Pulmonary immune response during (myco)bacterial infection
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Infectious diseases are the most common afflictions of mankind worldwide and the leading causes of death from disease. Bacterial and viral infections kill more people each year than all cancers and cardiovascular diseases. In The Netherlands eight out of ten disease cases are infectious diseases. They are predominantly located in the respiratory system due to the continuous exposure to the extracellular environment through breathing. Infections implicate a considerable amount of mortality, morbidity, and loss of economic productivity. The treatment of infectious diseases is often hampered by the increasing occurrence of multi-drug resistant bacterial strains, and the long term use of medicines in case of tuberculosis. It is therefore important that new host defense factors are defined that are essential for resistance against (myco)bacteria. The purpose of this thesis was to obtain more insights into host defense mechanisms that contribute to an adequate immune response against (myco)bacteria that invade the pulmonary compartment.

Extensive apoptosis of alveolar macrophages is found in lungs of tuberculosis patients. Alveolar macrophages have been implicated as the predominant inducers of protection against infections by phagocytosing and killing mycobacteria and by initiating an inflammatory response. However, macrophages are also the primary host cells for *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis. Inside the macrophage, tubercle bacilli can replicate and are protected from host defense mechanisms. It is unclear what role alveolar macrophages and apoptosis of these cells play in the pathobiology of tuberculosis. Therefore, we determined in chapter 2 the *in vivo* role of alveolar macrophage apoptosis in mice, using clodronate-liposomes after infection with *M. tuberculosis*. Interestingly, we found that mice with apoptotic macrophages were completely protected against lethality during a five month follow-up. In contrast, the survival of mice with an intact macrophage population decreased extensively with 90% mortality after 5 months. These results suggest that alveolar macrophages facilitate the growth of *M. tuberculosis* in lungs. Therefore, alveolar macrophage apoptosis as observed in tuberculosis patients could be an important host defense mechanism to restrict mycobacterial growth.

Chapter 2 suggested that alveolar macrophages facilitate the growth for intracellular living *M. tuberculosis*. This is rather unexpected since macrophages are generally considered as important phagocytes that are the first line of host defense. In
In chapter 3 we studied whether the dual role of macrophages could be due to the activation state of these cells. That is, activated macrophages inhibit mycobacterial growth \textit{in vitro}. To analyze the \textit{in vivo} role of activated macrophages in host defense against \textit{M. tuberculosis}, we depleted lungs of activated macrophages by treating transgenic mice expressing the human Fcy receptor I, that is upregulated on macrophages upon activation, intranasally with an immunotoxin directed to this receptor. To get a better reflection of the \textit{in vivo} situation in tuberculosis patients we induced apoptosis in activated macrophages after infection with \textit{M. tuberculosis}. For comparison with non-selective macrophage depletion, mice were intranasally treated with clodronate-liposomes as in our previous study. Whereas the non-selective induction of apoptosis in macrophages after infection with \textit{M. tuberculosis} led to an improved resistance, apoptosis of activated macrophages reduced host resistance. Activated macrophages can inhibit mycobacterial growth by displaying several direct antimycobacterial defense mechanisms like the production of toxic molecules. Together, these studies demonstrated that the dual role of pulmonary macrophages during tuberculosis is associated with the activation state of these cells. Resting macrophages promote the mycobacterial growth whereas activated macrophages serve as inducers of protection. The presence of alveolar macrophage apoptosis in tuberculosis patients could therefore be part of a host defense strategy, as long as these cells are not activated.

Alveolar macrophages seem to play another role in pulmonary infections induced with extracellular living bacteria. In chapter 4 we showed that mice depleted from alveolar macrophages have a higher mortality after infection with \textit{Streptococcus pneumoniae} than mice with an intact macrophage population. The increased mortality was not related to an impaired bacterial clearance. The classical role of alveolar macrophages as phagocytes of invading bacteria seems therefore less important during infection with \textit{S. pneumoniae} than with \textit{M. tuberculosis}. We also found that lungs of mice depleted from alveolar macrophages had a pronounced and prolonged influx of PMNs of which high proportions were apoptotic. This is likely the results of inefficiency of the normal resolution process in the absence of alveolar macrophages, thereby tipping the balance toward persistent inflammation and tissue injury. This resolution process prevents the release of potentially toxic or immunogenic intracellular contents. This study demonstrated that alveolar macrophages are important for host defense against extracellular \textit{S. pneumoniae} since they could end the inflammation by removing apoptotic PMNs.
Effective host defense against *M. tuberculosis* is primarily dependent on the interaction between macrophages, T cells and dendritic cells. This interaction requires migration of leukocytes which is dependent on the expression of adhesion molecules. The adhesion molecules that are important for migration of leukocytes during tuberculosis are not yet defined. CD44 is an adhesion molecule that is expressed on leukocytes and is known to be involved in migration of T cells to the site of inflammation. Little is however known about the *in vivo* role of CD44 during infections with pathogens. In *chapter 5* we studied the role of CD44 in the pathogenesis of tuberculosis by infecting mice deficient for CD44 with *M. tuberculosis*. We found that CD44−/− mice had a defect in migration of macrophages to the infected lungs early in the infection. This macrophage migration defect was confirmed in a delayed-type-hypersensitivity experiment in footpads of mice immunized for *M. tuberculosis*. Interestingly, CD44 appeared also a site on macrophages that mediates binding and phagocytosis of *M. tuberculosis*. The lack of CD44 resulted in an impaired resistance against *M. tuberculosis* as reflected by an increase in mycobacterial growth and a decrease in survival. In other studies, blocking of adhesion molecules other than CD44 did not influence the clearance of mycobacteria. Therefore, CD44 can be considered as a unique adhesion molecule in the host defense against *M. tuberculosis* that mediates binding and phagocytosis of mycobacteria, migration of macrophages and resistance during tuberculosis.

Cell-mediated immunity (CMI) is essential for host defense against *M. tuberculosis* and is tightly regulated by a balance between type 1 and 2 cytokines. IL-12 is a key regulatory cytokine produced by antigen-presenting cells such as macrophages. It supports Th1 and inhibits Th2 responses, and hence promotes CMI against intracellular pathogens. IL-12 is a heterodimeric protein composed of a p35 and p40 subunit. P40 is mainly produced as monomer and homodimer. *In vitro*, p40 homodimers inhibit IL-12 function by competing with the heterodimer for the IL-12 receptor. The *in vivo* role of p40 during mycobacterial infection has not been elucidated fully as p40 has been shown to have both agonistic and antagonistic effects on IL-12. To study the *in vivo* role of p40 during mycobacterial pulmonary infection we generated transgenic mice that overexpress p40 in their lungs (*chapter 6*). Infection of these mice with *M. tuberculosis* resulted in a reduced resistance as reflected by an enhanced mycobacterial outgrowth. Furthermore, we found a decrease in the amount of (agonists of) chemokines and numbers of leukocytes that are important for CMI in transgenic mice. These data
sugges that p40 acts as an antagonist for IL-12-mediated leukocyte migration to the *M. tuberculosis*-infected lungs by reducing chemokine production. One could hypothesize that p40 homodimers may act as physiological regulators of IL-12 mediated chemotaxis of inflammatory cells in order to restore pulmonary homeostasis after infection.

DNA of bacteria contains a higher frequency of unmethylated CpG motifs than vertebrate DNA as that of humans. Therefore, CpG motifs could signal the host for infection with pathogens. Indeed, CpG motifs can stimulate leukocytes to produce Th1 cytokines. Since a Th1 response is important for host defense against *M. tuberculosis* we studied the effect of CpG treatment on the immune response during tuberculosis in chapter 7. Administration of CpG motifs reduced mycobacterial outgrowth for up to 5 weeks after *M. tuberculosis* infection and was associated with a decrease in inflammation in lung tissue. The protective effect seemed mediated by the Th1 cytokine IFNγ. In light of the increasing amount of drug-resistant strains of *M. tuberculosis*, this study may provide a rationale for further development of CpG motifs as a new adjunctive therapy for tuberculosis.

Innate recognition of mycobacterial products is the first step in a chain of events that results in an effective host defense against *M. tuberculosis*. For this recognition animals have developed a immune-recognition mechanism based on Toll-like receptors (TLRs). These TLRs recognize microbial products and in this way can signal an infection with pathogens and activate immune responses that are important for host defense. *In vitro* experiments have demonstrated that TLR4 is involved in the recognition of *M. tuberculosis*. In chapter 8 we studied the *in vivo* role of TLR4 in host defense against *M. tuberculosis*. Mice with a nonfunctional TLR4 had an impaired resistance against *M. tuberculosis* as reflected by a moderate increased mycobacterial outgrowth and a reduced survival. The limited protective role of TLR4 in host defense against *M. tuberculosis* suggests that this pathogen is recognized by a repertoire of different receptors from the host.

The incidence of Gram-positive infections has increased considerably over the past few years. *Staphylococcus aureus* is the most frequently isolated Gram-positive pathogen in nosocomial infections associated with severe complications. Lipoteichoic acid (LTA) en peptidoglycan (PepG) are components of the bacterial cell wall that can induce inflammatory responses *in vitro*. In chapter 9 we studied the acute pulmonary inflammatory response caused by local exposure to LTA and PepG from *S. aureus* in order to understand how Gram-positive bacteria induce pulmonary inflammation *in vivo.*
IL-6 is a pleiotropic cytokine that is involved in regulation of inflammatory responses during Gram-positive bacterial infection. In this chapter we studied in addition the role of IL-6 in the pathogenesis of acute lung inflammation caused by LTA and PepG by using mice deficient for IL-6. Both LTA and PepG induced acute pulmonary inflammation in a dose dependent way, characterized by PMN influx and IL-6 production in the bronchoalveolar lavage fluid. Endogenously produced IL-6 attenuated inflammation induced by low dose LTA. This anti-inflammatory role of IL-6 is lost or even converted into a modest pro-inflammatory role during pulmonary inflammation induced by high dose LTA. Interestingly, endogenously produced IL-6 also plays a pro-inflammatory role in pulmonary inflammation induced by PepG. Together, these results suggest that the role of IL-6 in inflammation depends on the stimulus and/or the model of inflammation used. The different mechanisms by which LTA and PepG elicit inflammation could be the basis for the pleiotropic characteristics of IL-6.

The data from chapter 9 prompted us to examine the combined effects of LTA and PepG in mouse lungs in chapter 10. As found earlier, administration of LTA and PepG induced a rapid recruitment of PMNs to the bronchoalveolar space. PMN influx was however significantly greater after the combined administration of LTA and PepG than the additive effect of the two components alone. This suggests that LTA and PepG may act in synergy to cause PMN recruitment in the early phase of S. aureus pneumonia. This synergy may either function as a safety mechanism for the host by triggering an adequate innate immune response, or on the other hand may cause lung injury and dysfunction as observed during fulminant pneumonia.

CONCLUDING REMARKS

In this thesis we obtained insight into host defense mechanisms that contribute to an adequate response to (myco)bacteria invading the pulmonary compartment. Since the pathogenicity of (myco)bacteria is critically dependent on the outcome of the interaction between the macrophage and bacterium, we focused mainly on the role of macrophages during pulmonary infections. This thesis demonstrated that macrophages have very diverse functions during pulmonary infection with (myco)bacteria. Resting macrophages seem to provide M. tuberculosis with a good hiding and replication site and are therefore detrimental for host resistance against tuberculosis. In contrast, activated macrophages are inducers of protection against infection with M. tuberculosis, presumably by displaying several complex antimicrobial defense mechanisms and by inducing an
adequate immune response. When activation of macrophages is incomplete or not induced, macrophage apoptosis in lungs of tuberculosis patient could be a host defense mechanism. In this way mycobacterial growth is restricted by depriving mycobacteria from host cells and by constraintment of mycobacteria in apoptotic bodies that can be engulfed by newly recruited alveolar macrophages. Next to the initiation of an adequate immune response, alveolar macrophages also inhibit and dissolve infections in order to prevent lung tissue damage and to restore lung homeostasis.

Treatment of pulmonary infections is often hampered by the increasing incidence of multi-drug resistant bacterial strains and the long term use of medicines in case of tuberculosis. Knowledge of the pathogenesis of pulmonary infections is crucial for the development of new therapeutic strategies. This thesis has led to a better understanding of the basal mechanisms by which the host and especially macrophages interact with (myco)bacteria to inhibit bacterial growth and to prevent tissue damage. This understanding provides interesting guidelines for the treatment of pulmonary infections like tuberculosis. A strategy for the treatment of mycobacterial infections could for example be the induction of apoptosis in alveolar macrophages. In mice we have seen that alveolar macrophage apoptosis dramatically restricted mycobacterial growth. Precaution is however warranted since this approach could interfere with the enormous diverse functions of alveolar macrophages. Besides, pathogens other than \textit{M. tuberculosis} can take advantage of the impaired host immunity and in this way cause opportunistic pulmonary infections. Since depletion of activated macrophages appeared detrimental for host defense against intracellular \textit{M. tuberculosis}, the induction of apoptosis should take place in a selective way leaving activated macrophages alone. As yet, this seems technically difficult to realize. The activation of alveolar macrophages might be a better strategy for treating mycobacterial lung infections. Macrophages are simple to reach with liposomes, synthetic phospholipid vesicles that are easily and mainly taken up by these cells. By using liposomes as carriers of immunomodulators, macrophages can be influenced in a safe and directed manner. By encapsulating macrophage activators such as muramyl tripeptide analogs in these liposomes, the macrophage can be activated. This approach is already evaluated in patients with cancer metastasis to lungs. Intravenous administration of different liposome-encapsulated macrophage activators led to activation of alveolar macrophages, and eradicated lung metastases in several murine tumor systems and in spontaneous canine osteosarcoma (reviewed in ref 1). The destruction of metastases seemed directly mediated by activated macrophages. Next to liposomes
containing immunomodulators, CpG motifs could be used for the treatment of mycobacterial lung infections. Synthetic CpG motifs are able to activate macrophages to produce Th1 cytokines and to induce Th1 responses. The data from chapter 7 demonstrated that this was indeed advantageous in murine host defense against *M. tuberculosis*. The risk of activating macrophages would nevertheless be the induction of tissue damage, and the induction of systemic effects caused by the abundance of cytokines such as TNF-α. Furthermore, treatments could be hampered by the presence of lung granulomas in tuberculosis patients. Liposomes and activated macrophages may not be able to sufficiently infiltrate these lesions. Therefore, a possible therapeutic strategy directed against activation of alveolar macrophages should always be applied in combination with conventional therapy.

**Reference**