Pulmonary immune response during (myco)bacterial infection
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Citation for published version (APA):
Leemans, J. C. (2002). Pulmonary immune response during (myco)bacterial infection
CHAPTER 1

General Introduction and Outline of this Thesis
INTRODUCTION

1. INTRODUCTION

Infectious diseases are by far the greatest cause of morbidity and mortality worldwide (World Bank 1993). Indeed, respiratory infections and gastrointestinal infections cause more deaths than all other diseases added together. Microorganisms that cause these infections could thus be considered as the greatest threat to our survival. Although the epidemic spread of certain diseases suggested that some sort of agent was being transmitted, it was extremely difficult for people to imagine the existence of microorganisms that were too small to see, or to believe that they could harm large hosts. However, in 1674 a cloth merchant in Delft, Antonie van Leeuwenhoek, for the first time saw and described microorganisms with a simple one-lens microscope. In letters published by the Royal Society in London, he reported a wholly new previously invisible world of "animalcules" (Fig. 1). Later on these animalcules appeared to be protozoa and bacteria. Although bacteria are far simpler than eukaryotic cells, they are extremely efficient in their ability to cause human infections. Improved hygienics, antibiotic treatments, and immunization have however significantly reduced morbidity and mortality of bacterial infections nowadays. Then again, in the past few decades many new bacteria have been identified and many "old" bacterial pathogens, such as Mycobacterium tuberculosis and Staphylococcus aureus, have emerged with new virulence determinants in addition to new resistance patterns to antimicrobial therapy. This implies that a profound understanding of the basic mechanisms contributing to antibacterial host defense and pathogenesis of bacterial infections is important. The pathogenicity of bacteria is critically dependent on the outcome of the interaction between the macrophage and bacterium. Therefore, we focused the present thesis mainly on macrophages and their role in pulmonary bacterial infections in order to get more insight into these mechanisms.

Figure 1 In letters to the Royal Society, Antonie van Leeuwenhoek for the first time described the sizes, shapes, and even the motility of these bacteria. These are drawings from bacteria from the human mouth.
2. GENERAL ASPECTS OF PULMONARY IMMUNOLOGY

Lungs are exposed to a continuous flow of air and to aspiration of minor amounts of nasopharyngeal secretions throughout sleep (1). As a consequence infections occur more frequently in the lungs than in any other organ. An appropriate defense mechanism is therefore crucial to prevent and combat pulmonary infections and starts with innate immunity. In the upper and central respiratory tract mechanical defense mechanisms like filtration, sneezing, coughing and mucociliary clearance remove inhaled infectious agents. When microorganisms escape this mucociliary apparatus and reach the lower respiratory tract (distal airways and alveoli) they first encounter macrophages. Initially, these cells try to eliminate pathogens without amplifying the immune response by phagocytosis and several complex microbial activities. However, when mucociliary clearance and resident macrophages are over-powered, polymorphonuclear cells (PMNs) and monocytes are rapidly recruited to the lungs. PMNs phagocytose pathogens and degrade them using their powerful enzymes. The complement system enforces the function of these phagocytes by triggering a multicomponent enzyme cascade to increase the attraction of phagocytes to pathogens in preparation for phagocytosis.

Since microorganisms have evolved many ways to circumvent these innate immune defenses, specific acquired immunity is critical in the battle against pathogens in the lungs. Plasma cells derived from B-lymphocytes make antibodies that defend us against pulmonary infections by inactivating viruses and bacterial toxins and by recruiting the complement system and various cells to kill and ingest invading pathogens. Since many infectious agents like M. tuberculosis live inside host cells, it is impossible for antibodies to reach them. Therefore, another acquired immune system (cell-mediated immunity (CMI)) based on T-lymphocytes is critical to deal with threatening pathogens. A subpopulation of T cells, the T-helper cells, recognize and bind to antigen presented by dendritic cells and macrophages in a major histocompatibility complex (MHC)-restricted fashion. As a consequence they produce soluble regulatory proteins called cytokines that can activate macrophages to kill intracellular pathogens. Most current evidence indicates that dendritic cells are the most efficient antigen-presenting cells in the lungs stimulating naïve T cells (2). They are located within the pulmonary interstitium, and migrate to regional lymph nodes when they have acquired antigen, to interact with antigen-specific T cells.
The innate defense system is closely linked to the acquired defense system as depicted in Fig. 2. PMNs, complement and antibodies combat most extracellular pathogens while cytokines, macrophages, NK cells, dendritic cells (DCs) and T cells eliminate intracellular pathogens. The defense mechanisms depicted in Fig. 2 may be the most important for dealing with bacterial infection. However, inflammation is also an important aspect of bacterial pathogenesis since the inflammatory response induced by a pathogen can result in considerable damage to the host, and therefore be part of the pathology of bacterial disease.

Figure 2 Two pathways that link innate immunity with acquired immunity thereby providing the basis for humoral and cell-mediated immunity.

3. TOLL LIKE RECEPTORS

For the survival of an animal it is important that it detects infectious microorganisms in order to eliminate them without destroying its own tissues. The tremendous molecular diversity of pathogens makes detection a complex task for the host. To deal with this complexity, animals have evolved several distinct immune-recognition systems. Toll-like receptors (TLRs) take part in this recognition system since they recognize several conserved products unique to microbial metabolism (3). In this way TLRs can detect an infection and subsequently induce activation of antimicrobial innate immune responses (3). In addition, recognition of microbial products by TLRs of DCs triggers maturation of these cells and leads to the initiation of antigen-specific adaptive immune responses.
To date, ten TLRs (TLRs 1-10) have been reported (4). Here, we will focus on those that are most important for this thesis.

**TLR4**

TLR4 is expressed on a variety of cell types, most predominantly on immune cells, including macrophages and DCs (3). TLR4 functions as the signal-transducing receptor for lipopolysaccharide (LPS) (5). In addition to LPS, TLR4 is also involved in the recognition of several other ligands, including lipoteichoic acid from Gram-positive bacteria (6), and a heat-sensitive cell-associated factor derived from *M. tuberculosis* (7). In **chapter 8**, we determined the role of this receptor in host defense against pulmonary tuberculosis.

**TLR2**

TLR2 is involved in the recognition of many different microbial products. Peptidoglycan from Gram-positive bacteria (6), bacterial lipoproteins (8-10), mycobacterial cell-wall lipoarabinomannan (7, 11) a phenol-soluble modulin produced by *Staphylococcus epidermidis* (12), and yeast cell walls (13) are all recognized by this receptor. This uncommonly wide range of microbial products that are recognized by this receptor is partly explained by cooperation between TLR2 and at least two other TLRs: TLR1 and TLR6 (3). The formation of heterodimers between TLR2 and either TLR1 or TLR6 determines the specificity of ligand recognition (14, 15).

**TLR9 and CpG motifs**

Bacterial DNA differs structurally from vertebrate DNA since it has a higher frequency of CpG dinucleotides and lacks cytosine methylation. These unmethylated DNA sequences are called CpG motifs and are immunostimulatory (16). Because of the differences between eukaryotic and bacterial DNA, CpG motifs might be a signal for the presence of pathogenic agents. Indeed, in mice and humans, innate immune cells appear to recognize CpG motifs via TLR9 (17). CpG motifs are in particular recognized by DCs thereby activating these cells to upregulate co-stimulatory molecules and to produce Th1-polarizing cytokines, such as interleukin (IL)-12 and IL-18 (18). In this way, innate and acquired immunity is bridged by the interaction between TLR9 and CpG motifs. In **chapter 7**, we evaluated treatment with CpG motifs in a mouse model of tuberculosis.
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4. MACROPHAGES IN THE LUNGS

Lung macrophages are resident phagocytes of the alveoli, originating from blood monocytes and intrapulmonary proliferation (19). Macrophages keep the lungs sterile by eradicating particles and microorganisms through phagocytosis and the production of radicals and proteases. Phagocytosis is enhanced by molecules called opsonins that increase recognition and endocytosis through their binding to specific receptors. The mononuclear phagocyte system of the lung is comprised of cells in three compartments; airway and alveolar macrophages, interstitial macrophages, and in some species, intravascular macrophages (20). The alveolar macrophage is located in the alveolar space and is the best characterized macrophage because it can be readily recovered using the bronchoalveolar lavage technique. Interstitial macrophages are resident in the connective tissue of the lung and are not directly exposed to airborne pathogens like alveolar macrophages are. In intravascular structures there are also pulmonary macrophages present in several mammals, but not in rodents (21). Finally, airway macrophages are located in large and small airways. Few studies are currently available regarding the roles of interstitial, airway and intravascular macrophages. For convenience sake, we will therefore refer to alveolar macrophages when discussing lung macrophages since most information is available on this subpopulation. It should however be kept in mind that the pulmonary compartment harbors interstitial, intravascular, and airway macrophages that may differ from alveolar macrophages.

4.1 The alveolar macrophage

Alveolar macrophages (AM) are resident and predominant cells in the airway. They defend the lungs by phagocytosing and killing pathogens, and by their participation in the immune response by secreting cytokines. When AMs have engulfed bacteria they generally become activated to deliver hydrolytic enzymes, and produce toxic effector molecules like reactive nitrogen and oxygen intermediates in order to kill pathogens. Bacteria like *S. pyogenes* are fully susceptible for phagocytosis and the antimicrobial functions of AMs (22). However, intracellular bacteria like *M. tuberculosis* have AMs as primary host cells and are far more resistant to antibacterial activities. AMs are generally regarded as rather poor antigen-presenting cells (APC) (23-25). Their primary task is to acquire and eliminate pathogens to keep the alveoli sterile without amplifying the inflammatory response. It is even suggested that AMs inhibit amplification of immune
pathways (26-28). By inhibiting the inflammatory response, AMs are able to clear normal amounts of particles and pathogens from the lung without initiating an inflammatory response with subsequent damaging the delicate alveolar capillary membrane. However, when the infectious or antigenic load in the pulmonary compartment becomes too great for a quiescent elimination, other leukocytes are needed and recruited to the lungs.

4.2 Monocyte migration into the lungs

The recruitment of leukocytes is extremely essential during inflammatory processes. The process of monocyte migration to the lungs requires that cells adhere and migrate through the vascular endothelium, the extracellular matrix (ECM) of endothelial and epithelial cells, and finally the alveolar epithelial barrier. The interactions involved in the multistep process of leukocyte migration toward the lungs are well characterized for PMNs, but are only poorly defined for monocytes. To migrate across endothelium, monocytes utilize the sequential interaction of monocyte adhesion molecules selectins, \( \beta_2 \) (CD11/CD18) and \( \beta_1 \) integrins (very late Ag (VLA)-4 and VLA-5) (29) and platelet-endothelial cell adhesion molecule-1 (PECAM-1) with endothelial selectins, intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), and PECAM-1 (30, 31) (32). However, in the pulmonary microcirculation, monocytes may also use \( \beta_1 \) and \( \beta_2 \) integrin-independent pathways during emigration from the vasculature (33). CD44 is a member of the hyaluronate receptor family of cell adhesion molecules that is linked to cytoskeletal elements. In chapter 5 we studied the role of the adhesion molecule CD44 in monocyte migration to the M. tuberculosis infected lung.

5. TUBERCULOSIS

5.1 Introduction

Tuberculosis, caused by the intracellular pathogen M. tuberculosis, is one of the main threats to human civilization. Each year 2.2 million persons die from tuberculosis worldwide, which means that one person dies from this disease every 10 seconds (34, 35). As a consequence, M. tuberculosis causes more deaths annually than any other single infectious agent (36, 37). In addition, 8 million new cases of tuberculosis are identified yearly (34). The tubercle bacillus is a slow-growing acid-fast pathogen that is primarily transmitted from person to person via the respiratory route by inhaling aerosolized droplets, which arise during coughing, sneezing and talking of infected individuals.
Although \textit{M. tuberculosis} can infect any organ, the lungs are the prime target. Even though the risk of infection has been significantly reduced in developed countries, it remains high for malnourished individuals in impoverished areas and for patients infected with human immunodeficiency virus (HIV). In addition, ethnic and racial differences make one person more susceptible for tuberculosis than others. Africans, Native Americans, and Eskimos for example have a lower resistance against infection than Jews, other whites and Mongolians (38).

5.2 Infection with \textit{M. tuberculosis}

The primary route of entry of \textit{M. tuberculosis} is via the airways through the inhalation of respiratory droplets that are so small that they can easily reach the lower respiratory tract (39). Once tubercle bacilli have reached this site, they have several destinies (40). They can be eliminated by an effective initial host defense through which the host has no chance of developing tuberculosis; they can start replicating directly after the infection so that clinical disease (primary tuberculosis) develops; they can become dormant, with resultant latent infection that is only manifest by a positive tuberculin skin test; or latent bacilli can ultimately start growing, so that clinical disease develops, known as reactivation tuberculosis. Despite the enormous numbers of people infected with this organism, it is generally believed that persons infected with \textit{M. tuberculosis} without HIV infection have a 5 percent to 10 percent lifetime risk of developing active disease. The follow-up of a placebo group of a therapy trial even indicated that the chance of reactivation was as low as 1% over a 7-yr period (41). In severely immune compromised hosts such as HIV patients, there might be a chance of 7% to reactivate tuberculosis every year after latent infection is recognized (42).

6. THE INTERACTION BETWEEN MACROPHAGES AND \textit{M. TUBERCULOSIS}

6.1 Binding and phagocytosis of \textit{M. tuberculosis} by macrophages

After \textit{M. tuberculosis} bacilli have reached the lower respiratory tract they first will encounter AMs. AMs bind and then engulf tubercle bacilli via receptor-assisted phagocytosis involving several different cell-surface molecules (43, 44) (Fig 3). It is suggested that the receptor that is used by the macrophage to phagocytose tubercle
bacilli influences the cellular response of the host (44). While phagocytosis of bacilli via the Fc receptors induce reactive oxygen intermediates (ROI) and allows phagosome-lysosome fusion (45), complement receptor 3-mediated phagocytosis prevents the activation of the respiratory burst (46), and holds maturation of phagosomes (47). The latter would suggest that bacilli abuse this receptor in order to get inside their primary host. Recently, it was shown that in addition to receptors, mycobacteria also interact with cholesterol on the macrophage membrane to enter macrophages (48). In chapter 5, we studied whether the adhesion receptor CD44 on macrophages is involved in binding and phagocytosis of M. tuberculosis.

6.2 The interaction between macrophages and mycobacteria

Tubercle bacilli are intracellular pathogens that have macrophages as their primary host cell. Inside this cell, they can replicate and are protected from extracellular host defense mechanisms such as complement and specific antibodies (49). However, next to being host cells, macrophages are also the first line of defense. They phagocytose and kill this pathogen by displaying several complex antimicrobial defense mechanisms. Once bacilli are inside the macrophage through phagocytosis, an interaction between the pathogen and the macrophage is initiated of which the outcome determines the pathogenicity of M. tuberculosis. One mechanism of macrophages to degrade bacilli is by phagolysosome fusion. Phagosomes that arise during phagocytosis of viable bacilli are being delivered to lysosomes that contain potent hydrolytic enzymes (Fig. 3). The antimicrobial activity of the fused phagolysosome is mediated by these degrading enzymes and/or direct and indirect effect of acidification (49). Mycobacteria however, try to escape these antmycobacterial mechanisms by inhibiting the fusion of phagosomes with lysosomes. The TACO (tryptophan aspartate-containing coat) protein seems to be involved in this inhibition (50). TACO has been shown to be present in phagosomes that harbor viable bacilli, while it was absent in phagosomes containing killed bacilli. It was shown that TACO is actively preserved in the mycobacterial phagosome and in this way mycobacteria prevent maturation into or fusion with lysosomes, leading to enhanced mycobacterial survival.

Another antmycobacterial defense mechanism of macrophages is the production of ROI and reactive nitrogen intermediates (RNI) (Fig. 3). When macrophages become activated by the appropriate signals like IFNγ and TNFα, they generate ROIs such as H₂O₂ and O₂⁻ and RNIs such as nitric oxide (NO) and NO₂⁻. These compounds are
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found within phagolysosomes and are derived from L-arginine via an enzymatic pathway controlled by the enzyme inducible nitric oxide synthase (51). Whereas RNI are major killer molecules of the tubercle bacillus, ROI are regarded as less important (52). In answer to these highly toxic compounds, mycobacteria have acquired several mechanisms to resist ROIs and RNIs and to detoxicate them (52).

Mycobacteria need iron for their intracellular survival. In order to gain access to this essential nutrient, they have developed siderophores, iron-binding molecules, that transfer iron from host proteins to molecules in the cell wall of mycobacteria (53). However, iron is also an important cofactor for macrophages exerting their microbicidal effector functions (54). The outcome of the competition between the macrophage and the tubercle bacillus for the acquisition of this nutrient is therefore critical for clinical outcome.

In chapter 2 and 3 we studied the in vivo role of (activated) AMs in the pathogenesis of pulmonary tuberculosis in mice.

6.3 Macrophage apoptosis

Apoptosis is a highly regulated process of cell death that is found in response to infections with many pathogens, including M. tuberculosis (55). In vitro experiments
demonstrated that infection of human AMs with *M. tuberculosis* induced apoptosis in these cells (Fig. 3) (56). In addition, lungs from tuberculosis patients displayed an extensive apoptosis (50-70%) in tuberculous granulomas (56). Also, a significant increase in the number of apoptotic AMs was observed in bronchoalveolar lavage fluid from patients with active pulmonary tuberculosis (57, 58). Despite these clear observations, it is much less clear which role AM apoptosis plays in the pathobiology of this disease and whether it increases or decreases the mycobacterial load *in vivo*. Since macrophages are the primary host cell for mycobacteria but also the predominant inducers of protection, apoptosis of infected macrophages could respectively be a host defense mechanism or a pathogen-encoded virulence determinant. Several studies suggest that apoptosis may be a host defense mechanism to infection by mycobacteria. It has been anticipated that macrophages that go into apoptosis destroy the environment for intracellular replication and hiding (59). Furthermore, apoptotic bodies might contain mycobacteria from the extracellular environment because they maintain their plasma membrane integrity, and can later on be engulfed by newly recruited AMs (60). Apoptosis of human monocytes has indeed been shown to limit the growth of *M. avium* (60), *M. bovis* bacillus Calmette-Guérin (61) and *M. tuberculosis* (62) *in vitro*. However, *in vitro* data are not sufficient to determine the net effect of macrophage apoptosis *in vivo*. We therefore studied the role of (activated) macrophage apoptosis *in vivo* during *M. tuberculosis* infection in chapter 2 and 3. Since the role of AMs in respiratory infections by extracellularly growing pathogens could be different, we evaluated in chapter 4 the role of AMs during pneumococcal pneumonia.

7. T CELLS

The establishment of a protective immune response during the course of *M. tuberculosis* infection requires recruitment of T lymphocytes (63). T cells can be divided into a group of T helper cells (Th) that are CD4⁺ and a group of cytotoxic T cells (Tc), which are generally CD8⁺. These subsets of cells have similar and distinct functions during mycobacterial infections.

7.1 T helper cells

CD4⁺ T cells, provide T-cell help to other immune cells, and thereby regulate the immune response. Mycobacterial antigens are readily accessible to MHC class II
molecules on antigen-presenting cells (APCs), leading to the activation of antigen-specific CD4+ T cells. The importance of CD4+ T cells in controlling an acute *M. tuberculosis* challenge is demonstrated in studies in mouse models using antibody depletion (64-66), adoptive transfer (67), and transgenic mouse strains deficient in either MHC class II (68, 69) or CD4 (69). The principal mechanism by which CD4+ T cells mediate mycobacterial resistance is the production of cytokines ($\S$8.1).

In humans Th cells can display at least two phenotypes - Th1 and Th2 - which can be described mainly by the pattern of cytokines they secrete ($\S$8.1). All Th cells begin as naive Th cells that, after being activated, are capable of differentiating, into either Th1 or Th2 effector cells. Th1 cells are the principal regulators of type 1 immunity. Characteristic of Th1 cells is the production of interferon-γ (IFN-γ), which is the key effector cytokine in the host response to tuberculosis (Fig. 3). IFNγ gene-disrupted mice have been shown to succumb to tuberculosis, and the administration of this cytokine prolongs their survival (70, 71). Th2 cells stimulate high titers of antibody production and the cytokines they produce have been implicated in allergic and atopic reactions, as well as in airway inflammation (72).

### 7.2 CD8+ T cells

As already mentioned, CD4+ T cells are critical for the development of protective immunity during tuberculosis. However, a protective immune response to *M. tuberculosis* infection has also been shown to include CD8+ T cells. CD8+ T cells recognize peptide fragments that are processed and presented on cell surfaces in a MHC class I-restricted fashion, which then bind to the T-cell receptor. The mechanism by which mycobacterial proteins are processed and presented by MHC I molecules is not fully understood. That is MHC class I presentation is most efficient with cytoplasmic antigens while mycobacteria are captured into vacuoles. CD8+ T cells can also bind the CD1 molecule, another mode of antigen presentation, which is present on the surface of professional APCs (73). A protective role of MHC class I-restricted CD8+ T cells against tuberculosis, is demonstrated by studies showing that mice deficient for CD8+ T cells as a result of disruptions in the β2-microglobulin or TAP1 genes were more susceptible to infection with *M. tuberculosis* than wild-type mice (74, 75). CD8+ T cells probably contribute to immune protection against intracellular pathogens by producing cytokines and by exerting cytotoxic effects. Considerable numbers of activated CD8+ T cells with both cytokine-secreting and cytotoxic functions are found in the pulmonary compartment.
during tuberculosis infection in mice (69, 76-79). CD8+ cells can cause lysis of infected monocytes and macrophages (Fig. 3) by two pathways: Fas-FasL interaction and granule exocytosis (80, 81). After infected macrophages are lysed, mycobacteria are released and can be engulfed by freshly activated macrophages that then can efficiently kill the pathogen (82). When granules are released, perforin makes pores in the target cell thereby allowing other granule proteins to enter the cell and to induce lysis and apoptosis (83, 84). The contribution of this pathway to resistance against mycobacterial infection is however controversial since studies in mice with defects in perforin or granzymes indicate that these granule constituents are not needed for an effective immune response during the initial stages of infection (85, 86). In addition to lysis of infected macrophages, CD8+ T cells have also been shown to have microbicidal functions since they can directly kill mycobacteria by granulysin, a granule-associated protein (87). Granulysin itself was not capable of killing M. tuberculosis but needed perforin in order to get access to the target cell (87). Finally, CD8+ T cells contribute to host defense by being a source of IFN-γ and tumor necrosis-α (TNF-α) following antigen presentation (Fig. 3) (44). As already mentioned these cytokines are critical for the activation of macrophages to kill mycobacteria.

8. CYTOKINE CIRCUITS

Cytokines are a group of intercellular regulatory proteins that play an important role in immune defense mechanisms. They are produced by many different cell types and interact in complex ways with one another. They can activate and deactivate leukocytes, increase or decrease the functions of leukocytes, and promote or inhibit a variety of defense mechanisms. The same cytokine often has several functions that overlap with other cytokines.

8.1 Th1 and Th2 cytokines

The type 1 (Th1; including IFN-γ and IL-2) and type 2 (Th2; including IL-4, IL-5, and IL-10) cytokine patterns of immune response in mice were at first recognized by a panel of T helper cell clones, Th1 and Th2 cells. Since we understand that Th1 and Th2 cytokines are expressed by a variety of cells and that the functions of these cytokines are different it is suggested that an imbalance between Th1 and Th2 cytokines may be important in human disease (88). Indeed, patients with active tuberculosis have
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diminished IFN-γ production in response to purified protein derivative (PPD) in comparison with PPD-positive, uninfected case control subjects (89). Moreover, infected patients produced higher levels of IL-4 (90). These data are consistent with the notion that active *M. tuberculosis* infection is related to dominant type 2 immunity, whereas protected patients mount type 1 immune responses to the organism. In general, Th1-type cytokines stimulate CMI and activate leukocytes capable of inhibiting the growth of bacteria. IFN-γ is the protective Th1 key cytokine during mycobacterial infection since it stimulates bacterial phagocytosis, oxidative burst, intracellular killing, and MHCII and MHCII molecule expression that on their turn stimulate antigen presentation to T cells (91). Whereas Th1 immune responses are protective for mycobacterial infections, Th2 responses facilitate humoral immunity (92) and help to resolve the cell-mediated inflammation (72). It is believed that type 1 responses are used to protect the host against acute infection and are switched to type 2 responses later in the infection when the danger has disappeared, in order to reestablish homeostasis and protect the host from autoinflammatory destruction (72).

Although several factors are involved in Th differentiation, the local cytokine milieu is most important in this (72). IL-12 is a key regulatory cytokine produced by APCs, such as macrophages and DCs, that is involved in the differentiation of naïve T cells into Th1 cells (Fig.4). IL-12 plays a pivotal role in promoting Th1 while suppressing Th2 responses, and hence promotes CMI against intracellular pathogens like *M. tuberculosis* (93). On the other hand, IL-4 stimulates the differentiation into Th2 cells. Interestingly, Th1 and Th2 cells cross-regulate one another. IFN-γ directly suppresses IL-4 secretion and thus inhibits differentiation of naïve T cells into Th2 cells (72). On the other hand, IL-4 and IL-10 inhibit the secretion of IL-12 and IFN-γ, thereby inhibiting the differentiation of naïve T cells into Th1 cells (72).

But what triggers the host to preferentially produce IL-12 or IL-4 at the beginning of the immune response? Pathogens contain information within them that can directly trigger the secretion of IL-12 by phagocytic cells (Fig. 4), of which unmethylated CpG motifs are one good example (72). Despite the many conserved microbial constituents that induce IL-12 secretion, only few examples exist of the development of Th2 cells (94). This would suggest that a Th2 response might develop through a default pathway, when microbial structures are not available to stimulate the production of IL-12 and other Th1-inducing cytokines (94). The cellular sources of the initial production of IL-4, and
the mechanism for its induction, are still not clearly understood. In chapter 6, we evaluated the role of IL-12p40 overexpression in resistance against pulmonary *M. tuberculosis* infection.

**Figure 4** Model of induction of Th1 and Th2 cells. At the beginning of an infection naïve T cells can differentiate into Th1 or Th2 cells depending primarily on the cytokine milieu provided by macrophages and dendritic cells (DC1 or DC2). IL-12 promotes Th1 cells, whereas IL-4 drives Th2 cells. IFN-γ and IL-4 can act as autocrine growth factors and can inhibit the opposite Th subset. Th1 cells mediate the elimination of intracellular pathogens by inducing a strong CMI. Th2 cells are anti-helminthic and suppress CMI.

**8.2 Pro-inflammatory and anti-inflammatory cytokines**

Pro-inflammatory cytokines, like IL-1, IL-6, and tumor necrosis-alpha (TNF-α), promote the inflammatory response and lead to the synthesis of acute phase proteins. Other cytokines, such as IL-4, IL-10, IL-13, IFN-α and transforming-growth factor-β are recognized as anti-inflammatory cytokines that inhibit the release of pro-inflammatory cytokines and limit some pro-inflammatory activities. Although, this commonly used classification of pro- versus anti-inflammatory cytokines is straightforward, it is greatly simplified. Indeed, the cytokine concentration, the nature of the target cell, the nature of the activating signal, the nature of the produced cytokines, the timing, and the sequence of cytokine action greatly influence the anti- or pro-inflammatory properties of cytokines (95). IL-6 is a good example of the simplification of anti- versus pro-inflammatory since it has been reported to have both pro-inflammatory and anti-inflammatory effects (96, 97). In chapter 9 we studied the role of IL-6 during lipoteichoic acid- (LTA) and peptidoglycan (PepG) -induced pulmonary inflammation.
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9. MODELS FOR PULMONARY INFLAMMATION USED IN THIS THESIS

An enormous amount of literature exists describing individual elements of (myco)bacterial mechanisms and host immune responses to (myco)bacteria. However, still little is known about the combined interactions or the balance between these processes. To face the enormous complexity of the networks involved we used animal models since they display this enormous complexity. Mice were chosen because of the easiness in housing and handling, and the availability of well characterized strains and many immunological reagents.

9.1 Tuberculosis

There are numerous animal models of tuberculosis involving guinea pigs, rabbits and mice. In this thesis we chose the mouse model since this animal is able to generate a strong immune response to *M. tuberculosis* like most healthy humans. Within this model different routes of infection are possible. The most commonly used are the intravenous and aerogenic routes. Obviously the local administration of *M. tuberculosis* in the pulmonary compartment most closely mimics the reality of infection in humans. Since aerosol infection has the hazardous risk of contamination we chose to administer *M. tuberculosis* intranasally. Therefore we put droplets of *M. tuberculosis* suspension on the nares of mice which they inhale, causing pulmonary tuberculosis. We used the virulent and well-defined laboratory strain H37Rv which is also pathogenic in humans (chapters 2, 3, 5, 6, 7 and 8).

9.2 Pneumococcal pneumonia

*Streptococcus pneumoniae* is a leading causative pathogen of community acquired pneumonia (98) (99). Despite adequate antimicrobial therapy, pneumococcal pneumonia remains a major cause of morbidity and mortality worldwide. To study the role of AMs in *S. pneumoniae*-induced pneumonia we intranasally administered the Gram-positive bacterium *S. pneumoniae* serotype 3 to mice (chapter 4).

9.3 Pulmonary inflammation induced by bacterial cell wall components

*S. aureus* is the most frequently isolated Gram-positive pathogen in nosocomial infections
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associated with severe complications (100). In addition, *S. aureus* accounts for 6-33% of bacterial isolates from patients with hospital-acquired pneumonia (101)(102). Gram-positive inflammation is presumed to be due to bacterial cell wall components, such as LTA and PepG. In chapter 9 and 10, we studied the inflammatory responses of LTA and PepG after intranasal administration of these compounds.

10. AIM AND OUTLINE OF THIS THESIS

The general aim of this thesis was to obtain more insight into host defense mechanisms that contribute to an adequate response to microorganisms invading the pulmonary compartment. The specific objectives of each individual investigation is delineated in the respective chapters.

Tubercle bacilli are intracellular pathogens that have macrophages as their primary host cell. However, next to being host cells, macrophages are also the first line of defense. This raises questions as to the exact role of macrophages and macrophage apoptosis during mycobacterial infection. Hence, we studied the *in vivo* role of (activated) AMs and apoptosis of these cells in the pulmonary host response to *M. tuberculosis* in chapters 2 and 3. Since the *in vivo* role of AMs in pneumonia with extracellularly living pathogens has not been elucidated and could be quite different from pulmonary infection with intracellular pathogens, we investigated the contribution of AMs in host defense against *S. pneumoniae* in chapter 4. Phagocytosis of mycobacteria by macrophages and leukocyte migration are both important for controlling *M. tuberculosis* infection and are critically dependent on the reorganization of the cytoskeleton. Since CD44 is an adhesion molecule connected with the actin cytoskeleton and involved in inflammatory responses, we investigated the role of CD44 in both these processes in chapter 5. CMI is critical to host defense during tuberculosis and is regulated by a Th1/Th2 balance. IL-12 is a key regulatory cytokine composed of p35 and p40 that is involved in regulating this balance. In chapter 6, we studied the effect of p40 during tuberculosis by generating transgenic mice that overexpress p40 in lungs. Unmethylated CpG motifs from eukaryotic DNA can also regulate the Th1/Th2 balance by inducing the production of type-1 inducing cytokines. Since a type 1 response is protective during mycobacterial infection we evaluated in chapter 7 the influence of CpG motifs on pulmonary tuberculosis in mice. TLRs play an essential role in the innate recognition of microorganisms by the host. In chapter 8, we studied the role of TLR4 in host defense against lung tuberculosis. The
prevalence of Gram-positive infections has increased significantly in the past few decades. Since there are limited data available how Gram-positive bacteria like *S. aureus* can induce pulmonary infections, we studied the effect of two bacterial cell wall components, LTA and PepG in mouse lung in chapter 9 and 10, and assessed the role of IL-6 herein (chapter 9).

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