Endogenous danger signals in infectious diseases

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Chapter 13
Summary and General Discussion
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SuMMARy

During a lifetime, we encounter many different bacterial pathogens that are able to cause various infectious diseases. Bacterial infections present a significant burden on healthcare and cause considerable morbidity and mortality [1]. The emergence of bacterial strains that develop resistance to antimicrobial therapy makes treatment of these infections increasingly complicated [2]. In addition, antibiotic therapy may not be sufficient in severe infections to prevent mortality in many patients [3,4]. More insight in host defense mechanisms may identify complementary therapeutic options to further optimize treatment and improve outcome.

Microbes that invade the body are detected by pattern recognition receptors (PRRs) on innate immune cells, which trigger an inflammatory response. Inflammatory mechanisms can be perpetuated through the same PRRs after sensing of self-derived proteins, also known as alarmins, that are released upon cellular stress. Overwhelming release of alarmins can contribute to a disproportionate inflammatory reaction resulting in collateral tissue damage, that may be deleterious to the host [5-7]. Two of the most well characterized alarmins involved in the inflammatory response are High-mobility Group Box 1 (HMGB1) and the Myeloid-related protein 8/14 complex (MRP8/14 or calprotectin).

In this thesis, we focused on these proteins and their receptors (Receptor for Advanced Glycation Endproducts (RAGE) and Toll-like receptor (TLR)4) and their role in bacterial infection. Chapter 1 is a general introduction that describes the alarmins, the receptors and the infectious diseases relevant for the studies presented in this thesis.

In the first part of this thesis we focused on the role of RAGE and RAGE ligands during severe infections such as pneumonia and sepsis. In Chapter 2 we reviewed the literature concerning RAGE and its role during severe infection. In Chapter 3 we investigated the effects of RAGE-ligand neutralization in Escherichia (E.) coli induced abdominal sepsis. Administration of soluble RAGE (sRAGE; a decoy receptor for RAGE) and antibodies against HMGB1 led to enhanced bacterial dissemination to liver and lungs. In addition, sRAGE treatment was accompanied by increased hepatocellular injury and exaggerated systemic cytokine release. This chapter suggest that RAGE ligands, including HMGB1, improve host defense in Gram-negative abdominal sepsis. In Chapter 4 we report on the role of RAGE during Streptococcus (S.) pneumoniae bacteremia. Earlier investigations have shown that the absence of RAGE limits inflammation and bacterial dissemination, and increases survival in pneumococcal pneumonia derived sepsis [8]. We here directly injected S. pneumoniae into the circulation and thereby circumvented the extensive RAGE-expressing lung [9]. Absence of RAGE attenuated the systemic inflammatory response but did not influence bacterial loads or survival in this model. These results suggest that outcome of pneumococcal infection depends on the level of RAGE expres-
sion in the organ that is primarily infected (i.e., the lung). We conclude that while RAGE plays a harmful part in *S. pneumoniae* sepsis originating from lung, it has a limited role in the outcome of primary bloodstream infection. In chapter 5 we investigated the role of HMGB1 and its receptors RAGE and TLR4 in *Staphylococcus (S.) aureus* pneumonia. HMGB1 was released in the bronchoalveolar compartment and contributed to protein leak and lung edema in the early phase, while it did not influence neutrophil recruitment or bacterial clearance. RAGE deficiency was associated with reduced lung pathology and inflammation as well, and lower bacterial loads at the 6-hour time point. Although TLR4 has been implicated as the dominant proinflammatory receptor for HMGB1 [10], this receptor had only limited impact on the injurious host response during *S. aureus* pneumonia. In conclusion, both HMGB1 and RAGE have a distinct harmful role in the development of lung injury during the early phase of *S. aureus* pneumonia. Chapter 6 describes the role of RAGE during *Klebsiella (K.) pneumoniae* pneumonia. Intranasal *Klebsiella* infection induced pulmonary expression of RAGE and increased bronchoalveolar HMGB1 levels. RAGE deficient (*rage*⁻/⁻) mice had an impaired host defense in *K. pneumoniae* pneumonia derived sepsis as reflected by increased bacterial outgrowth in multiple organs and a worsened survival. Lung inflammation, cytokine and chemokine levels were hardly influenced. We conclude that RAGE contributes to an effective antibacterial host response during *K. pneumoniae* pneumonia, but plays an insignificant part in the lung inflammatory response. In chapter 7 we addressed the question whether RAGE is involved in local and systemic host defense in staphylococcal skin infection. We showed that RAGE plays a limited role in local host defense as shown by similar bacterial burdens and cytokine/chemokine levels at the primary site of infection. At distant sites however, *rage*⁻/⁻ mice displayed lower bacterial burdens. In accordance, direct intravenous infection with *S. aureus* was associated with lower bacterial loads in lungs and liver of *rage*⁻/⁻ mice. Together these data suggest that RAGE does not impact local host defense during *S. aureus* skin infection, but promotes bacterial growth at distant body sites.

In the second part of this thesis we focused on the role of the S100 proteins MRP8/14 (S100A8/9 or calprotectin) and S100A12 in the host response to various pulmonary pathogens, sepsis, and bacterial skin disease. In chapter 8 we show a protective role for MRP8/14 in a clinically relevant model of Gram-negative sepsis originating from the lung, using *K. pneumoniae*. MRP14 deficient (*mrp14*⁻/⁻) mice, unable to form MRP8/14 heterodimers, displayed enhanced bacterial dissemination accompanied by increased organ damage and a reduced survival. Inflammation during *Klebsiella* sepsis was not influenced by MRP8/14. Next to proinflammatory effects, extracellular MRP8/14 is able to reduce microbial growth as it binds divalent cations in infectious environments [11,12]. Previous investigations have shown that MRP8/14 is an important component of Neutrophil extracellular traps (NETs), which are composed of DNA-networks, decorated by antimicrobial proteins from the nucleus, granules and cytoplasm [13]. NETs are re-
leased by neutrophils to form a mesh as a strategy to contain and kill pathogens [14,15]. MRP8/14 most likely mediates its protective effect through binding of divalent cations in infected organs (e.g. in liver abscesses) and by enhancing the capacity of NETs to reduce *Klebsiella* growth.

In chapter 9 we investigated the role of MRP8/14 in clinical and experimental pneumococcal pneumonia. This study describes compartmentalized release of MRP8/14 in two patient populations with community-acquired pneumonia, as well as in the bronchoalveolar space of healthy subjects who were intrabronchially instilled with a component of gram-positive bacteria (lipoteichoic acid). We then linked these human data with functional studies on the role of MRP8/14 in murine pneumonia produced by *S. pneumoniae* infection. In sharp contrast to earlier reports [11-13,16], MRP8/14 enhanced pneumococcal outgrowth and worsened survival in this model. We here describe how the metal chelating capacities of MRP8/14 can be exploited by *S. pneumoniae* to induce invasive disease. In in vitro studies we show that MRP8/14 facilitates pneumococcal growth in the presence of physiological levels of zinc [17]. We conclude that high MRP8/14 is associated with enhanced bacterial outgrowth and lethality, most likely through binding of zinc by MRP8/14 and thereby reduction of zinc mediated toxicity towards the bacterium. In chapter 10 we focused on the role of MRP8/14 in *S. aureus* pneumonia. Previous studies have shown that MRP8/14 assists in the killing of *S. aureus* [11,12]. In the current study MRP8/14 deficiency had a limited effect on bacterial clearance. Surprisingly, absence of MRP8/14 was associated with increased cytokine levels in bronchoalveolar lavage fluid and aggravated lung histopathology. MRP8/14 deficiency in addition was associated with a diminished transmigration of neutrophils into bronchoalveolar lavage fluid. We conclude that MRP8/14 serves in an unexpected protective role for the lung in staphylococcal pneumonia. In chapter 11 we investigated the role of MRP8/14 in local host defense during skin infection caused by *S. aureus*, the most common causative pathogen in this disease. We here show that MRP8/14 facilitates *S. aureus* clearance from the skin. In addition MRP8/14 promoted neutrophil transmigration and the cutaneous inflammatory response. Together these data suggest that MRP8/14 is a protective mediator in *S. aureus* skin disease. In Chapter 12 we report on the extent of S100A12 and its soluble high-affinity receptor sRAGE [18] in patients with severe sepsis stratified to the three most common infectious sources [4] and in healthy human volunteers intravenously challenged with endotoxin. Patients with severe sepsis displayed increased circulating S100A12 concentrations. All severe sepsis subgroups had elevated serum S100A12, with the highest levels in patients with sepsis caused by pneumonia. Intravenous LPS injection in healthy volunteers elevated systemic S100A12 levels as well. In contrast to S100A12, sRAGE concentrations did not change during severe sepsis or human endotoxemia.
GENERAL DISCUSSION

Alarmins are released upon cellular stress and perpetuate inflammation in many disease models [5-7]. Preclinical landmark studies have indicated that in the setting of severe infection (i.e. sepsis and septic shock), alarmins dysregulate inflammatory processes and promote organ failure and death [19-22]. Some alarmins, like HMGB1, are associated with delayed and persistent release providing a clinically relevant timeframe for treatment measures [20]. As such, alarmins appeared to be a promising target for immunodulation in fulminant infections. In this thesis we demonstrate that the alarmin biology in (severe) infections is complex and variable. Alarmins are involved in a variety of innate immune mechanisms in different infectious diseases. Targeting alarmins may not always be of benefit for the host. The studies performed in this thesis contribute to the further understanding of alarmins and their receptors, which may eventually help in the development of new complementary treatment options in bacterial disease.

In the first part of this thesis we focused on the involvement of RAGE and RAGE ligands in pneumonia and sepsis derived from various sites. We described the expression of RAGE and RAGE ligands in mouse sepsis models and in patients with sepsis. RAGE is most abundantly expressed in lung epithelium [9] and its expression can be upregulated upon pulmonary infection [8] as shown in murine Klebsiella pneumonia (Chapter 6). RAGE expression was not enhanced after intravenous infection with S. pneumoniae (Chapter 4). In humans, sRAGE can be found in increased concentrations in the lung during acute lung injury [23]. We were unable however, to find enhanced levels of sRAGE in human sepsis or experimental endotoxemia (Chapter 12), which is in line with previous findings in the literature [23]. RAGE binds several ligands including HMGB1 and S100A12 [24,25]. In models of severe sepsis HMGB1 is released at a significant delay, compared to cytokines such as Tumor Necrosis Factor-α [20]. Nevertheless, HMGB1 kinetics may be distinct in different infectious disease models. We show a modest increase of plasma HMGB1 during murine S. pneumoniae bacteremia (Chapter 4). In addition, we show enhanced HMGB1 levels in the bronchoalveolar compartment in staphylococcal pneumonia reaching peak values at 24 hours (Chapter 5), while there is a transient increase in the course of pneumonia caused by Klebsiella (Chapter 6). In human patients, circulating S100A12 concentrations are increased in all forms of sepsis, for at least 3 days after admittance to the intensive care unit (Chapter 12).

RAGE has been implicated as an important receptor in perpetuation of inflammatory responses. Engagement of RAGE activates the NF-κB pathway, which in turn upregulates expression of RAGE, thus inducing sustained inflammation [24,25]. Deletion of the rage gene improved outcome in fulminant murine abdominal sepsis [19]. Our finding that blockade of RAGE may be ineffective or even harmful in some infectious conditions likely is mediated by distinct mechanisms. RAGE signaling appeared to be
important for an effective antimicrobial host defense in Gram-negative abdominal sepsis induced by E. coli (Chapter 3). Both inhibition of multiple RAGE ligands (by the administration of sRAGE, a decoy receptor for RAGE) and inhibition of HMGB1 resulted in enhanced E. coli dissemination from the primary site of infection. Previous studies have shown similar results in rage−/− mice and anti-RAGE treated mice: inhibition of RAGE signaling in severe Gram-negative abdominal sepsis persistently enhanced bacterial outgrowth, which led to enhanced local and/or systemic inflammation [26]. Similarly, rage−/− mice demonstrated a defective antibacterial response in the lungs and distant organs and a worsened survival in Gram-negative pneumonia derived sepsis induced by Klebsiella (Chapter 6). Recent evidence has indicated that next to alarmins, RAGE is able to interact with lipopolysaccharide [27], the endotoxin expressed in the cell wall of Gram-negative bacteria, which could explain impaired host defense of rage−/− mice in Gram-negative infection models [26,28]. In contrast however, deletion of the rage gene led to an improved outcome in the setting of Gram-positive pneumonia derived sepsis. Rage−/− mice displayed improved survival, lower bacterial loads and attenuated pulmonary inflammation compared to wild type mice in a model of sepsis caused by pneumococcal pneumonia [8]. It appears that the harmful role of RAGE in pneumococcal disease is primarily exerted in the pulmonary compartment as RAGE hardly impacted disease outcome when bacteria were directly injected in to the bloodstream (Chapter 4). Like in pneumococcal pneumonia, RAGE deficiency (modestly) improved bacterial clearance, reduced signs of lung pathology and decreased levels of cytokines in the bronchoalveolar compartment during staphylococcal pneumonia (Chapter 5). Similarly, rage−/− mice displayed transiently reduced bacterial outgrowth in lungs and livers upon intravenously injected staphylococci (Chapter 7). Together these data indicate that RAGE differentially impacts host defense, depending on the causative pathogen and the site of infection. Clearly, RAGE is not merely a receptor for alarmins that contributes to collateral tissue damage during severe infections; indeed, RAGE likely plays an important role in protective immunity during certain infections, which makes the potential use of RAGE blocking therapies in human sepsis difficult. Notably, we did not study the effect of RAGE or HMGB1 inhibition in the context of antibiotic therapy. It is conceivable that the beneficial effects of RAGE and HMGB1 on antibacterial defense are not or less obvious in hosts that are treated with antibiotics. Such studies should be the topic of future research, since these are expected to shed more light on the potential and risks of RAGE and/or HMGB1 inhibition in human sepsis.

Anti-HMGB1 treatment transiently attenuated pulmonary inflammation in S. aureus pneumonia (Chapter 5). Of notice, peak levels of bronchoalveolar HMGB1 at 24 hours did not sustain the cytokine response, which was most likely initiated via a TLR2-MyD88 dependent mechanism [29]. Recent investigations have shown that the functionality of HMGB1 is dependent on specific molecular conformations [10]. Future investigations on
the role of HMGB1 in infectious diseases should therefore take into account the several biological forms of this protein as well. Although TLR4 has been implicated as the dominant proinflammatory receptor for HMGB1 [30], this receptor had only limited impact on the injurious host response in this disease model.

In the second part of this thesis we focused on the role of MRP8/14 in pneumonia and sepsis. Our group previously reported elevated MRP8/14 serum levels in patients with sepsis and in healthy volunteers intravenously injected with endotoxin. In mice, MRP8/14 potentiated the fulminant systemic inflammatory response induced by endotoxin and E. coli administration, which contributed to organ failure and death. In this thesis we demonstrate that MRP8/14 impacts on various components of the innate host defense which distinctively influences the course and outcome of different types of bacterial pneumonia.

Patients with pneumonia and healthy volunteers intrabronchially challenged with lipoteichoic acid showed compartmentalized release of MRP8/14 in their airways (Chapter 9). Accordingly we demonstrate enhanced bronchoalveolar and systemic MRP8/14 levels in murine pneumonia caused by several pulmonary pathogens (Chapters 8-10). Extracellular MRP8/14 exhibits a proinflammatory role in various pathologic conditions [7,22,31], but despite high intrapulmonary MRP8/14 concentrations, we were unable to find such a role in bacterial pneumonia. Pulmonary inflammation was surprisingly enhanced in mrp14−/− mice during staphylococcal pneumonia (Chapter 10). Previous investigations have shown that mrp14−/− mice display an enhanced maturation state of dendritic cells, which release more cytokines [32,33]. Dendritic cells are important contributors of cytokine production in S. aureus pneumonia [34]. Our in vitro stimulation experiments suggest that the systemic inflammatory response is mainly driven by circulating bacterial burdens, independent from MRP8/14.

Several animal studies have implicated MRP8/14 as a mediator of neutrophil recruitment [35,36]. In Klebsiella and pneumococcal pneumonia, we found similar neutrophil numbers in total lungs in both wild type and mrp14−/− mice. In S. aureus pneumonia however, mrp14−/− mice displayed reduced neutrophil numbers in bronchoalveolar lavage fluid at the latest time points when most of the bacteria were already cleared (Chapter 10). These data suggest that MRP8/14 promotes transmigration of neutrophils into the bronchoalveolar compartment and that this may be compensated for by other mechanisms induced by viable bacteria at early time points.

Many previous investigations have established an antibacterial role for MRP8/14 in in vivo infection models [11,13,16], which is dependent on the chelation of both zinc and manganese [11,12]. Part of this antimicrobial effect is mediated through the release of NETs, which is particularly important in the host defense against infectious agents that are not easily phagocytosed (e.g. fungi and staphylococcal aggregates) [37]. We here demonstrate the contribution of MRP8/14 in NET-mediated growth inhibition of
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Staphylococci and *Klebsiella* *in vitro* as well (Chapter 8). The antibacterial role of MRP8/14 *in vivo*, however, is especially important in pyogenic infections [11]. Abscesses contain high concentrations of MRP8/14 [11,38], which may be important in bacterial control such as observed in the liver in a previous study during systemic *S. aureus* infection [11] and *Klebsiella* pneumonia derived sepsis in this thesis (Chapter 8). Surprisingly, we did not observe an antibacterial role for MRP8/14 in our model of *S. aureus* pneumonia (Chapter 10). Local staphylococcal loads did not grow and distant abscess formation did not occur in this study, which appear to be important conditions for an effect of MRP8/14 to take place. In the setting of pneumococcal pneumonia derived sepsis, lack of MRP8/14 unexpectedly reduced bacterial burdens in the host (Chapter 9). A previous study has shown that zinc is released at high concentrations in the nasopharynx and blood during pneumococcal infection [17]. Zinc interferes with uptake of manganese, which renders pneumococci more susceptible for oxidative stress and neutrophil killing [17,39]. We postulate that MRP8/14 protects *S. pneumoniae* against high zinc concentrations at infected body sites, through chelation of zinc, providing a benefit for the pathogen rather than for the host. Together these results exemplify the complex role of MRP8/14 in the host response to bacterial infection. MRP8/14 directly impacts bacterial growth by various mechanisms, whereby the net effect depends on the pathogen. Moreover, MRP8/14 can modulate the inflammatory response to invading pathogens, with an overall effect that varies depending on the causative pathogen, the type of infection and most likely the bacterial load.

**CONCLUSION**

The work presented in this thesis provides evidence for an important role for alarmins in the host response against bacterial infection. Targeting alarmins impacts on the complex host-pathogen interplay and can alter the course and outcome of infection. Alarmins promote organ failure and death in the setting of fulminant sepsis, but may be essential in other types of infection dependent on the pathogen and route of infection. These effects may not become apparent with concurrent antibiotic treatment, which is common practice in the clinical setting. Hence, extrapolation and translation of our experimental results into the complex human setting will remain a great challenge. Nonetheless, the advanced knowledge of the alarmin biology during infection may eventually provide new complementary therapeutic strategies for patients with life-threatening infectious disease.
REFERENCES

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