Mechanisms of immune activation during infection
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CHAPTER 1

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**Figure 1.** Diagrammatic outline of the theme. Note that the research described in chapter 11 is not present in the figure. This chapter investigates the role of the PAF receptor (and PAF) in the host response to respiratory tract infection induced by non-typeable *Haemophilus influenzae*. This chapter involves both pattern recognition and secreted inflammatory mediator.
Introduction and outline of the thesis

1. Introduction
Infectious diseases are a major cause of morbidity and mortality worldwide (1). Respiratory infections are the most frequent infectious cause of disease followed by infectious diarrhea, malaria and tuberculosis. Improved hygiene and the advent of antibiotics and immunization programs have successfully reduced the morbidity and mortality of many infectious diseases in the twentieth century. However, new infectious pathogens emerge continuously, forming a threat to mankind. Furthermore, the increasing incidence of bacterial resistance against antimicrobial therapy hampers adequate treatment and poses growing difficulties to the medical professional community (2-5). It is therefore mandatory to improve our understanding of the pathogenesis of (myco)bacterial infections and of the mechanisms contributing to the antibacterial host response. The research described in this thesis focuses on the induction and regulation of the inflammatory response to experimentally induced (myco)bacterial infection. For this we used several experimental models and interventions or genetically modified mice. In line with the diagrammatic presentation of the outline of the thesis presented in Figure 1, we will briefly discuss the following topics relevant for this manuscript: (1) induction of an innate inflammatory immune response, (2) experimental models used, and (3) interventions and mouse strains used.

2. Induction of an innate inflammatory immune response

2.1 Pattern recognition and Toll-like receptors
For the induction of a host immune response to invading microorganisms, the recognition of pathogens by cells of the immune system early in infection is of pivotal importance. Microorganisms share several highly conserved molecular structures called pathogen-associated molecular patterns (PAMPs). Examples of PAMPs are endotoxin (lipopolysaccharide, LPS), lipoteichoic acid (LTA), peptidoglycan (PGN) and lipoarabinomannan (LAM). Host immune cells recognize these PAMPs by certain receptors (pattern recognition receptors, PRRs) leading to intracellular signaling and ultimately resulting in activation of the immune system (6). CD14, a glycosylphosphatidylinositol-linked receptor expressed on the surface of phagocytic cells, has been widely accepted as a PRR for a variety of bacterial cell wall components (7-9, 16). However, CD14 does not
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contain a cytoplasmic domain and therefore cannot transduce activating signals across the cell membrane.

The Toll family of receptors, which is conserved throughout evolution from flies to humans, has been implicated to play a central role as PRRs in the initiation of cellular innate immune responses. The Toll receptor family most likely represents the connection between the extracellular compartment, where contact with and recognition of pathogens occurs, and the intracellular compartment, where signaling cascades leading to cellular immune responses are triggered. First discovered in the fruit fly, at present 10 human homologs of *Drosophila* Toll have been identified. This mamalian receptor family has been designated Toll-like receptors or TLRs. TLRs are distinguished from other PRRs by their ability to recognize, and more significantly, discriminate between different classes of pathogens. Ligands for at least 8 mammalian TLRs have been described, among which TLR4. TLR4 has been shown to be expressed by a variety of cells including dendritic cells and macrophages (10). TLR4 has been identified as the signal transducing receptor for endotoxin (11, 12). After binding of LPS to CD14, this complex interacts with TLR4 and the extracellular protein MD-2 resulting in activation of intracellular signaling cascades (13). TLR4 has also been implicated in the recognition of other ligands including LTA from Gram-positive bacteria (14) and a heat-sensitive cell-associated factor expressed by *Mycobacterium tuberculosis* (15).

LPS binding protein (LBP) is an acute phase protein that greatly enhances the presentation of microbial PAMPs to the pattern recognition receptor CD14 on immunocompetent cells, thereby facilitating subsequent signaling via TLR4 (and possibly TLR2). LBP has been described to bind LPS, PGN, LTA and LAM (8, 16-21).

2.2 P38 Mitogen Activated Protein Kinase

A vast range of extracellular signals is transmitted intracellularly via similar signaling pathways. Activation of these intracellular signal transduction pathways leads to gene expression and induction of immune responses. About ten years ago, a family of serine/threonine protein kinases, called Mitogen Activated Protein Kinases (MAPKs), was identified as an indispensable pathway in the induction of cellular responses to external inflammatory signals (22). A series of three protein kinases - a MAPK and two upstream components, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK) - forms the MAPK cascade. Once activated, the MAPKs can phosphorylate and activate other kinases or transcription factors (22). So far, three different MAPK pathways have been described in mammalian cells: the extracellular signal-regulated kinase (ERK) pathway (p42/p44 MAPK),
the c-Jun amino terminal kinase (JNK) pathway and the p38 MAPK pathway. In general, the ERKs are activated by mitogenic and proliferative stimuli, whereas the JNKs and p38 MAPKs act in response to environmental stress, including ultraviolet light, heat, osmotic shock and inflammatory cytokines. In addition, p38 MAPK is phosphorylated and activated in response to LPS (23). Activation of the p38 MAPK pathway is associated with the production of proinflammatory cytokines in phagocytes and T-lymphocytes. Moreover, p38 MAPK is also involved in other inflammatory responses including neutrophil activation (24, 25), apoptosis (26, 27) and the production of NO synthase (28). Four isoforms of p38 MAPK have been identified: p38α, p38β, p38γ and p38δ. Of these, p38α is the best characterized and perhaps the most relevant kinase involved in inflammatory responses (22). The characteristics of p38 MAPK as described above make p38 MAPK an interesting target for anti-inflammatory and anti-cytokine therapies.

2.3 Secreted proinflammatory mediators

After successful innate recognition of pathogens or PAMPs and the activation of intracellular signaling pathways such as p38 MAPK, the immunocompetent cell responds with the release of a plethora of inflammatory mediators. Cytokines are a family of small (8-80 kD molecular weight) proteins that play a pivotal role in the regulation of the host immune response (29). Cytokines function in a complex network in which they influence each other’s production and activity. Moreover, several cytokines have highly overlapping activities. Cytokines are produced by a variety of cells including leukocytes, epithelial cells and endothelial cells in response to a broad range of infectious and immunologic stimuli. They exert their effects by binding to a specific receptor expressed on the cell membrane of many different cell types. Historically, cytokines are classified into three groups: proinflammatory cytokines, anti-inflammatory cytokines and soluble inhibitors of proinflammatory cytokines. Proinflammatory cytokines stimulate inflammation and enhance the host immune response against invading microorganisms. Tumor necrosis factor-α (TNFα) interleukin (IL)-1, IL-12, IL-18 and interferon (IFN)γ are all examples of this group. In contrast, anti-inflammatory cytokines attenuate the host immune response by blocking the expression of proinflammatory cytokines or by inhibiting phagocytosis and microbicidal activity of macrophages and neutrophils (29). IL-4, IL-10 and IL-13 are members of the anti-inflammatory cytokine family. Soluble inhibitors, the third group, inhibit the activity of proinflammatory cytokines by binding to proinflammatory cytokines in the circulation (neutralization) or by competitive
binding to the membrane bound – proinflammatory - cytokine receptor without the induction of intracellular signaling. Examples of this group are soluble TNF receptors type I and II, IL-1 receptor antagonist (IL-1RA) and IL-18 binding protein (IL-18BP).

IL-12 is a heterodimeric proinflammatory cytokine (IL-12p70), formed by a p35 and p40 subunit, which is mainly produced by antigen-presenting cells (30). IL-12 is a potent stimulator of T cell functions, inducing the production of IFN-γ and facilitating the differentiation of naive CD4+ T cells into T helper 1 cells. Ample evidence exists that IL-12 also plays an important role in the pathogenesis of bacterial infection and the associated inflammatory response. IL-18, previously termed IFN-γ inducing factor, is a proinflammatory cytokine that was originally identified as a co-stimulatory factor in the production of IFN-γ during endotoxin shock in mice (31, 32). IL-18 is mainly produced by macrophages in response to a variety of microbial products (33). Although IL-18 alone is not a potent stimulator of IFN-γ production, it synergistically enhances IL-12 induced IFN-γ production. Besides the induction of IFN-γ, IL-18 has many other proinflammatory effects on T and NK cells, including the enhancement of cell proliferation and cytotoxicity and the production of TNF, IL-2 and TNF (34, 35).

Chemokines are a family of chemotactic proteins important for the recruitment and activation of leukocytes during inflammation (29, 36). They can be produced by all somatic cells in response to inflammatory stimuli. Chemokines are divided into four closely related polypeptide families. CXC, CC, C, and CX3C, based on the position of their cysteine residues. CXC and CC chemokines have been most extensively described. CXC chemokines can be further divided into two groups on the basis of the presence of the FLR motif. FLR-containing CXC chemokines like IL-8 and growth related oncogene (GRO)-α, act predominantly on neutrophils while FLR-negative CXC chemokines primarily target lymphocytes. CC chemokines mainly influence mononuclear cells (29). The murine analogues of human IL-8 are macrophage inflammatory protein (MIP)-2 and keratinocyte (KC).

Platelet-activating factor (PAF), a glycerophospholipid with proinflammatory properties, exerts its biological effects by interacting with the PAF receptor (PAFR) expressed on many different cell types including respiratory epithelial cells (37, 38). PAF is a potent proinflammatory mediator; in addition, the PAFR may play a role in the innate immune response that is independent of its interaction with PAF. Indeed, the PAFR specifically binds
phosphorylcholine (ChoP), the biologically active component of PAF, which is also a component of the cell wall of certain pathogens including non-typeable *Haemophilus influenzae* (NTHi). In vitro experiments showed that the PAFR facilitates the invasion of epithelial cells by NTHi (39-41).

3. Experimental models used

This thesis uses several models of infection and inflammation to obtain insight into the function of the immune pathways described above. Following the diagrammatic presentation of the outline of the thesis (Figure 1), the models used are lung tuberculosis and pneumonia in mice, and endotoxemia in healthy humans.

3.1 Tuberculosis

Tuberculosis, caused by the pathogen *Mycobacterium tuberculosis*, is an important health threat worldwide. One third of the world population is infected with *M. tuberculosis*, resulting in 8 million new cases of disease and over 2 million deaths per year (1, 42). The organism is primarily transmitted via the respiratory route and pulmonary tuberculosis is the most common disease manifestation. Tubercle bacilli are slow-growing acid-fast rods. They are intracellular pathogens and use macrophages as their primary host cell. *M. tuberculosis* bacilli contain LAM in their cell wall. LAM shares many physicochemical properties with LPS and is considered important for the induction of a proinflammatory immune response in *M. tuberculosis* infection (43). In chapters 6 and 7 we used a mouse model of pulmonary tuberculosis in which viable virulent *M. tuberculosis* is inoculated intranasally, resulting in a slowly developing disease resembling human tuberculosis.

3.2 Pneumonia

Pneumonia is a common and serious illness and the leading cause of death due to infectious diseases in the United States. In the USA the estimated annual incidence of pneumonia is 4 million, 20 % of which results in hospitalization. The mortality rate can be as high as 25%, especially in hospitalized patients (44). Pneumonia can be caused by a large number of pathogens. The most frequently identified pathogen is *Streptococcus pneumoniae* (45, 46). *S. pneumoniae* is a Gram-positive diplococcus of which over 90 serotypes have been described. Pathogenic serotypes have a capsule composed of polysaccharides with antiphagocytic properties. The pneumococcal cell wall is a potent inducer of inflammation. These proinflammatory properties are attributed to the cell wall components LTA and PGN (47,
Another factor that contributes to the virulence of the pneumococcus is pneumolysin. Pneumolysin is a toxin that lyases cholesterol containing membranes and activates the complement cascade (49). Furthermore, pneumolysin is implicated in the induction of a proinflammatory host response (50).

The Gram-negative bacterium *Klebsiella pneumoniae* is another causative pathogen of pneumonia. In contrast to *S. pneumoniae*, it is a rare cause of community acquired pneumonia but a frequent cause of hospital acquired pneumonia (45, 51, 52).

Pneumonia caused by NTHi is frequently seen in patients suffering from chronic obstructive pulmonary disease. NTHi is a Gram-negative bacterium that lacks a polysaccharide capsule ('non-typeable') in contrast to other *H. influenzae* isolates (53, 54).

In chapters 8-11 we used mouse models of pneumonia caused by either *S. pneumoniae*, *K. pneumoniae* or NTHi, in which viable bacteria were inoculated intranasally.

### 3.3 Human endotoxemia

Endotoxin or LPS is a major component of the outer membrane of Gram-negative bacteria. Endotoxin has strong proinflammatory properties and can activate multiple inflammatory cascades. It is therefore considered to play a key role in the toxic sequelae of Gram-negative sepsis. LPS is composed of a lipid moiety, designated lipid A, and a hydrophilic polysaccharide chain. In recent years the injection of endotoxin into humans has been employed to study the early inflammatory responses of the host to Gram-negative inflammation. Intravenous injection of low dose LPS has also been used as a model to study mechanisms that contribute to the activation of inflammation during other inflammatory conditions, including rheumatoid arthritis and Crohn's disease. The symptoms elicited by LPS may vary between study subjects. The majority of subjects complain of headache, generalized malaise and myalgia, sometimes accompanied by nausea. A monophasic fever, preceded by chills, is almost always registered, albeit the rise in body temperature varies. All signs and symptoms start to occur one hour to 90 minutes after the injection of LPS, are most prominent after two to three hours, and disappear within one to four hours thereafter, although some degree of fever may last a few hours longer. Intravenous injection of LPS induces activation of a number of inflammatory cascades, which can be measured with sensitive laboratory techniques. These changes include activation of the cytokine network, activation of leukocytes, activation of endothelial cells, and activation of coagulation and fibrinolysis.
In chapters 2-5 we used the human endotoxemia model to examine the activation and role of p38 MAPK in LPS-induced inflammation.

4. Interventions and mouse strains used: outline of the thesis

The general objective of this thesis is to gain more insight into the role of several factors implicated in the regulation of host immune responses in in vivo models of infection and inflammation.

We used the following experimental models, interventions and mouse strains to accomplish this objective. Note that we chose to first present the studies in humans challenged with LPS, followed by the mouse studies involving live pulmonary infections.

The first part of this thesis (chapters 2-5) evaluates the role of p38 MAPK during human endotoxemia. Chapter 2 analyzes the activation of MAPK pathways in humans intravenously injected with LPS. Chapter 3 describes the effect of a p38 MAPK inhibitor on p38 MAPK activity and the expression of cytokines during human endotoxemia. In chapter 4, the effect of p38 MAPK inhibition on LPS-induced activation of coagulation and fibrinolysis is investigated. Chapter 5 evaluates the role of p38 MAPK in the expression of chemokines and chemokine receptors important for the recruitment and activation of neutrophils during human endotoxemia.

The second part of this thesis (chapter 6-11) studies several factors important for host defense in models of (localized) pulmonary infection. In these experiments, mice were used. Pneumonia was induced by intranasal inoculation with Gram-positive S. pneumoniae, Gram-negative K. pneumoniae or Gram-negative NTHi bacteria. Similarly, pulmonary tuberculosis was induced by intranasal infection with M. tuberculosis. Chapter 6 evaluates the role of LBP in lung tuberculosis using LBP gene deficient mice. The same mouse strain was used to determine the role of LBP in pneumonia induced by S. pneumoniae and K. pneumoniae respectively (chapter 8). TLR4 mutant mice were used to study the role of TLR4 in pulmonary tuberculosis (chapter 7) and in Gram-positive and Gram-negative pneumonia (chapter 9). We assessed the contribution of IL-12 and IL-18 in the host defense against pneumococcal pneumonia in chapter 10. Finally, the role of the PAFR in pulmonary infection induced by NTHi was studied in chapter 11.
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Chapter 1


