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Citation for published version (APA):

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Chapter 11

Summary and general discussion of this thesis
“Cell-specific pattern recognition receptor signaling in antibacterial defense”

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Summary

Sepsis, the syndrome that describes infection complicated by acute organ failure, is most frequently caused by bacterial pneumonia and infection originating from the abdominal cavity and is a major cause of morbidity and mortality globally. Mortality rates remain high, despite the improvement of supportive therapies in the past decades. The global rise of antimicrobial resistance rates together with the fact that there currently is no prospect of the availability of new antimicrobial agents is alarming. A brisk and firm initial host response is needed for clearance of the pathogen, but on the other hand can induce tissue and organ injury. More insight in the initial immune response during pneumonia and sepsis potentially offers new targets for the development of therapeutic agents.

Chapter 1 is a general introduction describing the mechanisms of the initial host response and the role of pattern recognition receptors during infection and the relevant disease models that were used in this thesis.

In the first part of the thesis we focused on the role of Toll-like receptors (TLRs) and their intracellular adapter proteins myeloid differentiation primary response gene (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) during pneumonia with the gram-negative pathogens Klebsiella (K.) pneumoniae and Pseudomonas (P.) aeruginosa. Specifically, we investigated the role of these proteins in different body compartments and cell-types.

Chapter 2 describes the role of TLR2 and TLR4 during Klebsiella pneumonia. Tlr4−/−, but not Tlr2−/− mice demonstrated enhanced bacterial growth 24 hours after infection, indicating that TLR4 initially was more important for antibacterial defense than TLR2. However during later stage infection or after high dose infection, Tlr2−/− mice had higher bacterial loads than normal wild-type mice, and Tlr2/Tlr4 double deficient mice were more susceptible than Tlr4−/− mice. Moreover, using bone marrow chimeras, we demonstrated that hematopoietic TLR2 and TLR4 determined antibacterial capacity, while parenchymal TLRs did not contribute to a significant extent. This chapter shows that TLR4 is important for the initiation of the early host defense against K. pneumoniae, while TLR2 contributes to host defense against a higher infectious inoculum or during later stage infection. Moreover, TLR2 and TLR4 dependent signaling in hematopoietic cells is of primary importance to limit bacterial growth. In chapter 3 we report on the role of MyD88 and TRIF during K. pneumoniae infection and their respective roles in hematopoietic and non-hematopoietic cells. Both TLR adapters were crucial for antibacterial defense and survival, while Myd88−/− mice, that all died within 48 hours after infection, were even more susceptible than TRIF mutant mice. MyD88 in both hematopoietic and non-hematopoietic cells contributed to antibacterial defense during late stage infection and survival, while only TRIF in hematopoietic cells was protective. On the other hand, MyD88 in resident cells and TRIF in hematopoietic cells contributed to distant tissue injury. Early after infection, MyD88 in non-hematopoietic cells was crucial for the local production of chemokines and neutrophil attraction, while TRIF in both hematopoietic and non-hematopoietic cells was equally important. We here conclude that MyD88- and TRIF-dependent signaling have a different contribution to the host defense in different cell types that changes during the course of gram-negative infection.
In chapter 4 we further studied the role of specific cell MyD88 signalling during *K. pneumoniae* infection. Mice deficient for MyD88 in myeloid (LysM-Myd88<sup>−/−</sup>) and myeloid plus endothelial (Tie2-Myd88<sup>−/−</sup>) cells showed enhanced mortality and higher bacterial loads. Tie2-Myd88<sup>−/−</sup> mice reconstituted with control bone marrow, representing mice with a selective MyD88 deficiency in endothelial cells, showed an unremarkable antibacterial defense. Myeloid or endothelial cell MyD88 deficiency did not impact on lung pathology or distant organ injury during late stage sepsis, while LysM-Myd88<sup>−/−</sup> mice demonstrated a strongly attenuated inflammatory response in the airways early after infection. These data suggest that myeloid but not endothelial MyD88 is important for host defense during gram-negative pneumonia derived sepsis. In chapter 5 we further focused on the role of TRIF during gram-negative pneumonia and especially its role in the induction of interferon (IFN)-γ. The impaired antibacterial defense of TRIF mutant mice was associated with absent IFN-γ production in the lungs. Furthermore, in vitro IFN-γ production by splenocytes in response to *K. pneumoniae* was critically dependent on TLR4, MyD88 and TRIF. When TRIF mutant mice were reconstituted with recombinant IFN-γ via the airways, bacterial loads were reduced in lungs and distant body sites similar to levels measured in wild-type mice, while pulmonary cytokine levels were partially restored. The IFN-γ induced improved enhanced antibacterial response in TRIF mutant mice occurred at the expense of increased hepatocellular injury. These data indicate that TRIF mediates antibacterial defense during gram-negative pneumonia at least in part by inducing IFN-γ at the primary site of infection. In chapter 6 we investigated the role of MyD88 dependent signaling during airway infection with *P. aeruginosa*. Sftpccre-Myd88<sup>−/−</sup> mice, that are deficient for MyD88 in lung epithelial cells, demonstrated an impaired early bacterial clearance of *P. aeruginosa*, as well as impaired neutrophil recruitment and a selective impairment of CCL20 secretion. Tlr5<sup>−/−</sup> mice also demonstrated an impaired early antibacterial and inflammatory response that was dependent on non-hematopoietic cells in bone marrow chimeras, findings pointing to an interaction between TLR5 expressed by epithelial cells with flagellin (TLR5 ligand) expressed by *Pseudomonas*. Indeed, by the use of an unflagellated *Pseudomonas* mutant we could demonstrate that the detection of flagellin by MyD88 in lung epithelial cells is crucial for the initiation of the host response. Together, these data indicate that recognition of *Pseudomonas* flagellin by epithelial cell TLR5-MyD88 initiates host defense to induce clearance of *P. aeruginosa* from the airways.

In the next part of the thesis we focused on another component of the innate immune system demonstrated to be important for antimicrobial defense, the “NLRP3-inflammasome” (consisting of a protein complex formed by NLR family, pyrin domain containing 3 (NLRP3) and the adaptor apoptosis-associated speck-like protein containing a CARD (ASC)), during pneumonia caused by *Streptococcus* (*S.*) *pneumoniae*, the most common causative agent in community-acquired pneumonia. Chapter 7 describes the role of NLRP3 and ASC in the host response during pneumococcal pneumonia with the serotype 2 D39 strain. Both Nlrp3<sup>−/−</sup> and Asc<sup>−/−</sup> mice demonstrated impaired bacterial clearance from the lung, but only Asc<sup>−/−</sup> mice had increased mortality rates compared to wild-type mice. The early inflammatory response was disturbed in both genetically modified mouse strains as
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reflected by impaired cytokine secretion. Detailed analysis of the early inflammatory response in the lung by whole-genome transcriptional profiling identified several mediators that were differentially expressed between \( \text{Nlrp}^3\text{-/-} \) and \( \text{Asc}^\text{-/-} \) mice, possibly explaining the differences in lethality of these mice. These data confirmed a prominent role for the NLRP3-inflammasome during pneumococcal pneumonia and suggest that either ASC-dependent NLRP3-independent inflammasomes or inflammasome-independent ASC functions may be involved. In chapter 8, however, we demonstrate an opposite role of NLRP3 and ASC during infection with a serotype 3 pneumococcal strain. Notably, both \( \text{Nlrp}^3\text{-/-} \) and \( \text{Asc}^\text{-/-} \) mice showed a strongly improved host defense, as reflected by markedly improved survival rates and accompanied by diminished bacterial growth and dissemination. The early inflammatory response was slightly enhanced in \( \text{Nlrp}^3\text{-/-} \) and \( \text{Asc}^\text{-/-} \) mice, while lung inflammation and pathology were attenuated in \( \text{Nlrp}^3\text{-/-} \) and \( \text{Asc}^\text{-/-} \) mice during the late stages of the infection. Moreover, we investigated the contribution of MyD88 dependent signaling during infection with this specific virulent pneumococcal strain. Bacterial growth and survival were unaltered in \( \text{Myd}^\text{88/-} \) mice, although these mice demonstrated attenuated lung inflammation in the presence of high pneumococcal burdens. These data demonstrate that the contribution of proximal innate detection systems, such as the NLRP3-inflammasome and TLRs, can be dispensable depending on the pathogen and can vary between strains within the same bacterial species.

In chapter 9 the role of Single immunoglobulin IL-1 receptor-related molecule (SIGIRR), a negative regulator of TLR- and IL-1 receptor dependent inflammation during pneumococcal airway infection and sepsis is described. \( \text{Sigirr}^-\text{-/-} \) mice demonstrated delayed mortality and had significantly lower bacterial loads both during pneumococcal airway and bloodstream infection, while the inflammatory response was not different. However, in vitro, \( \text{Sigirr}^-\text{-/-} \) alveolar macrophages and neutrophils demonstrated higher phagocytic rates, possibly explaining enhanced bacterial defense.

Finally, we examined the effect of anti-TLR4 therapy, designed as an additional treatment to modulate excessive inflammation in human sepsis, in a model of murine abdominal sepsis. Chapter 10 shows that mice infected with a low infectious inoculum demonstrated higher bacterial loads after receiving anti-TLR4 therapy in a delayed treatment model of \( \text{E. coli} \) peritonitis, while organ injury was slightly preserved and survival was similar to the placebo treatment group. This illustrates that therapeutic targeting of TLR4 signaling can potentially interfere with bacterial clearance even in the context of antibiotic therapy, but on the other hand can help limit some aspects of tissue injury depending on the infectious dose and kinetics of the infection model.
General discussion

With the experimental studies presented in this thesis we intended to gain more insight in the role of innate immune receptor signaling, including TLR dependent and especially MyD88-dependent pathways as well as inflammasome receptors, in experimental models of infection and sepsis. The focus was on pneumonia since this is the most common cause of sepsis. Secondly, the aim was to gain more insight in the contribution of different cell types and body compartments to TLR and MyD88 dependent signaling during infection and sepsis, considering that innate immune sensors are widely distributed among different cell types. In sepsis, inflammation is a key element of host defense, but on the other hand can result in tissue damage, a severe systemic inflammatory response and organ failure. Different cell types may contribute differentially to these inflammatory processes; this is potentially interesting for the development of new therapies.

In the first part of the thesis, we focused on the role of TLR-dependent signaling during *K. pneumoniae* airway infection and sepsis. We observed a dynamic pattern in the contribution of different TLRs during the course of infection (chapter 2): TLR4 was essential for the early initiation of the host defense while TLR2 became important when bacterial loads were higher possibly (in part) by amplifying TLR4 signaling. These observations are in line with previous research on experimental *E. coli* peritonitis (1), despite the profound differences between these models of infection, underlining the importance of TLR4 and TLR2 during various infections. These findings are also in accordance with the fact that the phenotype of MyD88 deficient mice during *K. pneumoniae* infection was more severe than that of TRIF mutant mice (chapter 3), since MyD88 mediates the signals of both TLR2 and TLR4, while TRIF only participates in TLR4 dependent signaling. The observation that the expression of TLR2 and TLR4 on hematopoietic cells is sufficient to limit bacterial growth was corroborated by the finding that TRIF in hematopoietic cells determined bacterial growth during later stage infection and not the expression of TRIF in resident cells (chapter 3), reflecting the importance of TLR4 mediated signaling. However, the expression of MyD88 in both hematopoietic and resident cells was important for the antibacterial response during late stage *Klebsiella* infection (chapter 3), pointing to a role for MyD88 in the lung epithelium or other resident cells, similar as was demonstrated before in a model of *P. aeruginosa* airway infection (2). Indeed, during early stage infection MyD88 in resident cells was especially important for neutrophil attraction and cytokine production. We confirmed the importance of hematopoietic and especially myeloid MyD88 during late stage infection in chapter 4 and ruled out a contribution of endothelial MyD88 to antibacterial defense. However so far, we were not able to identify the resident cell type in which MyD88 dependent signaling confers protection in this model of gram-negative pneumonia since preliminary data show that MyD88 dependent signaling in type II lung alveolar cells or in Clara epithelial cells is not protective in this *K. pneumoniae* model. Possibly, our bone marrow chimera experiments were confounded by incomplete repopulation, or alternatively another (lung) cell type not targeted so far is more important. The finding that MyD88 in lung epithelial cells does
not contribute to host defense during *K. pneumoniae* infection is in contrast with our studies on *P. aeruginosa* airway infection (chapter 6). Here, we demonstrate that MyD88 in lung epithelial cells is crucial for the early initiation of the host response and the recruitment of neutrophils, and that the detection of flagellin is involved in this process. In line with this, we demonstrate that TLR5 (that detects flagellin) is involved in the initiation of early antibacterial defense and that its expression on resident cells is most important. This was the first time that the role of TLR5 on parenchymal cells in *Pseudomonas* airway infection was directly demonstrated. The observations above illustrate that host defense during airway infection is a dynamic process and that the way that different TLRs and their adapters in various cell types contribute during the course of the infection vary depending on the causative pathogen and the stage of the infection.

Our findings in chapter 7 and 8 further illustrate the complicated nature of the innate immune response, since we here demonstrate opposite roles for the inflammasome proteins ASC and NLRP3 during pneumococcal infection with two different bacterial strains: these proteins conferred protection in a model using one specific pneumococcal strain that is frequently used in experimental models, but played a detrimental role during infection with a virulent, clinically relevant *S. pneumoniae* strain. It is tempting to speculate that these different outcomes may depend on the net balance between the seemingly opposite roles of ASC and NLRP3 in key antibacterial responses, i.e., their ability to mediate bacteria-induced pyroptosis (an inflammatory form of cell death that may inhibit bacterial killing) and autophagy (a non-inflammatory form of cell death that is thought to attenuate pyroptosis) versus their ability to produce proinflammatory cytokines (favoring bacterial clearance); however the exact nature and effects of these processes have not been unraveled so far (3, 4). Our studies illustrate that the balance of benefit and harm that can result from these innate immune defense pathways can vary even after infection with pathogens within the same species. This is also illustrated by our finding that MyD88 dependent signaling during infection with a serotype 3 pneumococcus was not protective (chapter 8), while this was previously considered to be part of a protective host response after infection with moderately virulent pneumococcal strains (5); our group observed the same protective role of MyD88 during infection with the serotype 2 D39 strain (6).

Our observations in chapter 9 further illustrate the complicated interactions of innate immune receptor signaling. In this chapter we found a negative effect of the negative TLR and IL-1R regulator SIGIRR on survival and bacterial loads during pneumonia and blood-stream infection caused by the serotype 3 *S. pneumoniae* also used in chapter 8. SIGIRR is known to exert inhibiting activity on TLR4, TLR7, TLR9, IL-1R type I (IL-1RI), IL-18R, and ST2 (7). In earlier research mice deficient for TLR4, TLR9, IL-1R or IL-18R were reported to have an impaired bacterial response against *S. pneumoniae* (8-11) and therefore these receptors might be involved as targets for SIGIRR in this case. However when considering the observations in chapter 8 of this thesis, the here observed role of SIGIRR is still difficult to interpret, since all of its target proteins are (partially) dependent on MyD88 mediated signaling, except for ST2 that was demonstrated to have a limited role during infection with
the same pathogen (12, 13). Possibly, the observed effect of SIGIRR results from the combined inhibition of multiple receptors or unknown interactions or feed-back mechanisms between them. Together, this part of the thesis illustrates that the effect of the various innate immune receptors on antibacterial host defense results of a complicated interplay between various (anti)inflammatory processes that moreover may be specific for each pathogen and stage of the infection.

During infection, survival of the host is not only determined by the effectivity of the antimicrobial response and the clearance of the pathogen, but also by the severity of organ injury that is thought to occur as a result of a strong inflammatory response. This double edged sword character of the immune response during sepsis was clearly illustrated in some of our experimental models where the combat against bacteria occurred at the cost of organ injury. For example, during Klebsiella induced pneumonia derived sepsis organ injury was attenuated in mice deficient for MyD88 in resident cells as well as in mice deficient in TRIF in hematopoietic cells, while they had higher bacterial loads (chapter 3). Furthermore, improvement in antibacterial defense in TRIF deficient mice by the administration of IFN-γ (chapter 5) occurred at the expense of organ injury. Eventually, survival was determined by uncontrolled bacterial growth rather than the degree of organ injury in this experimental model of gram-negative pneumosepsis without antibiotic treatment and was therefore impaired in mice deficient for TLR2/4 pathways or their adapters MyD88 and TRIF. However, we observed a similar effect of attenuated TLR4 signaling on the balance between antibacterial defense and organ injury in the delayed treatment model of peritonitis that was used in chapter 11. Here, the selective targeting of TLR4 slightly improved organ injury but still resulted in higher peripheral bacterial loads despite antibiotic therapy.

Previously, there was some evidence pointing to a contribution of endothelial TLR-dependent signaling in the development of organ injury during sepsis as well as in the antibacterial defense (14-18); however, we could not confirm this in our model of gram-negative pneumosepsis in chapter 4. Then again, there were major differences between the infection models that were used and the way inflammatory pathways in endothelial cells were targeted, including the use of Cre-promoters that are not completely cell-specific. In our study we corrected for the leakage of the endothelial promoter Tie2 to the hematopoietic compartment via bone marrow transplantation, which is why we believe our observations truly reflect that in this model there is no role for TLR dependent signaling in the endothelium in antibacterial defense or in organ injury.

In conclusion in this thesis several key points on experimental sepsis were identified: 1) the crucial role of TLR2 and TLR4 and their intracellular adapters MyD88 and TRIF during gram-negative pneumosepsis and their respective contributions in different body compartments as well as during different stages of the infection. 2) the observation that the NLRP3 inflammasome can be both beneficial and detrimental for host defense in pneumococcal pneumonia, depending on the particular pathogen strain. 3) the inflammatory response contributes to organ injury, but on the other hand attenuation of this response by use of adjunctive anti-TLR4
directed therapy may result in less effective bacterial clearance.

Of course, the final question is how these findings in experimental murine sepsis relate to the pathophysiology and treatment of human sepsis. First of all, there are strong limitations in the translation of experimental sepsis studies to the human situation, but at the same time there is no other way to gather new insights. The available evidence is largely obtained from gene deficient mice, while the limitations of genetically modified mice as a model are well known. In humans, true genetic deficiencies for innate immune pathways are relatively rare, likely because they are under evolutionary pressure, but if they do occur the phenotype can be very different from that observed in mice (19). A strong example is provided by human MyD88 deficiency, which is associated with more severe and in a vast percentage even lethal infections in infancy and childhood albeit with only a small group of pathogens (S. pneumoniae, S. aureus and P. aeruginosa) (20). MyD88 deficient subjects are at a later age otherwise healthy, while murine MyD88 deficiency results in a higher susceptibility to virtually all pathogens. Furthermore, while several known human TLR polymorphisms may be associated with a somewhat higher susceptibility to some infectious diseases, this remains an issue of debate and in general do not explain a large part of the burden of sepsis (21, 22).

The large body of evidence that is available from in vitro, animal and pre-clinical studies, as well as genetic studies on the role of TLRs in sepsis, has resulted in the design of TLR-directed therapies. Two different TLR4 inhibiting agents were tested in phase III studies in severe sepsis patients; both failed to improve 28 day mortality rates and further studies were suspended (13, 23). These failures illustrate the problems that were encountered with many other adjunctive sepsis therapies as well; in pre-clinical studies many demonstrated promising results but once studied in a clinical setting therapeutic advantages could not be established. The most likely obstacle in studying adjunctive therapies is that sepsis describes a syndrome, and represents a heterogeneous spectrum of different diseases and pathophysiologies. As described in this thesis, the contribution of different innate immune receptors varies between different experimental sepsis models; between different pathogens and even between pathogens of the same species as well as during the course of the infection. In human sepsis, little is known about the actual activity of different innate immune receptors, let alone about their kinetics or contribution in specific conditions. Importantly, in clinical practice the causative pathogen is initially unknown since traditional cultures at least need 24 hours to yield a positive result and are known to lack sensitivity. Moreover, the stage of the inflammatory response may differ between patients, i.e., the course of the infection may not be similar in all sepsis patients, unlike in experimental sepsis studies. Additionally, the host may or may not have substantial co-morbidity that alters the host response and outcome. Finally, unlike in experimental sepsis models, the quality of standard supportive care has improved substantially and is in most cases sufficient to prevent mortality in the first days; however, severe sepsis patients that do survive initially remain at high risk for mortality due to organ failure and complications, even beyond 28 days (20, 24). For all these reasons, the concept of early intervention in the host innate immune response via targeting of just one receptor or pathway in the whole
group of severe sepsis patients may be too simplistic and therefore not result in measurable treatment effects. The first step in the improvement of additional sepsis treatments may very well be earlier identification of the pathogen, preferable including resistance characteristics, to improve antimicrobial therapy. In addition, enhanced insight in the specifics of the derailed host response in individual patients is warranted using (sets of) biomarkers to better direct and monitor targeted immune modulatory therapies. Future research should seek to integrate detailed observational studies in patients with sepsis in different stages of the disease course with data derived from experimental sepsis models, and link the knowledge obtained with the development of novel targeted interventions and rapid beside tests to identify patients who might benefit from these interventions and to monitor their effect.
References


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