophages (56, 57). LPS directly activates alveolar macrophages to secrete pro-inflammatory cytokines such as TNF-α and IL-1β; these, in turn, stimulate respiratory epithelial cells to produce, for example, chemokines (56). Respiratory epithelial cells have been repeatedly shown to be unresponsive when stimulated with LPS alone, although TLR4 mRNA is present in these cells (56, 58-60). In contrast, less is known about pulmonary TLR2, but this signaling receptor is expressed on alveolar macrophages and has gained much attention recently as part of a lipid raft receptor assembly at the apical side of airway epithelial cells (61). This finding strongly indicates the direct involvement of TLR2 in the sampling of and response to pathogens within the lungs. Given the high rate of colonization with *A. baumannii* in critically ill patients, it is tempting to speculate that the reduced inflammatory response we observed to occur in the presence of TLR2 might even prove beneficial for the host, by providing a delicate balance between situations requiring robust response for the rapid clearance of bacteria in individuals with high pulmonary bacterial counts and the risk of systemic dissemination (such as the model of acute infection described herein) and cases in which the low degree of colonization does not warrant such a vigorous response.

A more recent study by Benjamim *et al.* investigated the immunosuppression and higher susceptibility to nosocomial pulmonary infections following cecal ligation puncture (CLP). Among other findings, the authors described an increased expression of TLR2 within the lungs after CLP (62). Considering our observation of a weakened immune response to *A. baumannii* in the presence of TLR2, these data suggest that preceding insults such as CLP lead to molecular alterations that include the upregulation of TLR2 and are associated with an impaired ability to combat nosocomial bacteria such as *A. baumannii*.

In conclusion, we demonstrate that CD14 and TLR4 are indispensable for *A. baumannii* LPS-mediated signaling, resulting in the effective elimination of *A. baumannii* from the lungs *in vivo*, whereas TLR2 reduces the pulmonary inflammatory response and so delays elimination of bacteria from the lungs. Our model of *A. baumannii* pneumonia will be useful in further studies aimed at elucidating mechanisms involved in innate and adaptive immune responses to this increasingly important nosocomial pathogen.

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CHAPTER 17

Non-mannose-capped lipoarabinomannan induces lung inflammation via Toll-like receptor 2

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Abstract

Non-mannose-capped lipoarabinomannan (AraLAM) is part of the cell membrane of atypical mycobacteria. To determine the capacity of AraLAM to induce lung inflammation *in vivo*, and to determine the signalling receptors involved herein, wild-type (WT) mice, Lipopolysaccharide Binding Protein knock-out (LBP KO) mice, CD14 deficient (CD14 KO) mice, Toll-like receptor (TLR) 4 mutant mice or TLR2 KO mice were intranasally inoculated with purified AraLAM. AraLAM induced high lung levels of Tumor Necrosis Factor (TNF), Interleukin (IL-)1β, IL-6 and KC and an influx of neutrophils into the pulmonary compartment of WT mice. LBP KO, CD14 KO and TLR4 mutant mice displayed similar inflammatory responses as WT mice, whereas in TLR2 KO mice AraLAM-induced lung inflammation was strongly diminished. In addition, TLR2 KO mice, but not CD14 KO or TLR4 mutant mice, displayed a delayed clearance of pulmonary infection with the atypical AraLAM expressing *Mycobacterium* (*M.*) *smegmati*s. These data indicate that TLR2 is the signaling receptor for purified AraLAM in the lung *in vivo* and that this receptor contributes to an effective clearance of *M. smegmatis* from the pulmonary compartment.

Introduction

Toll-like receptors (TLRs) are of critical importance for the initiation of an efficient innate immune response (1-4). TLRs recognize pathogen associated molecular patterns (PAMPs), which are conserved motifs expressed by microorganisms but not by higher eukaryotes. One of the most prominent PAMPs connected with mycobacteria is the glycolipid lipoarabinomannan (LAM). LAM isolated from slowly growing and virulent strains like *Mycobacterium* (*M*.) *tuberculosis* or *M. leprae* are capped with mannose to varying degrees (ManLAM) (5, 6). In contrast, the arabinan termini from LAM of rapidly growing, atypical and avirulent mycobacterial species like *M. smegmatis* are uncapped (AraLAM) (7). This difference in biochemical structure of ManLAM and AraLAM accounts for the different inflammatory capacities of the purified components in vitro (8). Indeed, AraLAM but not ManLAM is a potent inducer of TNF expression in human and murine macrophages (8, 9).

In vitro experiments with transfected cell lines have established that AraLAM signalling is dependent on a functional TLR2 (9-11). Conversely, inhibition of endogenous TLR2 by overexpression of dominant negative TLR2 protein rendered macrophages unresponsive to AraLAM (12). CD14 likely functions as the ligand binding component of the AraLAM receptor (13), whereas lipopolysaccharide binding protein (LBP) has been found to facilitate the transfer of AraLAM to CD14 thereby enhancing the responsiveness of cells to AraLAM in vitro (10, 13).

The initial challenge of the innate immune system during mycobacterial infection is the first interaction with intact bacteria. Whole *M. tuberculosis* stimulates cells through TLR2 as well as TLR4 (9, 11); possible TLR2 ligands expressed by *M. tuberculosis* include the 19kDa lipoprotein, soluble tuberculosis factor and phosphatidylinositolmannan, whereas TLR4 ligands still await to be identified (14-19). Consistent with the fact that ManLAM does not activate TLRs, TLR activation by whole *M. tuberculosis* occurs independent of LAM (11). In line with these in vitro findings, chronically infected TLR2 or TLR4 deficient mice displayed a reduced clearance of mycobacteria from their lungs, and a diminished survival during *M. tuberculosis* infection in vivo (19-21). As observed for LAM, the TLR response to whole bacilli varies with the mycobacterial species. Indeed, *M. avium* activates cells via TLR2, but not TLR4 (22).

Knowledge of the inflammatory effects of AraLAM in vivo and the role of TLRs herein is not available. Therefore, in the present study, we sought to determine (1) whether AraLAM is capable of inducing an inflammatory response in the mouse lung in vivo, (2) the contribution of LBP, CD14, TLR2 and TLR4 in these effects, and (3) the role of CD14, TLR2 and TLR4 in the innate immune response to an AraLAM expressing fast-growing mycobacterium, *M. smegmatis*. Some of these results were presented in the form of an abstract (23).

Materials and Methods

Animals

LBP knockout (KO) mice (24) backcrossed 11 times to a C57Bl/6 background, and TLR2 KO mice (25), backcrossed 6 times to a C57Bl/6 background, were generated as described previously. CD14 KO mice, backcrossed 6 times to C57Bl/6 background, were obtained from the Jackson Laboratories (Bar Harbor, ME). Wild-type (WT) C57Bl/6 mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). C3H/HeJ and C3H/HeN mice were purchased from Charles Rivers (Someren, The Netherlands). The Animal Care and Use of Committee of the University of Amsterdam approved all experiments. Age and sex-matched animals were used in each experiment.

Reagents

AraLAM was isolated from rapidly growing Mycobacterium smegmatis and obtained from Dr. J.T. Belisle from the Colorado State University, Fort Collins, CO (under NIH Contract NO1-AI-75320). Endotoxin contamination was 0.373 ng/mg of AraLAM as determined by the Limulus Amebocyte lysate assay.

Experimental design

AraLAM was administered intranasally (i.n.) according to previously described methods (26, 27). In first experiments in WT mice, AraLAM was administered at 0, 0.1, 1 or 10 μg; after 6 h, mice were anaesthetized by intraperitoneal injection of Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium: active ingredients fentanyl citrate and fluanisone) and midazolam (Roche, Mijdrecht, The Netherlands) and sacrificed by bleeding from the vena cava inferior. Consecutively, WT mice were inoculated i.n. with AraLAM (10 μg) and sacrificed after 3, 6, 12 or 24h. In additional experiments, mice were inoculated i.n. with 10 μg of AraLAM and sacrificed after 6 h.

Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage and leukocyte differentiation were performed as described previously (26, 28). 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 3 times 0.5 mL aliquots of cold saline and collecting the fluid by aspiration. Approximately 1.3 mL of BAL fluid (BALF) was retrieved per mouse and total cell counts in each sample were determined using a hemocytometer. Differential cell counts were determined on cytospin preparations stained with hematoxilin and eosin.

Myeloperoxidase (MPO) activity assay

MPO activity was measured in homogenates as described previously (28). Lung tissue was homogenized in saline and pelleted at 4500 x g for 20 min. Pelleted cells were lysed in potassium phosphate buffer pH 6.0 supplemented with hexadecyltrimethyl

ammoniumbromide (HETAB) and 10 mM EDTA. MPO activity was determined by measuring the H2O2 dependent oxidation of 3,3'5,5' tetramethylbensidine (TMB). The samples diluted in potassium phosphate buffer were mixed with tetramethylbensidine substrate N,N'dimethylformamide. The reaction was stopped with glacial acetic acid followed and the OD655 was measured. MPO activity is expressed as activity per gram lung tissue per min. All reagents were purchased from Sigma.

Cytokine measurements

Lungs were homogenized for cytokine measurements exactly as described previously (26, 28, 29). Cytokines and chemokines were measured using specific enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The detection limits were 8 pg/ml for IL-1β, 31 pg/ml for IL-6 and TNF, 37 pg/ml for KC and 187 pg/ml for MIP-2.

Histologic examination

Unlavaged lungs were removed and fixed in 10 % buffered formalin in PBS for 24h and embedded in paraffin. Hematoxilin and eosin stained slides were coded and semi-quantitatively scored for the following parameters: interstitial inflammation, intra-alveolar inflammation, edema, endothelialitis, bronchitis, pleuritis and thrombi formation by a blinded pathologist. Each parameter was graded on a scale of 0 to 3, with 0: absent, 1: mild, 2: moderate and 3: severe. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 21 (30).

Model of infection

A laboratory strain of *Mycobacterium smegmatis* (ATCC 14468; Rockville, MD) was grown in liquid Dubos medium containing 0.01% Tween 80. Mice were infected i.n. with 2.5 x 10^5 colony forming units (CFU) of *M. smegmatis* as determined by viable counts on 7H11 Middlebrook agar plates. At 6 h and 1, 3 and 10 days after infection, mice were sacrificed, lungs were removed and homogenized. Ten-fold serial dilutions of homogenates were plated on 7H11 Middlebrook agar plates and after 4 days CFU were counted. In a different set of infection experiments, mice were i.n. infected with 10^4 or 10^6 CFU of *M. smegmatis* and sacrificed after 10 days.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Serial data were analyzed by Kruskall-Wallis test with Mann-Whitney U as post test using GraphPad Prism version 3.00, GraphPad Software (San Diego, CA). Two sample comparisons were done by Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

Results

AraLAM induces lung inflammation following i.n. inoculation

To determine if non-mannose capped LAM is capable of inducing an inflammatory response in the lung in vivo and to establish a dose-response relationship, we i.n. inoculated WT mice with three different doses of AraLAM (0.1, 1, 10 μg); control mice received PBS i.n.. Inoculation with AraLAM was associated with a dose dependent increase in TNF, IL-1β and KC levels in lung homogenates (Fig. 1) as well as BALF (data not shown) six hours after inoculation. Expression of the chemokine MIP-2 was not influenced by inoculation of AraLAM (data not shown). Compared to control mice, total cell count was significantly increased in BALF of mice inoculated with 10 μg of AraLAM (data not shown).

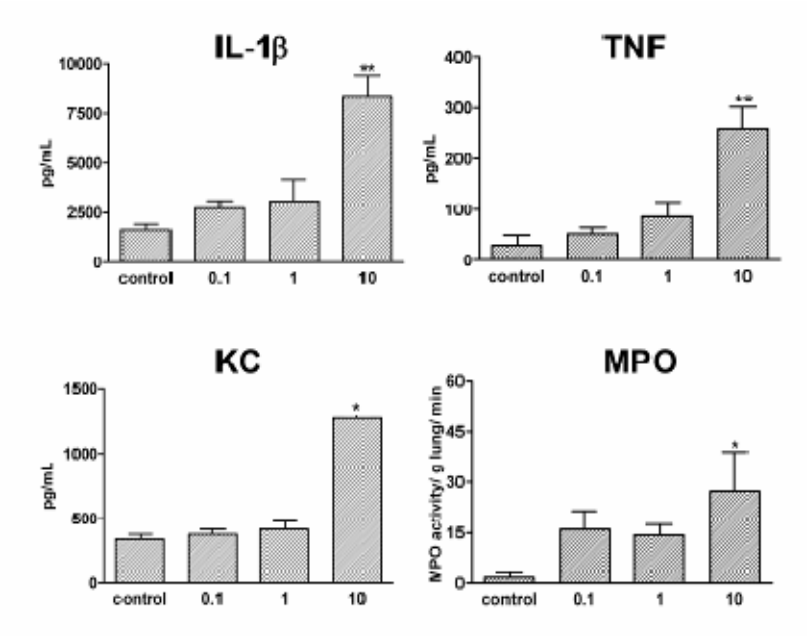

Fig. 1: AraLAM induces pulmonary inflammation. Groups of 4 to 8 WT mice were inoculated i.n. with three different doses of AraLAM (0.1, 1 and 10 μg). Control mice received sterile PBS. 6 h after inoculation mice were sacrificed and lungs were excised. IL-1β, TNF, KC and MPO activity as a measure of neutrophil influx were determined in lung homogenates. Data are means ± SEM; *, $P<0.05$ versus WT mice.

The increase in MPO activity (Fig.1) in lung homogenate as well as differential cell counts (data not shown) revealed that this increase was mainly due to the influx of neutrophils into the pulmonary compartment. Based on these results, additional experiments were performed with 10 μg of AraLAM. To further characterize the pro-inflammatory response induced by i.n. inoculation of AraLAM, we evaluated the kinetics of cytokine production and cell influx in the lungs of WT mice (Tables I and II). Increased levels of cytokines TNF, IL-1β and IL-6 were observed in lung homogenates already at 3 h after i.n. administration of AraLAM. Although the expression of chemokine KC increased in a similar way, no major changes in lung-associated MIP-2 levels could be detected. Total cell counts in BALF consistently increased during the first 24 h after i.n. inoculation, peaking after 6 h. Differential counts demonstrated that the influx was primarily composed of neutrophils (Table II).

cytokine	*control*	*3 h*	*6 h*	*12 h*	*24 h*
TNF	65 ± 22	526 ± 143**	363 ± 40**	162 ± 56*	169 ± 63*
IL-1β	1248 ± 115	5328 ± 979*	8416 ± 1011**	680 ± 132*	697 ± 180*
IL-6	77 ± 19	553 ± 101*	226 ± 28**	215 ± 70	197 ± 31
KC	443 ± 54	932 ± 91**	880 ± 58**	438 ± 25	495 ± 38
MIP-2	1079 ± 217	1384 ± 138	1747 ± 102	1489 ± 270	1600 ± 333

Table I: Cytokine and chemokine levels in lung homogenates of mice inoculated with AraLAM. Groups of 5 to 9 mice were i.n. inoculated with AraLAM (10 μg/ mouse). Control mice received sterile PBS. At the indicated time points, mice were sacrificed and lungs removed and cytokine and chemokine levels were measured in lung homogenates (pg/mL). Data are mean±SEM;*, P<0.05; **, P<0.01 versus control

time	*cells/ml* x10^3	*Macrophages* %	*PMNs* %	*Lymphocytes* %
control	11.6 ± 1.3	91.9 ± 1.5	5.5 ± 1.6	2.6 ± 0.3
3	23.6 ± 7.1	83.8 ± 5.3	13.2 ± 5.3	2.9 ± 0.1
6	44.8 ± 9.5**	46.9 ± 2.8*	51.0 ± 3.0*	2.1 ± 0.6
12	64.0 ± 8.8*	62.2 ± 9.5	34.6 ± 9.1	3.2 ± 0.4
24	68.0 ± 9.0*	51.9 ± 12.6*	45.3 ± 12.3*	2.7 ± 0.3

Table II: Effect of AraLAM on cellular subsets in BALF. Groups of 4 to 8 mice were i.n. inoculated with AraLAM (10 μg/ mouse). Control mice received sterile PBS. Lungs were lavaged at indicated time-points (h) and total cell counts and differential counts were determined in BALF and expressed as mean ± SEM. *, *P*<0.05; **, *P*<0.01 versus control

TLR2 but not CD14, LBP or TLR4, is crucial for AraLAM-induced lung inflammation

After having established that AraLAM induces pulmonary inflammation characterized by expression of proinflammatory cytokines and chemokines as well as the influx of neutrophils into the pulmonary compartment, we were interested in the role of TLR2, TLR4, CD14 and LBP herein. Therefore TLR2 KO, CD14 KO, TLR4 mutant and LBP KO mice were i.n. inoculated with 10 μg AraLAM and sacrificed after 6 h. In comparison with WT mice, TLR2 KO mice showed a strongly reduced inflammatory response after i.n. challenge with AraLAM. Indeed TLR2 KO mice displayed much lower lung concentrations of IL-1β, KC, IL-6 (Fig. 2 A-C) and TNF (data not shown), and an almost complete absence of neutrophil influx into the BALF (Table III). Accordingly, WT mice developed a significant lung interstitial inflammatory infiltrate in contrast to TLR2 KO mice which displayed normal lung histology (Fig.3).

	cells/ml	*Macrophages*	*PMNs*	*Lymphocytes*
	x10^3	%	%	%
WT	41 ± 8.0	44.8 ± 5.0	51 ± 5.1	2.9 ± 0.3
TLR2 KO	19 ± 2.5*	87.7 ± 2.5**	9.4 ± 2.6**	2.0 ± 0.2

TABLE III: Effect of TLR2 deficiency cellular subsets in BALF in response to AraLAM. Groups of 8 mice were i.n. inoculated with AraLAM (10 μg/ mouse). Lungs were lavaged after 6 h (h) and total cell counts and differential counts were determined in BALF and expressed as mean ± SEM. **P*<0.05; ***P*<0.01 versus control

By contrast, CD14, LBP and TLR4 deficient mice were indistinguishable from their respective WT mice with respect to the induction of TNF, IL-1β, IL-6 and KC in lungs (shown for IL-1β, KC and IL-6 in Fig. 2 D-L) and with respect to neutrophil influx in the BALF (data not shown).

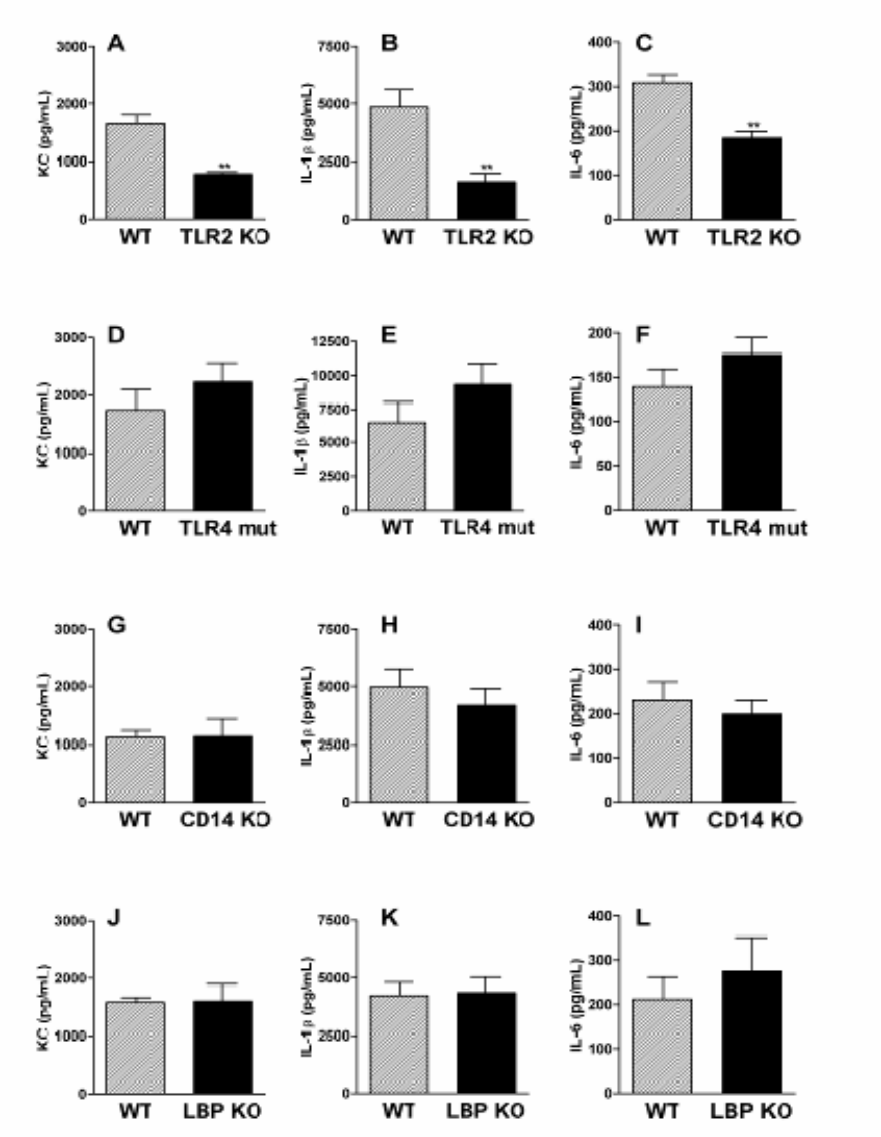

Fig. 2: Lack of inflammatory response in lungs of TLR2 KO mice. Groups of 6 to 8 WT, TLR2 KO (A-C), TLR4 mutant (D-F), CD14 (G-I) and LBP (J-L) KO mice were inoculated i.n. with 10 μg of AraLAM. After 6 h the mice were sacrificed and lungs were removed. IL-1β, KC and IL-6 levels were measured in lung homogenates by ELISA. Data are means ± SEM; ***P*<0.01 versus PBS controls.

These results demonstrate that TLR2 signaling is crucial for the induction of pulmonary inflammation after AraLAM challenge in vivo, and that CD14, LBP or TLR4 do not play a role of importance herein. Furthermore, from these results it can be concluded that the used AraLAM preparation was not contaminated with biologically relevant concentrations of LPS because no differences in response were detected between C3H/HeN and LPS unresponsive C3H/HeJ mice.

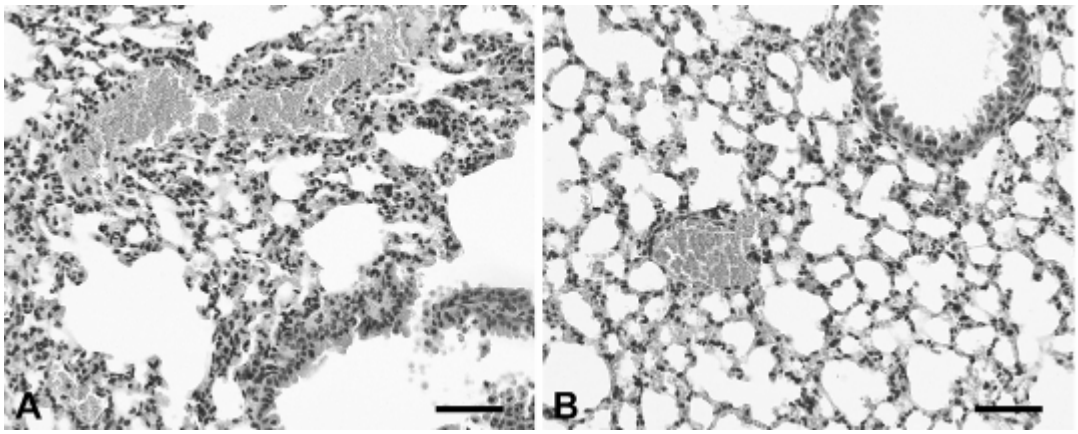

Fig. 3: Absence of inflammation in lungs of TLR2 KO mice. Representative lung histology of groups of 5 WT (A) and TLR 2 KO (B) mice sacrificed 6 h after i.n. inoculation with 10 μg of AraLAM. Hematoxilin & eosin staining, magnification x20. Bars represent 50 μm.

TLR2 deficiency delays the clearance of *M. smegmatis* infection

The cell wall LAM from avirulent fast growing mycobacterium *M. smegmatis* is predominantly non-capped (6). To determine the role of TLR2, CD14 and TLR4 in the host response to this myco-bacterium, we infected TLR2 KO, CD14 KO and TLR4 mutant mice with 2.5 x 10^5 CFU of *M. smegmatis* and sacrificed the mice after 6 h, 1, 3 and 10 days. Normal WT mice clear this infection during the first four weeks of infection (31). TLR2 KO mice displayed a delayed clearance of *M. smegmatis* when compared to WT mice (Fig. 4).

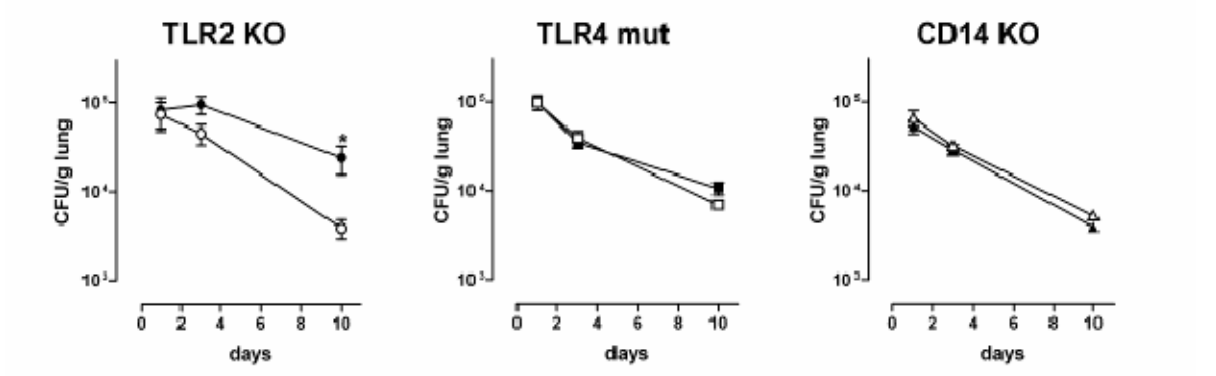

Fig. 4: Delay in clearance of *M. smegmatis* in TLR2 deficient mice. Mycobacterial outgrowth in lungs: WT (open symbols) and TLR2 or CD14 KO as well as TLR4 mutant mice (closed symbols) mice were infected i.n. with 2.5 x 10^5 CFU of *Mycobacterium smegmatis*. One, 3 and 10 days post-infection, mice were sacrificed and bacterial loads were determined in lung homogenates. Data are means ± SEM of 5-8 mice/ group. **P*<0.05.

Indeed, whereas at 6 h, 1 and 3 days post-infection no differences in bacterial outgrowth were found, at 10 days after infection the lungs of TLR2 KO mice contained approximately one log more *M. smegmatis* CFU than the lungs of WT mice. In accordance with the results obtained after AraLAM administration, the early cytokine response to *M. smegmatis* was reduced in TLR2 KO mice. Indeed, despite a similar bacterial burden 6 h post-infection, lung levels of TNF, IL-1β, IL-6 and KC were significantly reduced at this time-point, although MPO levels were similar in TLR2 KO and WT mice (Table IV).

	CFU/g lung x105	***MPO activity***	***TNF***	***IL-1β***	***IL-6***	***KC***
WT	3.4 ± 0.4	25.0 ± 6.3	2.1 ± 0.1	2.4 ± 0.3	0.4 ± 0.06	1.0 ± 0.1
TLR2 KO	3.7 ± 1.0	18.6 ± 4.3	1.6 ± 0.2*	1.6 ± 0.2*	0.3 ± 0.02*	0.6 ± 0.1**

TABLE IV: Effect of TLR2 deficiency on the early (6 h) response to *M. smegmatis*. Groups of 8 mice were i.n. inoculated with $2x10^5$ CFU of *M. smegmatis*. Six h post-infection, mice were sacrificed and bacterial load, MPO activity as well as cytokines and chemokines (ng/mL) were measured in lung homogenates. Data are expressed as mean ± SEM. *, $P<0.05$; **$P<0.01$ versus WT control.

After 1 and 3 days of infection influx of neutrophils to the interstitium was observed in both groups together with signs of pleuritis. After 10 days of infection, almost no inflammation was found despite the presence of mycobacteria. Regardless of differences in bacterial outgrowth, lung pathology was similar in WT and TLR2 KO mice although at 3 days post-infection, the inflammatory response in the TLR2 KO lung was enhanced (Semi-quantitative scores of WT 3.9 ± 1.5 versus 5.2 ± 1.1 of TLR2 KO mice respectively; $P<.05$; Fig. 5).

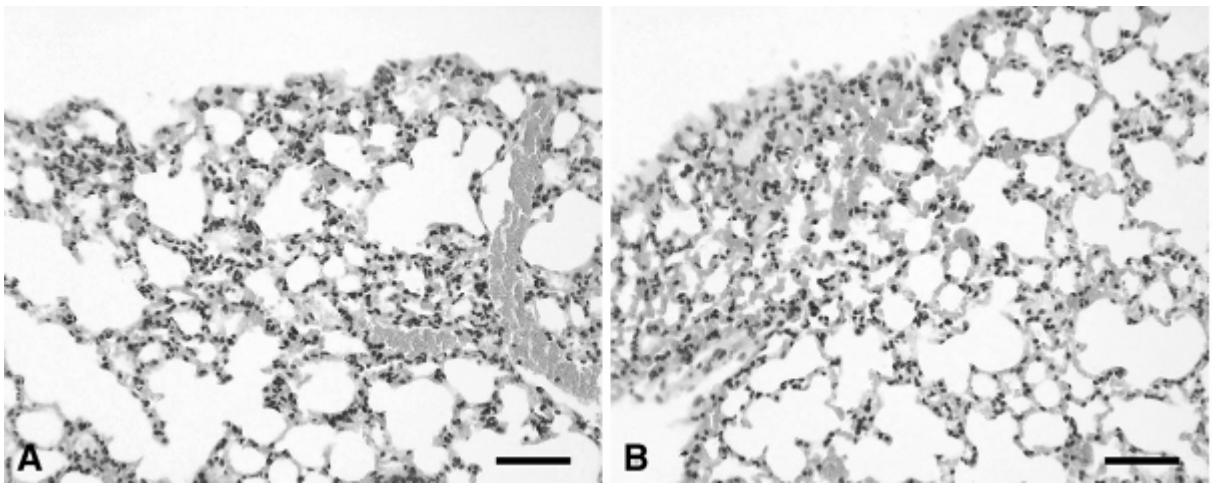

Fig. 5: Histopathology. Representative histologic sections of lungs of groups of 8 WT (A) and TLR 2 KO (B) mice sacrificed after infection with 2.5 x 10^5 CFU of *M. smegmatis*. Hematoxilin & eosin staining, magnification x20. Bars represent 50 μm.

In contrast, the number of *M. smegmatis* CFU (Fig. 4) and pathology (data not shown) did not differ between CD14 KO or TLR4 mutant mice with their respective WT mice at any of these time points. To obtain further insight into the role of TLR2 in host defense against *M. smegmatis*, we next i.n. infected TLR2 KO and WT mice with a lower (10^4 CFU) and a higher (10^6 CFU) mycobacterial inoculum and counted *M. smegmatis* CFU in lungs 10 days postinfection. Only 3/8 TLR2 KO mice infected with 104 CFU *M. smegmatis* had completely cleared the mycobacteria from their lungs 10 days postinfection versus 6/8 WT mice, all mice with detectable mycobacteria displayed 100-200 CFU/g lung (the detection limit being 100 CFU/g lung) with the exception of one TLR2 KO mouse that demonstrated 4500 CFU/g lung. After infection with 106 CFU *M. smegmatis*, all mice still had detectable mycobacteria in their lungs at 10 days, but TLR2 KO mice harbored significantly more *M. smegmatis* than WT mice (Fig. 6). Like in our studies using 2.5 x 10^5 CFU, an inflammatory response was barely present in lungs 10 days postinfection (data not shown).

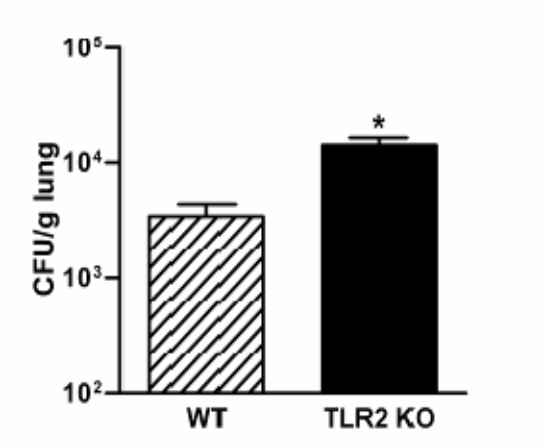

Fig. 6: Different dose of *M. smegmatis* in TLR2 deficient mice. Mycobacterial outgrowth in lungs: WT and TLR2 KO mice were infected i.n. with 10^6 CFU of *Mycobacterium smegmatis*. Ten days post-infection, mice were sacrificed and bacterial loads were determined in lung homogenates. Data are means ± SEM of 8 mice per group. **P*<0.05

Discussion

TLRs are the major receptors for the induction of the innate immune response after a microorganism enters the body. They recognize specific bacterial components, among which LAM prominently features in the mycobacterial cell wall. In the present study, we investigated the capacity of AraLAM from fast growing mycobacteria to elicit an inflammatory response in the lung. Thereby, we were particularly interested in signalling receptors involved in AraLAM-induced effects in the respiratory tract in vivo. We here demonstrate that AraLAM is able to induce a profound inflammatory response in the lungs of WT mice. The reaction observed was very similar to the response induced by i.n. LPS administration and characterized by rapid induction of pro-inflammatory cytokines and chemokines followed by a marked influx of neutrophils into the pulmonary compartment (32). In addition, by using a number of genetically modified or altered mouse strains, we established that TLR2, but not LBP, CD14 or TLR4, is important for triggering lung inflammation by AraLAM.

We chose to investigate the inflammatory response in lungs at 6 hours after i.n. administration of AraLAM for two reasons. First, from our kinetic study during 24 hours after administration, we found 6 hours most suitable for investigating both neutrophil influx and cytokine release. Second, several other studies in our laboratory and elsewhere challenging mice i.n. with different bacterial compounds (LPS, lipoteichoic acid, peptidoglycan or pneumolysin) have shown that 3-6 hours after challenge is an appropriate time point for studying pulmonary inflammation (26, 32, 33). Potential cells triggered after contact with AraLAM are macrophages, epithelial cells and most $CD11c^{hi}$ dendritic cells (34, 35). All of these cell types express TLR2 on their surface and all of them potentially contributed to the induction of inflammation observed in the current investigation. Of note, whereas AraLAM induced a rise in lung KC concentrations, it did not influence MIP-2 levels. Although a clear explanation for this finding is not available, it should be noted that earlier reports have suggested that the production of these two chemokines in the pulmonary compartment is regulated by at least partially different mechanisms (26, 36).

The inflammatory response in the lungs induced by AraLAM was dependent on TLR2 but not on LBP, CD14 or TLR4. With respect to the role of TLR2 in AraLAM signalling, our data are in line with in vitro observations (9-11). By engineering CHO-K1 fibroblasts, THP-1 human monocytic leukemia and RAW264.7 murine macrophage cell lines to express CD14, TLR4 or TLR2, Means et al. demonstrated that TLR2 but not TLR4 signalling is crucial for activating cells by AraLAM (10). Results obtained with peritoneal macrophages from TLR4 mutant mice also revealed that LPS and AraLAM use different recognition receptors for activation of host cells (37). Subsequent studies confirmed these data and added several other mycobacterial proteins to the list of TLR2 signalling PAMPs associated with mycobacteria: the 19kDa protein (16-18), soluble tuberculosis factor and phosphatidylinositol dimannoside from *M. tuberculosis* (PIM2) (11, 14) and lipomannan (LM) from *M. kansasii* (38). Furthermore, heat-killed as well as live whole mycobacteria stimulate macrophages through TLR2 (9, 21) and expression of a dominant negative TLR2 construct partially inhibited activation of mouse macrophages by heat-killed mycobacteria (12).

Interestingly, TLR2 can cooperate with different TLRs before transmitting a signal into the cytoplasm, in particular TLR1 and -6 (3, 39). This cooperation is important for discrimination between different microbial constituents and products. For example, TLR1 and -2 cooperate in the recognition of the 19 kDa peptide of *M. tuberculosis* (40). Recently, two studies demonstrated that also AraLAM utilizes both TLR2 and TLR1 for forming a functional signaling complex *in vitro* (41, 42). Therefore, it will be interesting to look at the *in vivo* role of TLR1 and/or TLR6 in pulmonary inflammation induced by AraLAM as well as in *M. smegmatis* infection. Such studies were not part of the present investigations since mice deficient for TLR1 or TLR6 (in the presence or absence of TLR2 deficiency) are not available to our laboratory at present.

Unlike the important role of TLR2, our data suggest that neither LBP nor cell associated CD14 nor soluble CD14 are crucial receptors for AraLAM. These findings are in conflict with several in vitro studies (10, 13, 43-47). These studies point out that AraLAM needs

binding of the LBP/CD14 complex in order to activate the cellular receptor. This discrepancy between the in vitro data and our in vivo findings possibly can be explained by the extent of LPS contamination of the AraLAM preparation used. LPS signaling is dependent on binding of LPS to LBP, CD14 or soluble CD14 and subsequent transfer to TLR4. In our study, AraLAM was used which contained 0.373 ng of LPS per mg of AraLAM, which results in intranasal inoculation of 3.73 pg of LPS. This amount of LPS does not induce a detectable inflammatory response in mouse lungs (48). Furthermore, no difference in inflammatory response was found in the lungs of the TLR4 mutant C3H/HeJ mice when compared to their WT C3H/HeN controls. If LPS contamination was the reason for the inflammatory response elicited by AraLAM administration, a suppressed or absent inflammatory response should have been seen in TLR4 mutant mice since they are LPS unresponsive (49).

Whole microorganisms contain several different PAMPs and the inflammatory response to the whole live microorganisms is most likely to be different from the effects of isolated bacterial components. For example, by using antibodies against LAM, it was shown that TLR dependent cellular activation by *M. tuberculosis* H37Ra was not purely mediated by LAM (11). *M. smegmatis* abundantly expresses non-mannose capped LAM on its surface (6). To facilitate comparisons with results obtained after AraLAM administration, we therefore studied the role of TLR2, CD14 and TLR4 in a model of pulmonary infection with *M. smegmatis*. In humans, *M. smegmatis* primarily is associated with soft tissue and wound infections. *M. smegmatis* rarely causes lung infection in the immunocompetent host (50). However, in severely immuno-compromised patients like patients with inherited IFNγ-R1 deficiency *M. smegmatis* infection can disseminate and can be lethal (51). In addition, *M. smegmatis* can cause pulmonary infections in patients with an underlying condition such as lipoid pneumonia (50, 52-54). Little is known about the pathogenesis of infection by either virulent or avirulent fast-growing mycobacteria (like *M. smegmatis*). It should be noted that it has not been established how much AraLAM is contained in *M. smegmatis*. It may therefore be possible that the amount of AraLAM in the different mycobacterial doses used was below the threshold for stimulating a measurable response by the mycobacterial AraLAM component in vivo. However, our data obtained in TLR2 KO mice early (6 h) after infection with *M. smegmatis* point to a possible role for AraLAM in the inflammatory response induced by this micoorganism. Indeed, at this time point TLR2 KO displayed significantly lower cytokine concentrations in their lungs than WT mice, which resembled the findings in TLR2 KO and WT mice administered with AraLAM.

An earlier study examined the role of TNF during intravenous infection of mice with *M. smegmatis* (31). In this and in our study, *M. smegmatis* was cleared from the main target organ, the liver in the intravenous model and the lung in our model. In the intravenous infection, the lack of TNF resulted in a delayed expression of cytokines and chemokines, reduced cellular recruitment and an associated delay in clearance of *M. smegmatis* (31). Different studies using TLR2 KO mice in infectious models with pathogenic mycobacterial strains like *M. tuberculosis*, *M. avium* and *M. bovis* BCG showed divergent results. Despite differences in host response against the pathogens in all studies like an altered inflammatory

response, enhanced bacterial outgrowth and/or reduced survival of the animals, these differences in outcome ranged from subtle to significant (20, 21, 55-58). These differences can be explained by the differences in routes of infection (intravenously versus aerosol), infection dose (low dose versus high dose) and/or mouse strains used. To our knowledge our study is the first to investigate the role of TLR2 in the defense against mycobacteria that are normally cleared by the immunocompetent host. In our model of mycobacterial infection, the host response against the non-pathogenic *M. smegmatis* was altered but TLR2 deficiency did not result in a greatly enhanced susceptibility, i.e. the clearance of this mycobacterium was delayed but still occurred. Hence, although TLR2 is involved in an effective clearance of *M. smegmatis* from the lungs, other receptors such as scavenger receptors and other members of the TLR family likely contribute as well. Indeed, although not established for *M. smegmatis*, heat-sensitive cell associated mycobacterial factors have been found to activate TLR4, whereas CpG-DNA is recognized by TLR9 (11, 19, 59). Unexpectedly, after 3 days of infection with 2x105 CFU, lungs of TLR2 KO mice displayed slightly enhanced inflammation. At this time-point, although not significantly different ($P = 0.1$), bacterial loads in the lungs of TLR2 KO mice were higher and the presence of other PAMPs of *M. smegmatis* bacilli may have triggered other TLRs like TLR4 or 9 (see also above) resulting in more stimulation of the inflammatory response than in WT mice. In line, Drennan et al. recently reported that although the production of proinflammatory cytokines induced by live *M. tuberculosis* in resident macrophages in vitro was strictly TLR2 dependent, during *M. tuberculosis* infection *in vivo*, TLR2 KO mice demonstrated an exaggerated inflammatory response (21). Of note, in our study hardly any inflammatory response was observed in lungs of either WT or TLR2 KO mice at 10 days after infection. Conceivably, the low numbers of nonpathogenic *M. smegmatis* were unable to elicit inflammation during that clearance phase of the infection.

In conclusion, we demonstrate that in vivo administration of the mycobacterial component AraLAM in the lungs triggered acute pulmonary inflammation characterized by induction of pro-inflammatory cytokines and in subsequent neutrophil influx. Furthermore, based on the results from several genetically modified mouse strains we conclude that TLR2 is indispensable for AraLAM signaling in the lung in vivo, and that this receptor contributes to an effective clearance of AraLAM expressing *M. smegmatis* from the pulmonary compartment

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CHAPTER 18

Cutting edge: Surface and soluble TREM-1 expression patterns in human endotoxemia

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Abstract

Triggering receptor expressed on myeloid cells (TREM)-1 is a recently identified molecule involved in the amplification of inflammation. To determine the regulation of TREM-1 we studied TREM-1 expression and soluble TREM-1 (sTREM-1) plasma-levels upon intravenous LPS challenge in healthy humans *in vivo* and *in vitro*.

Granulocyte TREM-1 expression was high at baseline and immediately downregulated upon LPS exposure along with an increase in sTREM-1. Monocytes displayed a gradual upregulation of TREM-1 upon LPS *in vivo* and *in vitro*. *In vitro* studies extended these findings to highly purified lipoteichoic acid (LTA) and *S. pneumoniae*. Non-bacterial TLR-ligands such as poly dI-dC and imidazoquinoline as well as the TLR9-ligand CpG did not impact TREM-1 expression. The LPS-induced alterations in TREM-1 surface expression were not a result of increased TNF-α or IL-10. Inhibitor studies disclosed a PI3K dependent pathway in LPS-induced upregulation of TREM-1 on monocytes while MAPK played a limited role.

Introduction

Sepsis is a leading cause of mortality and morbidity worldwide and characterized by a dysregulated host response to pathogens. The excessive release of inflammatory mediators such as TNF-α contributes to the multi organ failure and high lethality of septic patients (1, 2). Granulocytes and monocytes are key effector cells in sepsis by virtue of their capacity to recognize pathogen associated molecular patterns, which in turn leads to the induction of the inflammatory response following infection.

TREM-1 is a recently discovered cell surface molecule expressed on granulocytes, monocytes and a subset of macrophages (3, 4). It belongs to the immunoglobulin superfamily and interacts with the adaptor protein DAP12 for signaling and function (3). Engagement of TREM-1 results in the production of chemokines such as IL-8 and the proinflammatory cytokines TNF-α and IL-1β (3). Although the endogenous ligand is unknown, costimulation with TLR ligands LPS (TLR4-ligand), LTA or *Mycobacterium tuberculosis* 19kD lipopeptide (TLR2-ligands) synergistically enhances the inflammatory response (3, 5-7). The functional significance of TREM-1 has been underlined in murine models of LPS-induced shock and peritonitis since blocking TREM-1 downregulated inflammation and protected mice from lethality (5). Therefore, TREM-1 is considered an amplifier of inflammation in response to LPS and bacteria, making it an attractive therapeutic target in hyperinflammatory states like sepsis.

However, before potential therapeutic implications for TREM-1 in humans can be seriously considered, knowledge about the *in vivo* regulation and expression of TREM-1 has to be expanded. Investigations on TREM-1 expression are thus far limited to infected tissues obtained in later stages of infection. The *in vivo* expression of TREM-1 at the onset of inflammation is unknown. We therefore investigated the dynamics of surface and soluble TREM-1 during endotoxemia in humans.

Materials and Methods

Human endotoxemia

Endotoxemia was induced as described previously (8). Briefly, eight healthy males received an intravenous injection with *Escherichia coli* LPS, lot G (United States Pharmacopeial Convention, Rockville, MD) at a dose of 4ng/kg. For FACS analysis, EDTA anticoagulated blood was obtained before and 2, 4, 6 and 24 hours after LPS challenge.

The institutional scientific and ethics committees of the Academic Medical Center, Amsterdam, approved the study and written informed consent was obtained from all study subjects.

Soluble TREM-1 (sTREM-1) concentrations

sTREM-1 levels were measured with an immunoblot technique, as described previously (9, 10). 100ul of each plasma sample was dotted onto nitrocellulose membrane, dried, then

coated in phosphate-buffered saline supplemented with 3%bovine serum albumin and incubated with monoclonal anti-human TREM-1 (21C7)(3) for 60min. After thorough rinsing, the nitrocellulose sheet was incubated with goat-anti-mouse Ig (Dako, Glostrup, Denmark) for another 60min. Following a washing step with phosphate-buffered saline supplemented with 20% dimethylsulfoxide the membrane was incubated with horseradish peroxidase-conjugated streptavidin (BioRad, Marnes-la-Coquette, France) for 30min before the enzyme substrate Opti-4CN (BioRad) was added. Each sheet also contained calibration samples of a known concentration of sTREM (0 to 5.000 ng/ml). The staining intensity was colorimetrically compared to the standard curve with the help of a reflectance scanner and Quantity One Quantitation software (BioRad). All measurements were performed in triplicate and results are expressed as mean+/- SE. To ensure and control the specificity of the anti-TREM Ab and this technique, western blots were performed on a routinely basis (as depicted in Fig. 2B).

***In vitro* experiments**

Whole blood stimulations were performed as described (11) using aseptically collected blood from 5-6 volunteers. For some experiments PBMC were isolated using Histopaque (Sigma, St. Louis, MO). The following stimuli were used: LPS lot G (100, 1 or 0.01ng/ml), purified LTA (10µg/ml; *S. aureus*; kind gift of T. Hartung, Univ. Konstanz, Germany) (12), CpG-ODN (10µM, Eurogentec, Seraing, Belgium; TLR9-ligand), polyinosine-polycytidylic-acid (poly dI-dC) (50µg/ml, Sigma; TLR3-ligand), Imidazoquinoline S27609 (0.5µg/ml, 3M Pharmaceuticals, St. Paul, MN; TLR7-ligand), rhTNF-α (20ng/ml) and rhIL-10 (10ng/ml) (both Strathmann Biotec, Hannover, Germany), heat-killed *Streptococcus pneumoniae* (10^8CFU/ml, 6303, American type culture collection, Rockville, MD). All stimuli were independently tested for their bioactivity in our laboratory (unpublished data and (13, 14)). In some experiments cells were preincubated for 30min with the following inhibitors: p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (10µM), MAPK/ERK (extracellular signal-related kinase) (MEK) kinase inhibitor PD98059 (20µM), phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002 (10µM) (all Alexis, Leiden, Netherlands) or anti-TNF-α (25µg/ml; Etanercept, Wyeth, Hoofddorp, Netherlands). The dose of the respective kinase-inhibitors was chosen after ruling out additional cytotoxicity by the compounds themselves (data not shown). LY294002 was chosen as a PI3K-inhibitor due to its longer half-life as compared to other PI3K-inhibitors such as wortmannin. Stimulations were performed for the indicated time-points at 37ºC and repeated independently for at least two times.

FACS analysis

Erythrocytes were lysed and cells blocked for aspecific binding with 10% human Ig (Sigma) for 1h on ice. Leukocytes were incubated with anti-TREM-1 Ab (21C7) (3), followed by goat anti-mouse secondary Abs (FITC-labeled for *in vivo* studies and APC-labeled for *in vitro* studies) and finally anti-CD14 and anti-HLA-DR (all BDPharmingen, San Diego, CA).

Granulocytes were defined according to their scatter pattern and monocytes as $CD14^{high}$ and $HLA\text{-}DR^{pos}$. To correct for aspecific staining, appropriate isotype control Abs were used. Samples were analyzed by flow cytometry using a FACScan (Becton Dickinson).

Statistical analysis

Changes in time or between groups were analyzed by one-way analysis of variance, differences in response curves by repeated measurements. Data are presented as mean ± SE; $P<0.05$ was considered significant.

Results and Discussion

In light of the pro-inflammatory properties attributed to TREM-1 we studied the surface expression of TREM-1 on granulocytes and monocytes during the course of endotoxemia in humans. At baseline, TREM-1 was strongly expressed on blood granulocytes and monocytes. Endotoxemia induced a gradual up-regulation of TREM-1 on monocytes, peaking after 24h (MF 47.7±4.3 at t=0, 76.8±7 at t=24h; Fig. 1A; p=0.008).

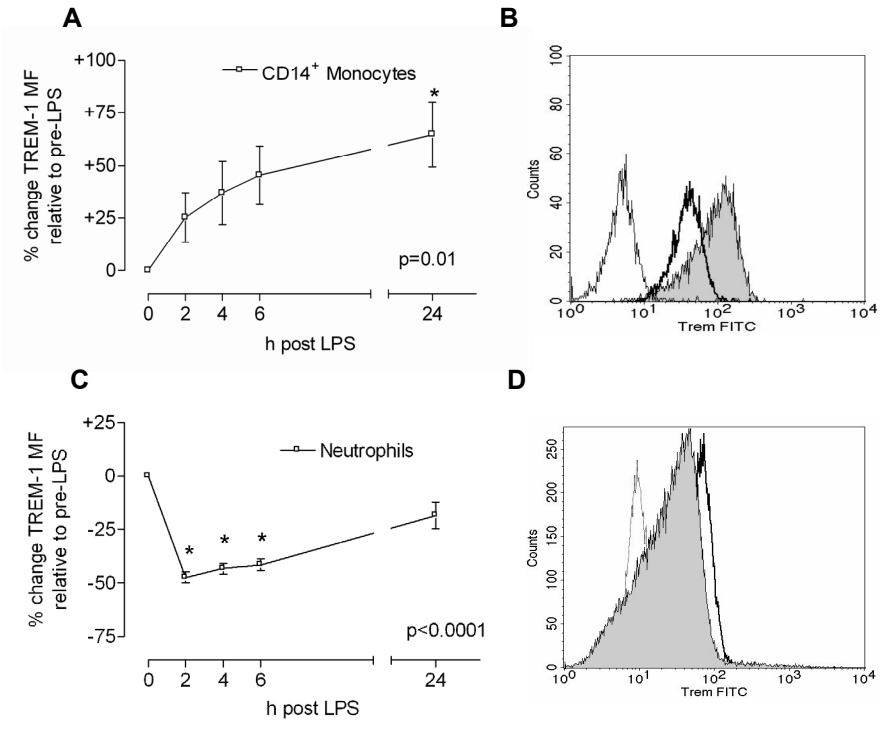

Fig. 1: TREM-1 cell surface expression *in vivo*. Subjects received an intravenous injection of LPS (4ng/kg) and the change in TREM-1 expression was monitored over 24h. Monocytes were defined as $CD14^{high}$ $HLA\text{-}DR^{pos}$ cells (A and B), granulocytes were gated according to their scatter pattern (C and D). Data shown represent results of two independent experiments. Representative histograms for monocytes (B) (t=0: bold line, t=24h: filled grey and isotype control: thin line) and granulocytes (D) (t=0: bold line, t=2h: filled grey and isotype control: thin line) are shown. Mean±SE of n=8 per time-point, * indicates $p<0.05$ vs. t=0; p-value of ANOVA is depicted in Fig.

Granulocytes displayed a different TREM-1 pattern, reflected by an immediate decrease in receptor expression that returned to baseline-values after 24h (MF 63±4.9 at t=0, 32.9±2.8 at t=2h, 49.7±2.7 at t=24h; Fig. 1B; $p<0.0001$). In addition, we measured sTREM-1

concentrations and identified a rapid increase in sTREM-1 that reached peak-levels 6h after LPS administration (Fig. 2, p=0.002).

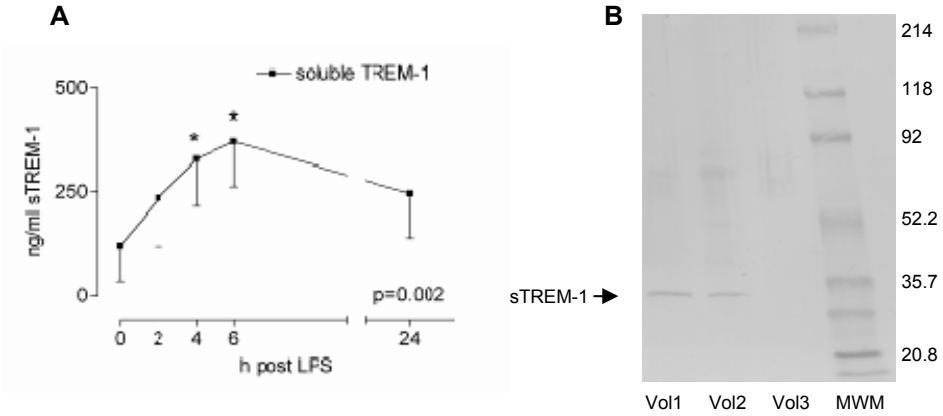

Fig. 2: Early increase in sTREM-1 levels *in vivo.* At indicated time-points soluble TREM-1 levels were measured in plasma samples from volunteers that received an intravenous injection of LPS (4ng/kg) (A). Data are mean±SE of n=8 per time-point, * indicates $p<0.05$ vs. t=0; p-value of ANOVA is depicted in Fig. A representative sTREM-1 blot of three volunteers ("vol") at t=2h is shown in B.

The latter finding adds to recent reports that demonstrated increased bronchoalveolar sTREM-1 levels in patients with pneumonia (9) and elevated sTREM-1 plasma concentrations in patients at risk for sepsis (10). Since administration of recombinant TREM-1 consisting of the extracellular domain effectively blocked septic shock (5), sTREM-1 might represent a negative feedback regulator of the inflammatory response during endotoxemia. Although evidence exists that sTREM-1 is a splice variant of TREM-1, the precise cellular source of sTREM-1 has not been clarified (4, 15). Since endotoxemia leads to an immediate drop in the number of circulating granulocytes (8) due to sequestration to organs such as the lungs (16), the possibility exists that primarily TREM-1^{high} cells are sequestered whereas TREM-1^{low} cells are released from the bone marrow, raising the impression of down-regulated TREM-1 expression on circulating granulocytes. To investigate this possibility, we stimulated whole blood with increasing concentrations of LPS *in vitro* and assessed changes in TREM-1 expression independent of potential sequestration. Indeed, irrespective of the LPS dose and incubation time, TREM-1 was not downregulated *in vitro* (data not shown). This observation fits well to the finding of maturation stage-specific TREM-1 expression of myeloid cells (4).
TREM-1 surface expression on monocytes was also increased after stimulation with LPS *in vitro*. We extended these findings and demonstrate that this LPS-triggered up-regulation occurs in a dose- and time-dependent manner with a maximum expression after 24h ($p<0.001$ for LPS 100 or 1 ng/ml vs. medium, n.s. for LPS 0.01ng/ml; Fig. 3A) (MF: t=0: 132.7±20.4; t=48h: 614.4±79.7 medium, 1340.3±108.4 LPS100ng/ml, 1048.2±99.1 LPS1ng/ml, 720.5±84.3 LPS0.01ng/ml). Comparable results could be obtained after stimulation with the TLR2 ligand LTA ($p<0.001$ vs. medium) (MF: t=48h: 933.9±98.6 LTA10µg/ml) and whole bacteria such as *S. pneumoniae* (p=0.001 vs. medium; Fig. 3B) (MF: t=48h: 939.4±81.9) and

flagellin (data not shown). As expected, the non-bacterial ligands poly dI-dC and Imidazoquinoline as well as CpG ODN did not alter TREM-1 expression on monocytes (Fig. 3B). This can be explained by the fact that TLR3, 7 and 9 are predominantly expressed on dendritic cells and B-cells (17, 18). The observation that TREM-1 is upregulated on monocytes following stimulation with bacterial ligands fits well with the fact that monocytes are a major source of proinflammatory mediators like TNF-α that are released during endotoxemia. The gradual but transient increase in TREM-1 expression could in turn explain why neutralization of TREM-1 in mice proved still protective when administered 4h after LPS administration (5).

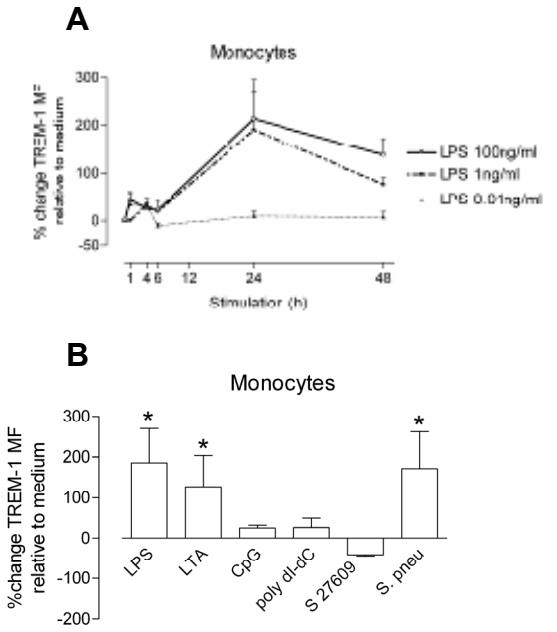

Fig. 3 Dynamic of TREM-1 surface expression *in vitro*. Whole blood stimulation was performed for indicated timepoints. Time- and dose-dependent TREM-1 upregulation on monocytes in response to 100, 1 or 0.01 ng/ml LPS (A). Dynamic of TREM-1 surface expression on monocytes incubated with indicated TLR-ligands for 24h: LPS (100ng/ml), LTA (10µg/ml), *S. pneumoniae* (10^8CFU/ml), CpG (10µM), Poly dI-dC (50µg/ml), Imidazoquinoline S-27609 (0.5µg/ml) (B). Data are mean±SE of n=6, differences were calculated with ANOVA, * indicates $p<0.05$ vs. medium, # indicates $p<0.05$ vs. LPS.

Since the interaction with TREM-1 augments the release of proinflammatory mediators, we asked whether TREM-1 upregulation following LPS challenge is mediated by TNF-α. However, stimulating whole blood in the presence of rhTNF-α did not significantly increase TREM-1 expression on monocytes, nor did the addition of anti-TNF-α alter the LPS-induced enhancement of receptor expression (Fig. 4). In addition, the anti-inflammatory mediator IL-10 did not affect TREM-1 expression levels (Fig. 4).

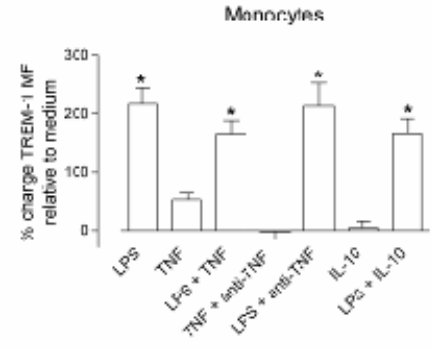

Fig. 4: TREM-1 surface expression is independent of TNF-α or IL-10. Whole blood stimulation was performed for 24h and TREM-1 upregulation was assessed on monocytes in the presence of LPS (100ng/ml) ± TNF-α (20ng/ml), anti-TNF-α (25µg/ml) or IL-10 (10ng/ml). Monocytes were defined as $CD14^{++}$ cells. Data are mean±SE of n=6, differences were calculated with ANOVA, * indicates $p<0.05$ vs. medium.

This finding is somewhat in contrast to an earlier report by Bleharski *et al.* who showed a modest influence of TNF-α and IL-10 that might be explained by the fact that we stimulated whole blood whereas they used primary monocytes (6).
TLR-signaling involves the activation of PI3K and MAP kinases that influence LPS-induced inflammatory responses (19-21). We therefore investigated their role in LPS-mediated TREM-1 upregulation with the use of synthetic inhibitors. Fig. 5 illustrates that PI3K-inhibition (LY294002) completely abrogated the LPS-mediated TREM-1 upregulation, while both MAPK inhibitors SB203580 (p38MAPK) and PD98059 (ERK) had a modest effect on TREM-1 expression levels. Reports on the role of PI3K in LPS-induced inflammatory responses are conflicting; whereas some authors report that LPS induces cytokine production via the activation of PI3K in RAW macrophages (22) other groups have reported that LPS-induced cytokine production in THP-1 cells is negatively regulated by PI3K (20). However, we have found a positive role for PI3K in LPS-induced TREM-1 expression in isolated monocytes. It is currently not known why p38 MAPK and p42/p44 MAPK inhibitors only partially inhibit LPS-induced TREM-1 upregulation, but a certain degree of redundancy may exist between these two kinases.

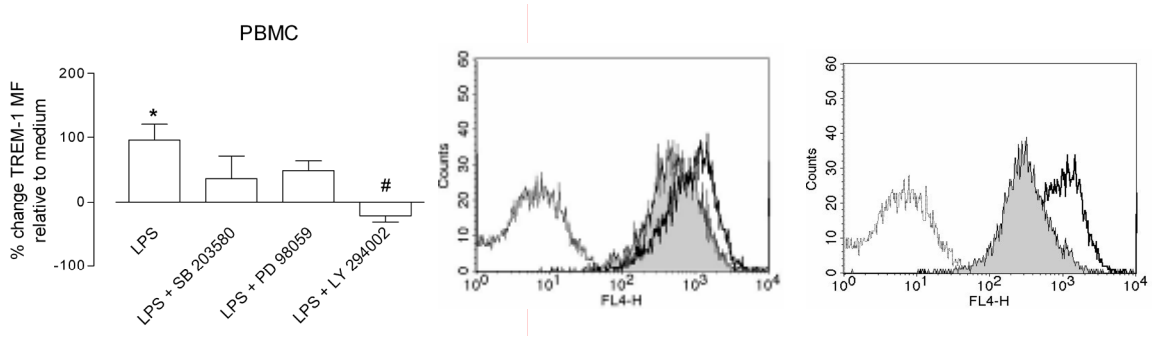

Fig. 5: PI3K-inhibition abrogates LPS-induced TREM-1 upregulation. Influence of SB203580, PD98059 or LY294002 on LPS-induced TREM-1 upregulation on PBMCs after 16h. Monocytes were defined as $CD14^{++}HLA\text{-}DR^{++}$. Data shown represent results of two independent experiments. Mean±SE of n=5, differences were calculated with ANOVA, * indicates $p<0.05$ vs. medium, # indicates $p<0.05$ vs. LPS in the absence of inhibitor. Representative histograms illustrate the influence of LPS stimulation (bold line) as compared to medium (grey filled) (B) and the impact of LY294002 (grey filled) on LPS (bold line) induced TREM-1 expression (C).

In conclusion, TREM-1 is upregulated on monocytes during human endotoxemia together with an increase in sTREM-1. Receptor upregulation is confined to bacterial molecular patterns but not a result of TNF-α production and involves a PI3K-dependent mechanism.

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CHAPTER 19

Summary and general discussion

Summary

Bacterial infections are a major cause of morbidity and mortality worldwide. The innate immune system is regarded the first line of defense against bacterial infections. **Chapter 1** is a general introduction discussing the clinical relevance of bacterial infections and the most frequent causative pathogens that have been investigated in this thesis.

In the **first part** of this thesis we examined specific host defense factors during respiratory tract infections. **Chapter 2** gives an introduction to murine pneumonia models and an overview of the innate immune response during respiratory tract infections. This article highlights the role of specific cytokines and host cells during lung inflammation. In addition, pneumonia models induced by various pathogens, such as *Streptococcus* (*S.*) *pneumoniae, Pseudomonas (P.) aeruginosa, Klebsiella (K.) pneumoniae* or *Acinetobacter (A.) baumannii*, are described. This chapter also stresses the observation that the requirement for specific host defense mediators differs during pulmonary infections caused by different pathogens. As an example, while TNF-α improves the outcome of *S. pneumoniae* pneumonia, this mediator does not contribute to host defense against *P. aeruginosa*. Together these observations underline the importance and need of further studies to gain closer insights into host defense pathways during respiratory tract infections.

Alveolar macrophages (AM) are resident phagocytes within the pulmonary compartment that are considered the first cells to encounter inhaled bacteria and to initiate the inflammatory response aimed at the effective elimination of bacteria. In **chapter 3** we investigated the *in vivo* role of AM during pneumococcal pneumonia. Using a technique to deplete lungs of AM we discovered that the initiation of the inflammatory response was not altered despite the lack of these cells. However, mortality was higher in AM-depleted mice, which could not be attributed to impaired bacterial clearance. We then disclosed AM to represent key effector-cells in the resolution process by virtue of their capacity to phagocytose apoptotic neutrophils. We found tremendously increased numbers of apoptotic neutrophils and prolonged inflammation in mice lacking AM, which in turn resulted in impaired survival. Thus, AM importantly contribute to the resolution of pneumococcal pneumonia *in vivo*.

Host defense against respiratory pathogens involves the coordinated interaction of cells such as macrophages, neutrophils, epithelial and endothelial cells. To allow for a coordinated immune response, these cells secrete mediators that in turn instruct and assist other cells to perform their task. Granulocyte colony-stimulating-factor (G-CSF) is one of the mediators released by macrophages upon activation by bacteria. **Chapter 4** discusses the role of endogenous G-CSF during pneumococcal pneumonia. G-CSF is known to exert diverse effects such as enhancement of phagocytosis by neutrophils and down-regulation of pro-inflammatory cytokines. This attractive profile led to the initiation of clinical studies that studied the effectiveness of G-CSF during pneumococcal pneumonia. However, the role of endogenous G-CSF during *S. pneumoniae* pneumonia *in vivo* had never been investigated. We first verified the local, pulmonary synthesis of G-CSF during pneumonia in mice and humans. Using neutralizing anti-G-CSF antibodies we found that endogenous G-CSF contributes to the

attraction and activation of neutrophils as well as the downregulation of pulmonary TNF-α, KC and IL-1β in mice *in vivo*. However, blocking endogenous G-CSF had no impact on bacterial outgrowth or survival. Thus, pulmonary, endogenous G-CSF exerts both pro- and anti-inflammatory properties during *S. pneumoniae* pneumonia but does not contribute to survival *in vivo*.

IL-18 is a pro-inflammatory cytokine produced by monocytes, macrophages and dendritic cells in response to various stimuli. Just like IL-1, IL-18 is produced as a pro-from that needs further cleavage by interleukin-1 converting enzyme (ICE, caspase-1) to become functionally active. In models of *S. pneumoniae* pneumonia, both IL-1 and IL-18 proved to importantly contribute to inflammation and bacterial clearance. However, we knew from earlier studies that the requirement for distinct pro-inflammatory mediators differs in models of *S. pneumoniae* versus nosocomial pneumonia. In an attempt to understand the role of IL-18 and caspase-1, respectively, during nosocomial pneumonia we therefore studied the role of these factors in models of *P. aeruginosa* (**Chapter 5**) and *A. baumannii* (**Chapter 6**) infection in mice. Using IL-18$^{-/-}$ and wild type mice we noticed reduced bacterial outgrowth and dissemination in IL-18 gene-deficient mice infected with *P. aeruginosa*. This finding was associated with decreased pulmonary inflammation and cytokine concentrations in IL-18$^{-/-}$ animals *in vivo*. Moreover, treatment of wild type mice with a neutralizing IL-18-binding protein confirmed these observations. Hence, IL-18 promotes inflammation and bacterial outgrowth during *P. aeruginosa* pneumonia in mice. *A. baumannii* is a Gram-negative pathogen that is, similar to *P. aeruginosa*, increasingly encountered during nosocomial pneumonias in humans. With the help of caspase-1$^{-/-}$ and wild type mice, we observed an enhanced bacterial outgrowth in wild type animals. In addition, caspase-1$^{-/-}$ mice displayed reduced lung concentrations of TNF-α, IL-1β, IL-6 as well as KC and MIP-2 early after infection with *A. baumannii*. Concomitantly decreased pulmonary inflammation was discovered by histological examination of infected lungs. The administration of a selective caspase-1 inhibitor to wild type mice confirmed these observations *in vivo*. Hence, caspase-1 importantly contributes to pulmonary inflammation during *A. baumannii* pneumonia in mice.

Nosocomial pneumonia is almost invariably preceded by critical illness. One major insult occurring on intensive care units is the aspiration of gastric contents. The impact of acid aspiration on the development of nosocomial pneumonia is poorly understood. We therefore developed a “two-hit” model of acid aspiration followed by infection with *K. pneumoniae*; the results are described in **Chapter 7**. Preceding aspiration pneumonitis followed by *Klebsiella* infection was associated with increased pulmonary cytokine/chemokine production and cell influx together with an increased outgrowth of bacteria. The same phenotype was observed when mice were treated with *Klebsiella* LPS after acid aspiration. *Ex vivo* stimulations of AM from mice that underwent acid aspiration disclosed enhanced TNF-α release as compared to cells of sham mice. Treatment with anti-TNF-α led to increased chemokine concentrations and bacterial outgrowth in lungs of *Klebsiella*-infected sham mice but did not alter chemokine concentrations or bacterial counts in the acid aspiration groups. Together, acid aspiration

primed the host for an exaggerated inflammatory response to secondary *Klebsiella* infection that was associated with increased bacterial outgrowth.

The **second part** of this thesis focuses on the role of pattern recognition receptors during bacterial infections. The recent discovery of Toll-like receptors (TLR) disclosed an important family of pattern recognition receptors that revolutionized our understanding of the innate immune response to pathogens. **Chapter 8** provides an introduction to this part of the thesis and discusses the importance of CD14, LPS-binding protein (LBP) and TLRs. This chapter gives insight into the broad role of these receptors during infectious diseases. LBP is an acute phase protein that transfers LPS of Gram-negative bacteria to the cellular LPS-recognition complex consisting of CD14/TLR4/MD-2. Thereby, LBP is known to enhance the inflammatory response to LPS and as a result, $LBP^{-/-}$ mice are resistant to LPS-induced lethality. However, the precise *in vivo* role during Gram-negative infections with viable bacteria was less well understood and we therefore studied the biological function of LBP during *Escherichia (E.) coli* peritonitis in mice. **Chapter 9** presents the results of this study. Using $LBP^{-/-}$ and wild-type mice we were able to show that $LBP^{-/-}$ animals succumbed quickly following infection with *E. coli*. This phenotype was explained by an impaired chemokine production and consecutively reduced neutrophil recruitment to the site of infection that in turn led to accelerated bacterial dissemination and outgrowth. Together, LBP crucially contributes to the initiation of a protective immune response against *E. coli*. In line with this finding we then studied the *in vivo* role of oxidized phospholipids (OxPL) during *E. coli* peritonitis. Based on previously published reports we knew that OxPL exert anti-inflammatory effects due to their capacity to inhibit the interaction of LPS with LBP and/or CD14. Data presented in **chapter 10** reveal our findings obtained from *in vivo* studies in wild-type and $CD14^{-/-}$ mice infected intraperitoneally with *E. coli* and treated with OxPL at the onset of infection. We disclosed that CD14 gene-deficient mice were more susceptible to *E.coli* peritonitis, illustrated by increased bacterial outgrowth and impaired survival when compared to wild type mice. The administration of OxPL transformed wild type mice into a $CD14^{-/-}$ phenotype, whereas no additional effect was observed in $CD14^{-/-}$ animals treated with OxPL. However, the underlying mechanisms differed between OxPL treated wild type mice and $CD14^{-/-}$ animals. While the absence of CD14 prevented the initiation of a protective immune response, the early cytokine/chemokine response and resulting influx of neutrophils was unaltered in OxPL treated mice as compared to controls. We revealed that OxPL inhibited the phagocytosis of *E. coli* by professional phagocytes, which in turn explained increased bacterial counts and early death.

LBP, besides its important role as an acute phase protein, has been demonstrated to be secreted by respiratory epithelial cells. However, the precise *in vivo* role of LBP during lung inflammation was poorly understood. We therefore investigated the function of pulmonary LBP during LPS-induced lung inflammation (**chapter 11)**. After having established the presence and LPS-dose-dependent increase of LBP within bronchoalveolar lavage fluid, we examined the biological role of LBP using gene-deficient mice. Much to our surprise, we

discovered that pulmonary LBP, in contrast to its reported properties during systemic inflammation, inhibited the inflammatory response to medium to high LPS doses within the respiratory tract *in vivo*. While lung inflammation induced by low-dose (1ng) LPS was augmented in the presence of LBP, the cytokine/chemokine response and neutrophil influx were greatly reduced in wild type mice inoculated with LPS doses above 10ng as compared to $LBP^{-/-}$ mice. The exogenous administration of LBP to $LBP^{-/-}$ mice reversed this phenotype and prevented overwhelming inflammation, thus confirming the specificity of this finding.

While LPS is the major immunogenic determinant of Gram-negative bacteria, lipoteichoic acid (LTA) is considered one of the most important immunogenic molecules expressed by Gram-positive pathogens. From (predominantly) *in vitro* studies it was known that TLR2 is the receptor signaling the presence of LTA. Moreover, LBP and CD14 have been considered co-receptors for LTA-signaling *in vitro*. Because information regarding the *in vivo* role of these recognition receptors was very limited, we decided to study the role of LBP, CD14, TLR2 and 4 as well as the platelet activating factor receptor (PAF-R) in response to LTA *in vivo* (**chapter 12**). Using LBP, CD14, TLR2, TLR4 and PAF-R gene-deficient mice and highly purified LTA (from *S. aureus*) we investigated the specific role of each molecule during LTA-induced lung inflammation *in vivo*. We thereby disclosed that LBP does not contribute to the inflammatory response, and that CD14 plays an only minor role. As expected, TLR2 turned out to be the main receptor and in the absence of TLR2 the LTA-induced pulmonary inflammation was almost completely abrogated. However, rather surprising, we revealed an important role for TLR4 and PAF-R as illustrated by greatly reduced cytokine/chemokine response and decreased cell-influx in $TLR4^{-/-}$ and $PAF\text{-}R^{-/-}$ animals. Together, while TLR2 is the major LTA-receptor, TLR4 and/or PAF-R importantly contribute to pulmonary inflammation induced by LTA. Whether LTA is a direct ligand for TLR4 and/or PAF-R or rather induces endogenous TLR4 and/or PAF-R ligands that synergistically promote inflammation remains to be determined.

The next set of papers investigated the role of pattern recognition receptors during *S. pneumoniae* pneumonia. In **chapter 13** we explored the function of CD14 during pneumococcal pneumonia. CD14 has been suggested to contribute to the inflammatory response induced by Gram-positive pathogens. We therefore used wild type and $CD14^{-/-}$ animals and infected them intranasally with *S. pneumoniae*. Rather unexpected we discovered that $CD14^{-/-}$ mice were protected against lethal infection as illustrated by improved survival rates. Further experiments revealed that $CD14^{-/-}$ mice had lower bacterial loads in their lungs and – even more pronounced – almost no detectable bacteria in blood at a time when almost all wild type mice were bacteremic. In addition, both cytokine/chemokine release and influx of neutrophils was decreased in $CD14^{-/-}$ compared to wild type mice. To study whether the effects of soluble (s) CD14 could explain our findings, we first established the presence and increase of sCD14 within the alveolar compartment during pneumococcal pneumonia. Next, we demonstrated that the intrapulmonary administration of sCD14 resulted in invasive infection of $CD14^{-/-}$ mice and concomitantly increased lethality. Together, CD14 renders the host more susceptible to invasive pneumococcal pneumonia. In **chapter 14** we studied the

role of TLR2 during pneumococcal pneumonia. TLR2 has been regarded *the* recognition receptor signaling the presence of Gram-positive bacteria due to the fact that major Gram-positive molecules such as peptidoglycan, LTA or lipopeptides are ligands for TLR2. The biological role of TLR2 during pulmonary tract infections was not known at this point. Using $TL2^{-/-}$ and wild type mice we found that TLR2 had no major impact on mortality during *S. pneumoniae* pneumonia. Irrespective of the size of the inoculum, we were not able to observe altered bacterial outgrowth or survival rates. However, significantly lower chemokine concentrations were measured in lungs of $TLR2^{-/-}$ mice and histological scoring of pulmonary inflammation revealed less pronounced infiltrates in these mice. Hence, TLR2 does not contribute to antibacterial defense or survival but plays a role in the attraction of neutrophils during pneumococcal pneumonia. Arguing that other TLR ligands might be of importance during *S. pneumoniae* pneumonia, we also studied the role of TLR4. **Chapter 15** presents results from experiments that investigated the role of TLR4 during pneumococcal and *Klebsiella* pneumonia in mice. Low dose infections with *S. pneumoniae* rendered C3H/HeJ mice, which display a mutant non-functional TLR4, more susceptible to pneumonia as illustrated by increased mortality rates and higher bacterial loads within their lungs. Infections with higher doses of *S. pneumoniae* did not result in altered outcome. A more impressive phenotype was observed when C3H/HeJ mice were infected with the Gram-negative pathogen *K. pneumoniae*. Significantly higher mortality rates and bacterial outgrowth were found in TLR4-mutant mice irrespective of the dose mice were infected with. Together, TLR4 contributes to a protective immune response during *S. pneumoniae* pneumonia and even more so during *Klebsiella* pneumonia in mice.

In **chapter 16** we studied the role of CD14, TLR2 and TLR4 during *A. baumannii* pneumonia. *A. baumannii* is an emerging pathogen that causes nosocomial pneumonias. Due to the unavailability of proper models, *in vivo* host defense pathways against this pathogen had not been investigated thus far. We therefore started to set up an appropriate mouse model of *A. baumannii* pneumonia. Using CD14, TLR 2 and 4 gene-deficient mice we were able to demonstrate that both CD14 and TLR4 contribute to a protective immune response against *Acinetobacter*. Rather surprisingly, the absence of TLR2 was associated with an accelerated bacterial clearance. Moreover, an accelerated cell-influx and chemokine response was observed in $TLR2^{-/-}$ mice. Additional experiments disclosed that CD14 and TLR4 recognize the LPS from *A. baumannii.*. A similar approach was undertaken in the next chapter. **Chapter 17** describes results obtained from *in vivo* studies that investigated the importance of pattern recognition receptors during non-mannose-capped lipoarabinomannan (Ara-LAM) induced pulmonary inflammation. Ara-LAM is part of the cell membrane of atypical mycobacteria and it was our aim to elucidate host defense pathways against this molecule within the lungs. Our experiments disclosed that LBP, CD14 and TLR4 are of minor importance, whereas TLR2 importantly contributes to lung inflammation induced by Ara-LAM. Moreover, $TLR2^{-/-}$ mice displayed a delayed clearance of the Ara-LAM containing atypical *Mycobacterium smegmatis*. Hence, these data illustrate that TLR2 is the signaling receptor for Ara-LAM within the lungs *in vivo*.

The last chapter of this thesis discusses a receptor that is closely linked to effects exerted by TLRs. In **Chapter 18** we examined the regulation of surface expression of triggering receptor expressed on myeloid cells (TREM)-1 during human endotoxemia. TREM-1 is a receptor expressed on myeloid cells whose involvement synergistically enhances the inflammatory response elicited by TLR-ligands. TREM-1 up-regulation has been proposed to be selectively associated with bacterial and fungal infections. Moreover, soluble TREM-1 has been suggested as a valuable diagnostic marker in body fluids of patients suffering from bacterial/fungal infections. We studied the time-course of TREM-1 expression on neutrophils and monocytes during endotoxemia and revealed a gradual receptor up-regulation on monocytes and a fast down-regulation on neutrophils. Concomitantly, soluble TREM-1 plasma concentrations rose in response to LPS. We furthermore showed that TREM-1 up-regulation on monocytes can be reproduced *in vitro* and that this effect was also observed after stimulation with TLR ligands such as LTA (TLR2) or whole bacteria (*S. pneumoniae*). However, non-bacterial TLR ligands did not affect receptor expression. Using cytokines, antibodies and pharmacologic inhibitors we also demonstrated that TREM-1 up-regulation was independent of TNF-α, but involved PI3K pathways.

General discussion

The innate immune response to bacterial pathogens is the crucial first line of defense. While much has been learned over the last years, the precise defense mechanisms against specific pathogens are still not entirely understood. With the experimental studies described in this thesis, we intended to get further insights into distinct host defense mechanisms during clinically important infections. We believe that this knowledge will help us one day to optimize therapies targeted against specific pathogens.

In the first part of this thesis we focused on respiratory tract infections caused by diverse pathogens such as *S. pneumoniae*, *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*, Our findings clearly add to the notion that host defense pathways against different pathogens, although all of them cause pneumonia, rely on distinct mechanisms. While others showed that AM are important phagocytes that eliminate bacteria such as *P. aeruginosa*, we instead found that these cells predominantly contribute to the clearance of inflammation during pneumococcal pneumonia. Likewise, while others showed that IL-18 or IL-1 help to prevent bacterial outgrowth during *S. pneumoniae* pneumonia, we here describe that bacterial clearance is accelerated in the absence of IL-18 during *Pseudomonas* pneumonia or in the absence of caspase-1 during *Acinetobacter* pneumonia. These examples underline the observation that respiratory pathogens may cause the same disease, i.e. pneumonia, but underlying pathways differ substantially. While this statement seems obvious at first, it is still quite surprising how sophisticated pathogens and host structures behave by requiring almost opposite host defense mechanisms. One explanation can be deducted from clinical observations. While pneumococcal pneumonia predominantly occurs in otherwise healthy

individuals, *Pseudomonas* or *Acinetobacter* pneumonia are found in hospitalized patients that suffer from underlying diseases. Any systemic inflammation, such as induced by peritonitis, influences immune surveillance mechanism in the lung. In other words, nosocomial pathogens such as *P. aeruginosa* or *A. baumannii* encounter host-structures that are altered when compared to lung defense mechanisms in the previously healthy host. It seems reasonable that the altered pulmonary immune status together with ongoing antibiotic therapy facilitate the invasiveness of multi-drug resistant bacteria such as *Pseudomonas* and *Acinetobacter*. We hereby provide some mechanistic explanations for the clinical observation of nosocomial pneumonia. Furthermore, to better imitate the clinical situation we developed a two-hit model where bacterial infection was preceded by acid aspiration, a frequent problem among intensive care patients. We thereby confirmed our hypothesis that prior damage and/or inflammation primes the host for subsequent bacterial pneumonia.

While *in vitro* experiments offer valuable tools to discover specific biological functions and pathways, the final proof of these findings requires *in vivo* reproducibility. We encountered some unexpected findings when investigating mechanisms that we believed to be thoroughly explored by *in vitro* experiments. For instance, LBP has been shown to augment the inflammatory response to LPS in numerous experiments over the years. However, when we studied $LBP^{-/-}$ mice that were inoculated intranasally with LPS, we observed the rather surprising finding that LBP inhibits LPS-induced pulmonary inflammation. This discovery is definitely associated with the localization of inflammation, i.e. the pulmonary compartment, but nevertheless quite unanticipated given the numerous reports from AM stimulations by LPS that report increased TNF-α release in the presence of LBP. Since studies that investigated the role of LBP during systemic inflammation (including our experiments during *E. coli* peritonitis) confirmed the pro-inflammatory properties of LBP, evidently, localized pulmonary factors help neutralize LPS in a LBP-dependent manner.

We also encountered rather unexpected findings by studying the *in vivo* role of CD14 and Toll-like receptors during bacterial infections. While TLR2 has been suggested as the prime pattern recognition receptor for Gram-positive pathogens, we found TLR2 to mainly contribute to the chemokine response and cell-influx but not bacterial clearance during *S. pneumoniae* pneumonia. At the same time, TLR4 proved to be involved in the control of bacterial outgrowth and survival during this Gram-positive infection. Apparently, the complex interplay between different pattern recognition receptors finally leads to the optimal host defense that is required to survive bacterial infections. On the other hand, CD14, which is mostly considered a co-receptor for some TLRs and thus involved in protective host defense mechanisms, seems to exert a variety of alternative functions. While CD14 (as well as TLR4) positively influences bacterial clearance in a model of Gram-negative pneumonia (*Acinetobacter*), its role during pneumococcal pneumonia is converse. CD14 contributes to invasiveness of *S. pneumoniae* within the pulmonary compartment and thus negatively impacts outcome during pneumonia.

These data underline the importance of *in vivo* studies, since most of these observations, and almost all of our unexpected findings, would not be known without the use of animal models.

However, despite genetic similarities between rodents and humans, we should be cautious in extrapolating results to the human situation. Numerous examples, such as those aimed at developing treatments for sepsis, underline these limitations. However, we should not forget that exactly these ‘failures’ taught us to be more specific in defining sepsis in general. In former days sepsis models were predominately based on intravenous injections of bacteria, whereas presently it becomes clear that localized infections eventually disseminate and cause sepsis, which immensely grows closer to imitating the clinical situation and might therefore be more appropriate. We consider it reasonable to regard the models investigated in this thesis as the ‘next generation’ of infectious disease and sepsis models, because all of them attempt to mimic clinical reality. We certainly hope that results obtained by the above means may indeed contribute to the development of new treatment strategies against infectious diseases.

Furthermore, while the use of knock-out mice is an elegant approach to study the function of specific molecules, we must keep in mind that compensatory mechanism exerted by genetically modified mice may play a varying role. Newer approaches like siRNA or the generation of inducible knockout mice may help us to minimize some of these factors.

In closing, although the road seems endless and its conduits numerous, our quest to find pathways and mechanisms for future therapies of infections leaves us with a daunting yet very exciting task.

Samenvatting en algemene discussie

Samenvatting

Bacteriële infecties zijn een belangrijke oorzaak van morbiditeit en mortaliteit. De aangeboren afweer wordt beschouwd als de eerste verdedigingslinie tegen bacteriële infecties. **Hoofdstuk 1** is een algemene introductie waarin de klinische relevantie van bacteriële infecties besproken wordt, alsmede de meest frequente pathogenen die deze infecties veroorzaken.

In het **eerste deel** van dit proefschrift onderzochten wij specifieke afweer mechanismen tijdens luchtweg infecties. **Hoofdstuk 2** geeft een introductie van muis modellen van pneumonie en een overzicht van de aangeboren afweer bij luchtweg infecties. Dit artikel stipt de rol van cytokinen en afweer cellen aan bij de ontstekingsreactie in de long tijdens pneumonie. Bovendien worden muis modellen met verschillende respiratoire pathogenen besproken, zoals modellen met *Streptococcus (S.) pneumoniae, Pseudomonas (P.) aeruginosa, Klebsiella (K.) pneumoniae* en *Acinetobacter (A.) baumannii.* Dit hoofdstuk benadrukt dat verschillende afweer mechanismen van belang zijn tijdens luchtweg infecties door verschillende bacteriën. Zo speelt TNF-α een belangrijke rol bij de afweer tegen *S. pneumoniae* pneumonie, maar niet bij luchtweginfecties door *P. aeruginosa*. Deze observaties tonen aan dat er behoefte is aan meer studies naar de afweer tegen pneumonie.

Alveolaire macrofagen (AM) bevinden zich in alveoli van de "rustende" long, waar ze dienen voor vroege detectie van geïnhaleerde bacteriën. In **hoofdstuk 3** onderzochten wij de rol van AM bij pneumococcen pneumonie. Gebruik makend van een methode om AM te depleteren ontdekten wij dat de initiatie van de ontstekingsreactie niet veranderde in afwezigheid van deze cellen. Toch namen wij een verhoogde sterfte waar bij muizen waarbij AM gedepleteerd waren, hetgeen niet veroorzaakt werd door een verminderde klaring van bacteriën. Ons onderzoek toonde aan dat AM essentieel zijn voor de resolutie van de ontstekingsreactie, vooral doordat ze apoptotische neutrofiele granulocyten fagocyteren. Het gevolg van AM depletie was een sterke ophoping van apoptotische neutrofiele granulocyten en een verlengde ontstekingsreactie in de long, hetgeen resulteerde in een verminderde overleving. De conclusie van dit werk was dan ook dat AM essentieel zijn voor de resolutie van pneumococcen pneumonie.

De afweer tegen respiratoire infecties vindt plaats door een gecoördineerde interactie tussen verschillende celtypes, waaronder macrofagen, neutrofiele granulocyten, epitheelcellen en endotheelcellen. Teneinde de immuunreactie gecoördineerd te laten verlopen, scheiden deze cellen mediatoren uit die andere cellen instrueren hun specifieke taken naar behoren uit te voeren. Granulocyte colony-stimulating factor (G-CSF) is zo´n mediator, die na activatie van macrofagen door bacteriën geproduceerd wordt. **Hoofdstuk 4** behandelt de rol van endogeen G-CSF bij pneumococcen pneumonie. G-CSF heeft een aantal eigenschappen die de afweer tegen pneumonie kunnen beïnvloeden, zoals versterking van de fagocytose door neutrofiele granulocyten en remmen van de productie van proinflammatoire cytokinen. Dit aantrekkelijke profiel leidde tot klinische studies waarin de effectiviteit van G-CSF bij pneumonie geëvalueerd werd. De rol van endogeen G-CSF was echter nog nimmer onderzocht. Wij stelden vast dat G-CSF locaal in de longen gesynthetiseerd wordt tijdens pneumonie bij

muizen en mensen. Behandeling met een neutraliserende anti-G-CSF antistof toonde aan dat endogeen G-CSF bijdraagt aan de migratie en activatie van neutrofiele granulocyten als ook aan de remming van de productie van TNF-α, KC en IL-1β in de longen van muizen met pneumococcen pneumonie. Neutraliseren van endogeen G-CSF had geen effect op de groei van bacteriën of op de overleving. Deze studies toonden aan dat endogeen G-CSF zowel anti- als proinflammatoire effecten heeft tijdens pneumonie, maar dat het niet bijdraagt aan de uiteindelijke uitkomst.

IL-18 is een proinflammatoir cytokine dat geproduceerd wordt door monocyten, macrofagen en dendritische cellen. IL-18 wordt, net als IL-1β, geproduceeerd als een pro-eiwit dat gekliefd moet worden door "IL-1 converting enzyme" (ICE, caspase-1) teneinde functioneel actief te worden. Eerder onderzoek toonde aan dat IL-1 en IL-18 belangrijk zijn voor de afweer tegen pneumococcen pneumonie. Omdat de rol van deze cytokinen in modellen van pneumococcen pneumonie mogelijk anders is dan in modellen van nosocomiale pneumonie, onderzochten wij de bijdrage van IL-18 en caspase-1 aan de weerstand tegen luchtweginfectie veroorzaakt door *P. aeruginosa* (**hoofdstuk 5**) en *A. baumannii* (**hoofdstuk 6**). IL-18 deficiënte muizen bleken een verminderde bacteriële groei te hebben ten opzichte van wild-type muizen tijdens *Pseudomonas* pneumonie, hetgeen geassocieerd was met een mindere ontstekingsreactie en lagere cytokine concentraties in de longen van eerst genoemde dieren. Deze bevindingen werden bevestigd door behandeling van wild-type muizen met een IL-18 bindend eiwit. Blijkbaar bevordert endogeen IL-18 de bacteriële uitgroei en ontsteking van de long tijdens pneumonie veroorzaakt door *P. aeruginosa*. *A. baumannii* is een Gram-negatief micro-organisme dat, net als *P. aeruginosa*, in toenemende mate geïsoleerd wordt als verwekker van nosocomiale pneumonie. Caspase-1 deficiënte muizen bleken in vergelijking met wild-type muizen een verminderde uitgroei van deze bacterie, alsmede lagere cytokine concentraties en minder ontsteking in hun longen te hebben. Deze bevindingen konden gereproduceerd worden door behandeling van wild-type muizen met een selectieve caspase-1 remmer. Deze studies toonden derhalve aan dat caspase-1 een belangrijke regulator is van long inflammatie tijdens *Acinetobacter* pneumonie.

Nosocomiale pneumonie treedt vooral op bij patiënten met een preëxistente ziekte. Een belangrijke voor pneumonie predisponerende aandoening is aspiratie pneumonitis, een steriele ontstekingsreactie van de long ten gevolge van aspiratie van zure maaginhoud. Het mechanisme waardoor aspiratie het optreden van pneumonie bevordert is grotendeels onbekend. Daarom ontwikkelden wij een "two-hit" model waarin aspiratie pneumonitis gevolgd werd door pulmonale infectie met *K. pneumoniae* (**hoofdstuk 7**). Het bestaan van een aspiratie pneumonitis leidde na infectie met *Klebsiella* tot een hogere productie van cytokinen en chemokinen en een verstekte influx van neutrofiele granulocyten; tevens was de bacteriële uitgroei versterkt. Toediening van gezuiverd *Klebsiella* LPS leidde ook tot een versterkte ontstekingsreactie bij preëxistente aanwezigheid van aspiratie pneumonitis. AM geoogst van muizen die zuur geaspireerd hadden produceerden meer TNF-α na "ex vivo" stimulatie met LPS dan gezonde muizen. Toediening van anti-TNF-α leidde in muizen die geen zuur geaspireerd hadden tot een toegenomen uitroei van *Klebsiella* en verhoogde

chemokine concentraties in de longen; dezelfde behandeling had echter in de muizen met aspiratie pneumonitis en secundaire pneumonie geen additioneel effect. Deze resultaten maakten aannemelijk dat preëxistente aspiratie pneumonitis de patient predisponeert voor een overmatige ontstekingsreactie in de long bij secundaire infectie door *Klebsiella,* hetgeen aanleiding geeft tot een sterkere bacteriële groei.

Het **tweede deel** van dit proefschrift richtte zich op de rol van "pattern recognition receptors" tijdens bacteriële infecties. De kennis omtrent het functioneren van de aangeboren afweer is de laatste jaren enorm toegenomen door de ontdekking van Toll-like receptoren (TLR´s), een belangrijke familie van "pattern recognition receptors". **Hoofdstuk 8** introduceert dit deel van het proefschrift en geeft een overzicht van CD14, LPS-binding protein (LBP) en TLR´s en hun rol bij de afweer tegen infecties. LBP is een acuut fase eiwit dat het LPS van Gram-negatieve bacteriën transporteert naar het LPS-receptor complex bestaande uit CD14/TLR4/MD-2. LBP versterkt zo de ontstekingsreactie op LPS, hetgeen ondermeer blijkt uit het feit dat LBP deficiënte muizen resistent zijn tegen LPS-geïnduceerde letaliteit. Omdat de rol van LBP bij infectie met intacte Gram-negatieve bacteriën nauwelijks onderzocht was, bestudeerden wij in **hoofdstuk 9** de biologische functie van LBP tijdens *Escherichia (E.) coli* peritonitis. LBP deficiënte muizen bleken snel te overlijden na intraperitoneale infectie met *E. coli* waarschijnlijk ten gevolge van een verminderde locale chemokine productie en een verlaagde influx van neutrofiele granulocyten naar de buikholte. Dit leidde tot een sterke groei en verspreiding van bacteriën. Blijkbaar speelt LBP een eminente rol bij de presentatie van *E. coli* aan het aangeboren immuun systeem. In het verlengde van deze bevindingen bestudeerden wij de effecten van geoxideerde fosfolipiden (OxPL) bij *E. coli* peritonitis. Eerdere onderzoeken hadden al aangetoond dat OxPL ontsteking inhiberende eigenschappen hebben door hun capaciteit de interactie tussen LPS en LBP/CD14 te remmen. In **hoofdstuk 10** rapporteren wij onze experimenten met wild-type en CD14 deficiënte muizen, die al of niet behandeld werden met OxPL, tijdens *E. coli* peritonitis. CD14 deficiënte muizen bleken een verminderde afweer te hebben tegen *E. coli* peritonitis, zich uitende in een verhoogde bacteriële uitgroei en een verminderde overleving ten opzichte van wild-type muizen. Het toedienen van OxPL aan wild-type muizen deed hen qua fenotype veranderen in CD14 deficiënte muizen, terwijl OxPL in CD14 deficiënte muizen geen additionele effecten had. Opvallend was dat de mechanismen die bijdroegen aan de verminderde afweer ten gevolge het ontbreken van CD14 enerzijds en de behandeling met OxPL anderzijds verschilden: terwijl CD14 deficiëntie de initiatie van de vroege immuun response belemmerde, bleek OxPL de fagocytose van *E. coli* door professionele fagocyten te remmen.
LBP heeft behalve zijn rol als acuut fase eiwit mogelijk ook een rol in de long gezien het feit dat respiratoire epitheelcellen in staat zijn LBP te produceren. Omdat de bijdrage van LBP aan longontsteking grotendeels onbekend was, onderzochten wij in **hoofdstuk 11** de rol van dit eiwit bij pulmonale inflammatie geïnduceerd door locale toediening van LPS. LPS toediening via de neus leidde tot een dosis afhankelijke toename van LBP concentraties in de bronchoalveolaire lavage vloeistof. Verrassenderwijs bleek dat dit "long LBP", in

tegenstelling tot de proinflammatoire eigenschappen tijdens systemische inflammatie, de ontstekingsreactie op gemiddelde en hoge LPS doses in de luchtwegen remde: terwijl LBP deficiënte muizen minder ontsteking genereerden dan wild-type muizen na toediening van een lage LPS dosis (1 ng), produceerden ze veel meer cytokinen en chemokinen en vertoonden ze een versterkte influx van neutrofiele granulocyten na toediening van LPS doses boven de 10 ng. Dit fenotype van LBP deficiënte muizen werd teniet gedaan door suppletie met recombinant LBP, hetgeen de specificiteit van deze bevinding bevestigde.
Terwijl LPS beschouwd wordt als de krachtigste immunologische determinant van Gram-negatieve bacteriën, is lipoteichoïne zuur (LTA) een van de sterkste ontsteking inducerende moleculen in Gram-positieve bacteriën. *In vitro* studies hebben aangetoond dat TLR2 de effecten van LTA mediëert, waarbij LBP en CD14 mogelijk als coreceptoren optreden. De *in vivo* rol van deze receptoren bij LTA geïnduceerde ontsteking was onbekend, hetgeen aanleiding was LTA (van *Staphylococcus aureus*) via de neus toe te dienen aan muizen die deficiënt waren voor LBP, CD14, TLR2, TLR4 of de platelet-activating-factor receptor (PAF-R) (**hoofdstuk 12**). Deze studies toonden aan dat LBP niet betrokken is bij LTA geïnduceerde longontsteking en dat CD14 een beperkte rol speelt. TLR2 bleek de meest belangrijke receptor te zijn gezien het feit dat TLR2 deficiënte muizen geen ontstekingsreactie genereerden onder invloed van LTA. Verrassenderwijs bleken ook TLR4 en PAF-R bij te dragen aan de inflammatie in de long na toediening van LTA: TLR4 en PAF-R deficiënte muizen vertoonden een sterk gereduceerde reactie gekarakteriseerd door lagere concentraties van cytokinen en chemokinen, alsmede een verminderde influx van neutrofiele granulocyten in de long. Deze resultaten suggereren dat TLR2 de voornaamste LTA receptor in de long is, terwijl TLR4 en/of PAF-R een additionele rol vervullen. Nader onderzoek is nodig om vast te stellen of LTA een directe ligand is van TLR4 en/of PAF-R, of dat LTA de productie van endogene liganden voor deze receptoren induceert.
De volgende serie manuscripten onderzocht de rol van "pattern recognition receptors" bij pneumonie veroorzaakt door *S. pneumoniae*. In **hoofdstuk 13** werd de rol van CD14 bij pneumococcen pneumonie geëvalueerd. Eerder onderzoek had gesuggereerd dat CD14 mogelijk een rol speelt bij infecties door Gram-positieve bacteriën. Daarom vergeleken wij de afweer van CD14 deficiënte en wild-type muizen tegen pneumococcen pneumonie. Onverwacht bleken CD14 deficiënte muizen beschermd te zijn tegen deze infectie: ze hadden minder bacteriën in hun longen en – nog indrukwekkender – ze hadden niet of nauwelijks positieve bloedkweken. Bovendien waren de concentraties van cytokinen en chemokinen en de influx van neutrofiele granulocyten naar de longen van CD14 deficiënte muizen verminderd. Teneinde vast te stellen of de effecten van oplosbaar CD14 deze waarnemingen konden verklaren, toonden we eerst aan dat de concentraties van oplosbaar CD14 stegen tijdens pneumococcen pneumonie. Tenslotte toonden we aan dat het toedienen van recombinant oplosbaar CD14 via de neus, CD14 deficiënte muizen net zo gevoelig maakten voor pneumococcen pneumonie als wild-type muizen: ze ontwikkelden nu ook invasieve infectie en vertoonden een toegenomen sterfte. Deze resultaten maakten aannemelijk dat CD14 de gastheer meer gevoelig maakt voor invasieve pneumococcen pneumonie. In

hoofdstuk 14 bestudeerden wij de rol van TLR2 bij *S. pneumoniae* pneumonie. TLR2 wordt algemeen beschouwd als *de* "pattern recognition receptor" die de aanwezigheid van Gram-positieve bacteriën detecteert gezien het feit dat TLR2 belangrijke bestanddelen van dergelijke micro-organismen, zoals LTA en peptidoglycaan, herkent. Omdat het aandeel van TLR2 bij luchtweginfecties onbekend was, vergeleken wij het beloop van pneumococcen pneumonie bij TLR2 deficiënte en wild-type muizen. TLR2 deficiëntie bleek geen belangrijke invloed te hebben op de uitkomst van de infectie: beide groepen muizen hadden een vergelijkbare bacteriële uitgroei en overleving na infectie met verschillende doses van *S. pneumoniae*. Wel bleek dat TLR2 deficiënte muizen lagere chemokine concentraties en minder tekenen van ontsteking in hun longen hadden. Deze gegevens maakten aannemelijk dat TLR2 geen significante rol speelt bij de antibacteriële afweer tijdens pneumococcen pneumonie, maar wel een aandeel heeft in de regulatie van de ontstekingsreactie. Teneinde de rol van andere TLR´s en TLR liganden te onderzoeken, infecteerden wij C3H/HeJ muizen (die een niet functionerend TLR4 tot expressie brengen) en C3H/HeN muizen (met een normaal TLR4) met *S. pneumoniae* of *K. pneumoniae* (**hoofdstuk 15**). C3H/HeJ muizen vertoonden na inoculatie met lage doses *S. pneumoniae* meer bacteriële uitgroei en een hogere mortaliteit dan C3H/HeN muizen; deze verschillen waren niet aanwezig na infectie met hogere doses *S. pneumoniae*. TLR4 bleek een belangrijkere rol te spelen bij Gram-negatieve pneumonie: C3H/HeJ muizen toonden een sterkere uitgroei van *K. pneumoniae* en een hogere mortaliteit na infectie met verschillende bacteriële doses.

In **hoofdstuk 16** bestudeerden wij de rol van CD14, TLR2 en TLR4 bij *A. baumannii* pneumonie. Deze bacterie is een steeds vaker voorkomende verwekker van nosocomiale pneumonie. Door het ontbreken van adequate dier modellen, was de kennis over de afweer tegen deze infectie beperkt. Dit was reden een muis model van *A. baumannii* pneumonie te ontwikkelen. Dit model werd vervolgens gebruikt om de afweer tegen deze luchtweginfectie te vergelijken in normale muizen en muizen met een genetische deficiëntie van CD14, TLR2 of TLR4. CD14 en TLR4 bleken bij te dragen aan een adequate afweer, terwijl verrassenderwijs TLR2 deficiëntie leidde tot een versnelde klaring van *Acinetobacter* uit de longen, hetgeen geassocieerd was met een sterkere chemokine productie en neutrofielen influx. Aanvullende experimenten maakten aannemelijk dat CD14 en TLR4 het LPS van *A. baumannii* herkennen. **Hoofdstuk 17** beschrijft vergelijkbare studies die als doel hadden de rol van "pattern recognition receptors" bij de herkenning van "non-mannose-capped lipoarabinomannan (Ara-LAM), onderdeel van de celwand van atypische mycobacteriën, in de long vast te stellen. Aangetoond werd dat LBP, CD14 en TLR4 niet belangrijk waren, terwijl TLR2 een eminente bijdrage leverde. Bovendien vertoonden TLR2 deficiënte muizen een vertraagde klaring van *Mycobacterium smegmatis*, een atypische mycobacterie die Ara-LAM tot expressie brengt. Deze experimenten toonden aan dat TLR2 de signalerende receptor is voor Ara-LAM in de long.

Het laatste hoofdstuk van dit proefschrift handelt over een receptor die betrokken is bij de effecten van TLR´s. In dit **hoofdstuk 18** onderzochten wij de regulatie van de expressie van "triggering receptor expressed on myeloid cells-1" (TREM-1) tijdens humane endotoxinemie.

TREM-1 is een receptor op myeloide cellen die de response van TLR´s op TLR liganden versterkt. Eerdere onderzoeken suggereerden dat TREM-1 expressie selectief plaats vindt tijdens infecties door bacteriën of schimmels. Bovendien is gesuggereerd dat de concentraties van oplosbaar TREM-1 een waardevolle diagnostische parameter kunnen zijn voor dergelijke infecties. Wij bestudeerden het tijdsbeloop van TREM-1 expressie op neutrofiele granulocyten en monocyten na toediening van LPS aan gezonde vrijwilligers. Terwijl de expressie van TREM-1 op eerst genoemde cellen snel afnam, trad er op laatste genoemde cellen geleidelijk een verhoogde TREM-1 expressie op. Tegelijkertijd was er sprake van een stijging van de plasma concentraties van oplosbaar TREM-1 na LPS injectie. De opregulatie van TREM-1 expressie op monocyten kon in vitro worden gereproduceerd na stimulatie met verschillende TLR liganden zoals LTA of bacteriën (*S. pneumoniae*). Niet-bacteriële TLR liganden beïnvloedden de expressie van TREM-1 niet. Experimenten met cytokinen, antistoffen en farmacologische remmers toonden aan dat de verhoogde TREM-1 expressie onafhankelijk van TNF-α en afhankelijk van PI3K mechanismen verliep.

Algemene discussie

De aangeboren afweer vormt de eerste, cruciale, verdedigingslinie tegen bacteriën. Hoewel onze kennis de laatste jaren sterk is toegenomen, is nog veel onbekend over de exacte mechanismen die ten grondslag liggen aan de afweer tegen specifieke pathogenen. De studies die in dit proefschrift beschreven worden hadden als doel meer inzicht te verschaffen in afweer mechanismen tijdens klinisch belangrijke infecties. Dergelijke kennis kan ons in de toekomst helpen betere en doelgerichtere therapieën te ontwikkelen tegen specifieke ziekte verwekkers.

In het eerste deel van dit proefschrift richtten we ons op luchtweginfecties door diverse pathogenen, zoals *S. pneumoniae*, *P. aeruginosa*, *A. baumannii* en *K. pneumoniae*. Onze bevindingen illustreren eens te meer dat de afweer mechanismen tijdens pneumonie door verschillende respiratoire pathogenen divers zijn. Terwijl anderen hadden aangetoond dat AM belangrijk zijn voor de eliminatie van *P. aeruginosa*, vonden wij dat AM tijdens pneumococcen pneumonie voornamelijk bijdragen aan de resolutie van de ontstekingsreactie. Bovendien toonden wij aan dat IL-18 en caspase-1 deficiëntie leidt tot een versnelde klaring van respectievelijk *P. aeruginosa* en *A. baumannii*, terwijl anderen eerder rapporteerden dat de aanwezigheid van IL-1 en IL-18 de antibacteriële afweer tegen pneumococcen pneumonie juist verbeterden. Hoewel het voor de hand liggend lijkt dat verschillende pathogenen op verschillende wijzen de lagere luchtwegen kunnen infecteren, is het integrerend om vast te stellen dat bescherming tegen pneumonie soms door tegengestelde mechanismen tot stand komt. Een mogelijke verklaring ligt in het feit dat *S. pneumoniae* voornamelijk infecties veroorzaakt in tevoren gezonde mensen, terwijl *Pseudomonas* en *Acinetobacter* vrijwel uitsluitend ziekte veroorzaken in gehospitaliseerde patiënten met preëxistente morbiditeit. Een systemische ontstekingsreactie, zoals optreedt bij peritonitis, leidt tot een verandering in de

immunologische status quo in de long die, in combinatie met het langduring toedienen van breed spectrum antibiotica, het optreden van infecties door nosocomiale multi-drug resistente pathogenen als *P. aeruginosa* en *A. baumannii* bevordert. Het vrijwel altijd bestaan van preëxistente ziekte bracht ons ook tot het ontwikkelen van een zogenaamd "two-hit" model, waarbij de rol van een aspiratie pneumonitis (een frequente complicatie bij patiënten op een Intensive Care) op de gevoeligheid van de gastheer voor *K. pneumoniae* pneumonie onderzocht werd.

Hoewel *in vitro* experimenten zeer waardevol zijn voor de ontdekking van specifieke biologische functies en activatie mechanismen, zijn *in vivo* studies van eminent belang om de relevantie van dergelijke bevindingen te toetsen. In dit proefschrift deden wij enkele verrassende bevindingen in muizen die op grond van beschikbare *in vitro* gegevens onverwacht waren. Zo hebben vele experimenten aangetoond dat LBP de effecten van LPS potentiëert. Wij vonden echter, gebruik makend van LBP deficiënte muizen, dat LBP in de long de effecten van LPS remt. Deze LBP functie is waarschijnlijk orgaan specifiek. Studies waarin de rol van LBP tijdens systemische ontstekingsreacties onderzocht werd toonden een proinflammatoire functie van dit eiwit aan (zo ook onze experimenten met *E. coli* peritonitis). Blijkbaar zijn er in het long compartiment stoffen die LPS neutraliseren op een LBP afhankelijke wijze.

We stuitten ook op verrassende bevindingen toen we de rol van CD14 en TLR´s onderzochten bij bacteriële infecties. Hoewel TLR2 in de literatuur gekenschetst wordt als *de* receptor die de afweer tegen Gram-positieve pathogenen initieert, vonden wij een beperkte rol voor TLR2 bij de reactie van gastheer op pneumococcen pneumonie, terwijl TLR4 wel de groei van *S. pneumoniae* en de sterfte beperkte. CD14, dat een coreceptor is voor sommige TLR´s en waaraan vooral een beschermende rol werd toebedacht tijdens infecties, bleek diverse functies te hebben: terwijl CD14 de klaring van *A. baumannii* bevorderde, bleek deze receptor een belangrijke factor in het ontstaan van invasieve ziekte tijdens *S. pneumoniae* pneumonie. Deze bevindingen benadrukken het belang van *in vivo* studies: de meeste van deze observaties zouden zonder het gebruik van adequate diermodellen onbekend blijven. Hierbij dient wel aangetekend te worden dat voorzichtigheid geboden is bij de extrapolatie van proefdier resultaten naar de situatie in patiënten. Talloze voorbeelden, waaronder de vele mislukte pogingen om nieuwe sepsis therapieën te ontwikkelen, illustreren deze beperking. Dit falen heeft geleid tot een betere definitie van sepsis en het heroverwegen van de waarde van diermodellen van sepsis: terwijl vroeger sepsis modellen veelal gebaseerd waren op het intraveneus toedienen van grote hoeveelheden bacteriën, worden tegenwoordig steeds meer modellen gebruikt van gelokaliseerde infecties die in een later stadium tot disseminatie leiden. Deze laatste modellen hebben meer relevantie voor de klinische praktijk en in dit opzicht draagt dit proefschrift bij aan de validatie van de "nieuwe generatie" van infectieziekten c.q. sepsis modellen. Het is onze hoop dat dergelijke modellen zullen bijdragen aan het ontdekken van effectievere therapieën voor ernstige infectieziekten.

Het gebruik van "knockout" muizen is een elegante manier om de biologische functie van een bepaald eiwit vast te stellen. Wij dienen ons er echter van bewust te zijn dat genetisch

gemodificeerde muizen compensatoire mechanismen ontwikkeld kunnen hebben die de resultaten kunnen beïnvloeden. Nieuwere benaderingen, zoals het toedienen van si RNA of het genereren van induceerbare “knockout” muizen, kunnen behulpzaam zijn.
De weg naar het ontrafelen van afweer mechanismen en het ontwikkelen van nieuwe therapieën bij ernstige infectieziekten schijnt eindeloos. Het is juist daarom uitdagend en opwindend dit pad af te gaan.

Dankwoord

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