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Chapter 11

Summary and general discussion
Summary
Infectious diseases are a major cause of morbidity and mortality worldwide. The lung is prone to become infected due to constant exposure of pathogens through the large contact area of the lung with inhaled air. Bacteria have the ability to continuously adapt to the immune system and antibiotics, and the increasing antibiotic resistance demands more insight into factors involved in the innate immune response during infection. Although the host inflammatory response is needed for effective clearance of an infection, it can also cause damage to tissues, not only after infection but also during non-infectious inflammation. Chapter 1 is a general introduction that describes the known functions of CD44 and osteopontin (OPN), and the infectious and inflammatory diseases relevant for the studies presented in this thesis.

In the first part of this thesis we examined the role of CD44 during the inflammatory response either induced by hyperoxia or by infection using CD44 knockout (KO) mice. Chapter 2 describes the role of CD44 during hyperoxia induced lung inflammation. CD44 KO mice showed a reduced resistance as indicated by 37.5% mortality within the 72-hour observation period whereas all wild-type (WT) mice survived. In addition, CD44 deficiency was associated with a profound influx of neutrophils into the bronchoalveolar space, enhanced proinflammatory cytokine and chemokine release and increased vascular leak. Strikingly, CD44 protected against bronchial epithelial cell death. OPN KO mice were indistinguishable from WT mice during exposure to hyperoxia for up to 72 hours. This chapter shows that CD44 is protective during hyperoxia induced lung injury by a mechanism that does not rely on its interaction with OPN. In chapter 3 we report on the role of CD44 during Escherichia (E.) coli induced abdominal sepsis. CD44 KO mice demonstrated enhanced CXC chemokine and cytokine levels in peritoneal lavage fluid. In accordance, CD44 KO peritoneal macrophages released enhanced levels of these mediators upon stimulation with E. coli or lipopolysaccharide (LPS) in the presence of autologous serum. In contrast, CD44 KO blood leukocytes secreted similar amounts of these mediators upon ex vivo incubation with E. coli or LPS. The proinflammatory phenotype of CD44 KO macrophages was not associated with an altered expression of inhibitors of Toll-like receptor (TLR) signaling, whereas it could be partially reversed by addition of WT serum. We here conclude that CD44 differentially influences cytokine and chemokine release by different leukocyte subsets. In chapter 4 we studied the function of CD44 during gram-positive pneumonia. During lethal Streptococcus (S.) pneumoniae induced pneumonia CD44 KO mice showed a prolonged survival, which was accompanied by
diminished pulmonary bacterial growth, reduced bacterial dissemination to distant body sites and increased early neutrophil influx into the lungs of these animals. We confirmed a detrimental role of CD44 in defense against this pathogen during sublethal pneumonia, as demonstrated by an improved capacity of CD44 KO mice to clear a low infectious dose. In addition, CD44 appeared important for the resolution of lung inflammation and the clearance of hyaluronic acid (HA) during sublethal pneumonia. These data are in line with the results of the study described in chapter 5 in which we examined the role of CD44 during gram-negative pneumonia induced by Klebsiella (K.) pneumoniae infection: CD44 KO mice demonstrated reduced bacterial growth and dissemination during lethal infection, increased neutrophil recruitment, reduced gene expression of the negative regulators of TLR signaling interleukin-1R-associated-kinase (IRAK)-M, A20 and suppressor of cytokine signaling 3, and impaired resolution of HA and inflammation after sublethal K. pneumoniae infection or after intrapulmonary delivery of LPS derived from Klebsiella. Interestingly, in contrast to S. pneumoniae infection, CD44 KO mice demonstrated no survival benefit during lethal K. pneumoniae infection, probably due to the enhanced pulmonary inflammation in these mice also during lethal Klebsiella pneumonia. In Chapter 6 we examined the function of IRAK-M during bacterial pneumonia. The absence of IRAK-M resulted in a strongly improved host defense as reflected by reduced bacterial growth in the lungs and diminished dissemination to distant body sites, as well as higher survival rates, which was accompanied by an increased extent of lung inflammation shortly after K. pneumoniae or S. pneumoniae infection. These data indicate that IRAK-M impairs host defense during both gram-negative and gram-positive pneumonia.

In the second part of this thesis we focused on the role of OPN in the host response to various pulmonary pathogens. In chapter 7 we examined the function of OPN during K. pneumoniae infection. We showed that pulmonary and plasma OPN levels are increased during Klebsiella pneumonia. OPN KO mice appeared more susceptible to infection with K. pneumoniae as indicated by higher bacterial loads in these mice. Early neutrophil recruitment into the bronchoalveolar space was impaired in the absence of OPN after intrapulmonary delivery of either intact Klebsiella or Klebsiella LPS. Moreover, recombinant OPN induced neutrophil migration into the bronchoalveolar space, which was independent from the presence of CD44. This chapter demonstrates that OPN in the bronchoalveolar space serves a chemotactic function towards neutrophils, thereby facilitating an effective innate immune response. In contrast, OPN impaired host defense during pneumococcal pneumonia (chapter
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8). OPN KO mice showed a prolonged survival after infection with *S. pneumoniae* via the airways, which was accompanied by diminished pulmonary bacterial growth and dissemination to distant body sites, and reduced pulmonary inflammation. In contrast to pneumococcal pneumonia, OPN deficiency did not influence bacterial growth in primary pneumococcal sepsis induced by direct intravenous infection, suggesting that the detrimental effect of OPN on antibacterial defense during pneumonia primarily is exerted in the pulmonary compartment. Importantly, OPN was constitutively present in lungs of naive WT mice and OPN concentrations increased during *S. pneumoniae* induced pneumonia. Interestingly, recombinant OPN stabilized *S. pneumoniae* viability in vitro, suggesting that this pathogen misuses OPN in the airways for optimal growth and to cause invasive disease after entering the lower airways. In chapter 9 we demonstrated that OPN also impairs host defense during gram-negative sepsis due to pulmonary *Burkholderia pseudomallei* infection (melioidosis). We observed that plasma OPN levels were elevated in patients with severe melioidosis, and that these levels correlated with mortality and successful therapy. In experimental melioidosis in mice plasma and pulmonary OPN levels were also increased. OPN KO mice were protected from melioidosis as demonstrated by delayed mortality, reduced bacterial numbers in lungs, diminished pulmonary tissue injury, and decreased neutrophil infiltration in these mice. In chapter 10 we show that OPN is not crucial for protective immunity during murine tuberculosis and that during the late phase of tuberculosis it may even be detrimental for the host. There was no significant difference between WT and OPN KO mice in terms of bacterial outgrowth, pulmonary inflammation or inflammatory cell recruitment to the lungs during the first five weeks after infection. Despite an unaltered immune response in the early phase of tuberculosis, OPN KO mice showed a modest survival advantage, and both pulmonary bacterial loads and lung inflammation were reduced in these mice during the late phase of tuberculosis.

**General discussion**

With the experimental studies described in this thesis we intended to obtain further insight into the functional role of CD44 and its ligand OPN during infection and inflammation especially in the lung. While interpreting the results one has to keep in mind that although inflammation is a key element of the antibacterial host defense, excessive or prolonged presence of inflammation can result in tissue damage, a severe systemic inflammatory response syndrome and ultimately multiple organ
failure. Thus, a carefully balanced response is vital in order to survive and recover from a severe infection.

In the first part of this thesis we focused on the role of CD44 during sterile pulmonary inflammation and during infections caused by the diverse pathogens E. coli, S. pneumoniae and K. pneumoniae. Our finding that CD44 is protective during hyperoxia induced lung injury is in accordance with the result that during Klebsiella pneumonia CD44 limits lung inflammation, which however, was not the case during lethal pneumococcal pneumonia. This result clearly adds to the notion that host defense pathways against different pathogens, although both cause pneumonia, rely on distinct mechanisms. On the other hand, CD44 facilitated bacterial growth and dissemination of both K. pneumoniae and S. pneumoniae. The finding that the presence of CD44 accelerated mortality only during lethal pneumococcal and not Klebsiella pneumonia is probably due to the enhanced pulmonary inflammation counteracting the reduced bacterial growth in lungs from CD44 KO mice during Klebsiella pneumonia. Importantly, after infection with lower Klebsiella doses, that were not associated with an impact of CD44 on bacterial growth, CD44 KO mice but not WT mice demonstrated mortality, further illustrating the importance of a balanced inflammatory response. Interestingly, in all these models neutrophil recruitment into the pulmonary compartment was enhanced in CD44 KO mice, despite the reduced bacterial loads in these mice during pneumonia, thus strongly suggesting that CD44 impairs neutrophil recruitment into the lung. On the other hand, intrapulmonary administration of Klebsiella derived LPS resulted in decreased neutrophil recruitment to the lungs of CD44 KO mice, suggesting a positive role for CD44 in neutrophil migration. Several other in vitro and in vivo studies add to this confusion (1-6); a possible explanation for this discrepancy might be that the contribution of CD44 to neutrophil migration from the circulation into tissue depends on vascular integrity and shear stress, and thus on the severity of tissue damage. Moreover, the resolution of inflammation and of HA due to sublethal pneumonia, either induced by S. pneumoniae or K. pneumoniae, or due to sterile inflammation induced by Klebsiella derived LPS, was impaired in the absence of CD44. As a functional role for CD44 in the resolution of inflammation and the clearance of HA had been described for sterile pulmonary inflammation (6, 7), our results underline this function of CD44 and extent this to infection induced lung inflammation.

In vitro experiments offer valuable tools to discover specific biological functions and pathways; however, the final proof of these findings requires in vivo experiments.
especially when it concerns infection with bacteria that can expand in the host. We demonstrated enhanced cytokine and chemokine release by CD44 deficient peritoneal macrophages ex vivo and confirmed this finding in vivo as indicated by elevated cytokine and chemokine levels at the primary site of infection in CD44 KO mice during *E. coli* induced peritonitis. Despite this significant effect on proinflammatory mediators CD44 deficiency did not affect bacterial growth, neutrophil recruitment or organ injury during this infection, underlining the importance of in vivo studies to study the net result of several processes occurring together and influencing each other during infection. Interestingly, cytokine and chemokine release by leukocytes in whole blood was not altered, and in contrast to alveolar macrophages (6), the proinflammatory phenotype of CD44 KO peritoneal macrophages was not due to impaired expression of negative regulators of TLR signaling. Together, our studies on CD44 clearly demonstrate that CD44 potentially affects several inflammatory processes but that the net effect on the course and outcome of the infection is dependent on the pathogen, the dose of infection, and the organ involved. Our result that IRAK-M impairs the antibacterial response during *Klebsiella* and pneumococcal pneumonia is in accordance with previous results demonstrating that IRAK-M dampens TLR and IL-1/IL-18 induced proinflammatory responses (8-11). The fact that elimination of a protein that has evolved to inhibit excessive inflammation, results in an improved outcome during pneumonia further illustrates the complexity and importance of a balanced immune response.

In the second part of this thesis we focused on the role of OPN during pulmonary infection. Our findings that OPN deficiency is beneficial in one pneumonia model and detrimental in the other, clearly adds to the notion that OPN mediated host defense against different pathogens, although all of them cause pneumonia, relies on distinct mechanisms. While OPN KO mice demonstrated reduced bacterial loads during pneumonia induced by the gram-positive pathogen *S. pneumoniae*, the same mouse strain showed enhanced bacterial growth during (gram-negative) *Klebsiella* pneumonia. The reduced burdens of *S. pneumoniae* in the absence of OPN are probably due to a stabilizing effect of OPN on pneumococcal viability. On the other hand, the phenotype during *Klebsiella* pneumonia appeared to result from impaired early neutrophil recruitment in the absence of OPN, and indeed we demonstrated that OPN is chemotactic in the lung. In correspondence, gram-positive bacteria induce different mechanisms that regulate neutrophil recruitment to the lung than gram-negative bacteria do (12, 13). In addition, neutrophil appearance and bacterial
growth occurred faster during *Klebsiella* pneumonia than during pneumococcal pneumonia. As such, it is likely that the chemo-tactic activity of OPN, that is crucial during *Klebsiella* induced pneumonia, also occurs during pneumococcal pneumonia and vice versa, but that the net effect of OPN is determined by the process that dominates during infection by that particular pathogen.

In view of the known effect of OPN on T-helper (Th)1 mediated immune responses, we also encountered some unexpected findings by studying the *in vivo* role of OPN during *B. pseudomallei* and *M. tuberculosis* infection. *A priori*, we hypothesized that OPN would have a marked influence on the immune response that is induced by these (myco)bacteria as the development of a strong Th1 mediated immune response is considered of main importance for host defense against these pathogens (14-17), and several *in vitro* and *in vivo* studies have suggested that OPN induces Th1 responses (18-21). Much to our surprise, we found that OPN impairs host defense during melioidosis and that OPN is not crucial for a protective immune response during tuberculosis. Interestingly, although *B. pseudomallei* like *K. pneumoniae* is an gram-negative pathogen, we did not find an effect of OPN early during infection, but rather demonstrated that sustained production of OPN impairs host defense during melioidosis. As neutrophils are the main infiltrating cells during melioidosis (22) and the area of affected lung is reduced in OPN KO mice in a later phase of infection, a plausible explanation would be that pulmonary OPN in WT mice continues to attract neutrophils to the lung thereby enhancing tissue damage. Otherwise, reduced neutrophil recruitment can also be a result of decreased bacterial loads, which in turn can result in less necrotic tissue that provides a niche for bacterial expansion. In accordance with our findings for melioidosis, OPN did not affect the early phase of tuberculosis, whereas in the late phase bacterial loads and pulmonary inflammation were reduced in the absence of OPN. Again, the diminished mycobacterial loads in OPN KO lungs might be a consequence of less mycobacteria-containing macrophages available. However, considering that late phase tuberculosis implies several months of disease, additional ‘restore’ processes that can be affected by OPN, such as tissue remodelling and fibrosis formation (23-26), might now interfere with the primary antibacterial response.

In our investigations we used experimental mouse models to study diseases that are of human importance. The high degree of homology between the mouse and human immune system makes the mouse a scientific proper model for studying inflammation and infection. Nevertheless, when interpreting our data we have to
be cautious in extrapolating data obtained from mouse experiments to the human situation, as there are discrepancies between the experimental models we used and infections occurring in humans. Although we tried to infect the animals as ‘naturally’ as possible, we are limited to administer a relatively high number of bacteria by intranasal inoculation, whereas in the human situation pneumonia usually occurs by inhalation of smaller numbers of bacteria or results from prior colonisation of the upper respiratory tract, after which the infection gradually develops in time. Moreover, to study the role of CD44, IRAK-M or OPN we used animals with a single gene deficiency, which on the one hand is an elegant approach to determine the role of these specific proteins, but on the other hand may introduce bias due to development of compensatory mechanisms in these genetically modified mice that do not directly relate to the genetic deletion (27). Our studies on the function of CD44 and OPN during Klebsiella and pneumococcal pneumonia demonstrate that, although OPN is a ligand for CD44, the absence of either protein affects the immune response differently. Moreover, the protective effect of CD44 during hyperoxia induced lung injury is OPN independent. Indeed, CD44 is not the only receptor for OPN (28), and OPN is not the single ligand for CD44 (29). It would be highly interesting to further explore which receptor-ligand interactions are involved in the distinct functions of CD44 and OPN described in this thesis. In addition, our results clearly demonstrate that the net effect of eliminating CD44 or OPN during pulmonary infection is dependent on the pathogen involved and on the stage of the infection. Therefore, it is too early to introduce interventions targeting these molecules as therapeutic tools. However, we have shown that for melioidosis patients OPN plasma levels can be used as an indicator for mortality or therapy success. Knowledge derived from this research can further be extended to specify effects during particular infectious and inflammatory diseases, and especially to fine-tune timing of possible new treatment strategies.
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