RAGE and the innate immune response in Infection and Inflammation

van Zoelen, M.A.D.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Summary, general discussion and future perspectives for research and therapy
**Summary**

Infectious diseases are great threats causing major morbidity and mortality worldwide. Bacterial pathogens have and will keep having a remarkable ability to resist antibiotics and other drugs and the idea that a new influenza pandemic might occur in the near future has triggered renewed interests in viral pathogens. From a treatment perspective, insight in factors involved in the innate immune system during infection is needed to identify possible new therapeutic targets. In addition, the inflammatory (host) response will not always be of net benefit for the host: it can be damaging, not only during infection but also during non-infectious severe inflammation. RAGE has been suggested to be involved in the inflammatory response in several inflammatory models and via several ways (amongst others induction of NF-κB, interaction with leukocyte β2-integrins leading to neutrophil recruitment). Therefore, in this thesis, we focused on RAGE and its ligands as potential therapeutic targets during infection and inflammation.

**Chapter 1** is a general introduction. It describes several RAGE ligands and the infectious and inflammatory diseases relevant for the studies presented in this thesis.

In **part I** of this thesis we examined the role of RAGE in the host defense during severe infections using RAGE deficient (RAGE\(^{-/-}\)) mice. **Chapter 2** studies the role of RAGE during *Escherichia (E.) coli* induced peritonitis. Here we demonstrate that in mice with *E. coli* sepsis, RAGE expression is upregulated in the liver. RAGE\(^{-/-}\) mice showed a reduced resistance, as indicated by an enhanced bacterial outgrowth in their peritoneal cavity and increased dissemination of the infection, accompanied by increased hepatocellular injury and exaggerated systemic cytokine release and coagulation activation after administration of *E. coli*. In addition, Wild-type (Wt) mice treated with anti-RAGE antibodies also displayed a diminished defense against the growth and/or dissemination of *E. coli*. RAGE was important for the initiation of the host response as reflected by a reduced immune and procoagulant response early after intraperitoneal injection of *E. coli* lipopolysaccharide (LPS). This chapter shows that intact RAGE signaling contributes to an effective antibacterial defense during *E. coli* sepsis, thereby limiting the accompanying inflammatory and procoagulant response. In **chapter 3** we aimed to determine the role of RAGE in the innate immune response to *Streptococcus (S.) pneumoniae* pneumonia. *S. pneumoniae* pneumonia resulted in an upregulation of constitutively present RAGE expression in murine lung tissue, especially in the interalveolar septae. RAGE\(^{-/-}\) mice showed an improved survival, which was accompanied by a lower bacterial load in the lungs in an early phase and a decreased dissemination of the bacteria to blood and spleen in a later phase after inoculation of *S. pneumoniae*. RAGE\(^{-/-}\) macrophages showed an improved killing capacity of *S. pneumoniae* *in vitro*. Lung inflammation was attenuated in
RAGE-/- mice in a later phase, as indicated by histopathology and cytokine/chemokine levels. Neutrophil migration to the lungs was mitigated in the RAGE-/- mice. In addition, in RAGE-/- mice activation of coagulation was diminished. Additional studies examining the effect of RAGE deficiency on the early inflammatory response to S. pneumoniae did not reveal an early accelerated or enhanced immune response. We here conclude that RAGE plays a detrimental role in the host response to S. pneumoniae pneumonia by facilitating the bacterial growth and dissemination and concurrently enhancing the pulmonary inflammatory and procoagulant response. Chapter 4 investigates the role of RAGE in the host response to Klebsiella (K.) pneumoniae pneumonia. K. pneumoniae pneumonia induced an increased pulmonary expression of RAGE. RAGE deficiency impaired host defense as reflected by a worsened survival, increased bacterial outgrowth and dissemination in RAGE-/- mice. Furthermore, mice deficient in RAGE infected with K. pneumoniae showed similar lung inflammation, and slightly elevated - if any - cytokine and chemokine levels and unchanged hepatocellular injury. In addition, RAGE-/- mice displayed an unaltered response to intranasally instilled Klebsiella LPS with respect to pulmonary cell recruitment and local release of cytokines and chemokines. To sum up, RAGE contributes to an effective antibacterial host response during K. pneumoniae pneumonia, but plays an insignificant part in the lung inflammatory response to either intact Klebsiella or Klebsiella LPS. Data presented in chapter 5 reveal our findings obtained from studies in Wt and RAGE-/- mice infected with live virulent Mycobacterium (M.) tuberculosis. While lungs of uninfected Wt mice expressed RAGE, in particular on endothelium, M. tuberculosis pneumonia was associated with an enhanced expression of pulmonary RAGE. Lung inflammation was increased in RAGE-/- mice, as indicated by histopathology, percentage of inflamed area, lung weight and cytokine and chemokine levels. In addition, lung lymphocyte and neutrophil numbers were increased in RAGE-/- mice. RAGE-/- mice displayed higher mycobacterial loads in the lungs in an early phase, while they showed similar loads in the liver in an early and later phase. This chapter also shows that RAGE-/- mice displayed body weight loss and a worsened M. tuberculosis induced mortality. Hence, these data illustrate that RAGE plays a beneficial role in the host response to pulmonary tuberculosis. In chapter 6 we explored RAGE involvement in pneumonia caused by influenza A virus which can have devastating effects, resulting in respiratory failure and death. Whereas RAGE was constitutively expressed in the lungs of uninfected Wt mice, in particular on endothelium, influenza pneumonia was associated with enhanced expression on endothelium and de novo expression on bronchial epithelium. We found that RAGE-/- mice are relatively protected from influenza induced mortality and have an improved viral clearance and enhanced cellular T cell response and activation of neutrophils. Taken together, RAGE renders the host more susceptible to influenza pneumonia.
Part II of this thesis focuses on the (effects/) role of soluble RAGE, RAGE ligands and damage-associated molecular patterns (DAMPs, also referred to as alarmins) during severe infection and inflammation. Soluble (s) RAGE is a truncated (or spliced at the RNA level) form of full-length RAGE, that can compete with cell-surface RAGE for ligand binding and therefore may contribute to the removal/neutralization of circulating ligands as a decoy. The emerging concept of pattern recognition involves RAGE and Toll-like receptors (TLRs) in sensing not only “pathogen–associated molecular patterns” (PAMPs) but also (endogenous) DAMPs. This “novel” group of DAMP molecules has been introduced as important pro-inflammatory factors of innate immunity. Infection commonly is associated with the release of these endogenous proteins that serve to warn the host for eminent danger. Examples of alarmins and established ligands for RAGE are high mobility group box 1 (HMGB1) and some members of the S100 family (e.g. S100A8 (myeloid-related protein 8, MRP8), S100A9 (MRP14) or S100A12 (MRP6). In chapter 7 we aimed to characterize the effects of sRAGE treatment in experimental abdominal *E. coli* sepsis. We found that sRAGE treated mice demonstrated an enhanced bacterial dissemination to liver and lungs accompanied by increased hepatocellular injury and exaggerated systemic cytokine release after intraperitoneal administration of *E. coli*. Remarkably, lung inflammation was unaffected. Furthermore, HMGB1 release was enhanced during peritonitis and anti-HMGB1 treatment was associated with higher bacterial loads in liver and lungs. Together, these data indicate that RAGE ligands, including HMGB1, limit bacterial dissemination during gram-negative sepsis.

In chapter 8 we sought to determine the extent of HMGB1 release in patients with sepsis stratified to the three most common infectious sources and to determine HMGB1 concentrations at the site of infection during peritonitis or pneumonia. Patients with severe sepsis due to pneumonia, peritonitis or urinary tract infection displayed elevated circulating HMGB1. HMGB1 concentrations did not differ between survivors and non-survivors and were not correlated to either disease severity or concurrently measured cytokine levels. In line, although intravenous LPS injection clearly elevated plasma cytokine levels, HMGB1 remained undetectable. In patients with peritonitis, HMGB1 concentrations in abdominal fluid were >10-fold higher than in concurrently obtained plasma. In pneumonia patients, HMGB1 levels were higher in bronchoalveolar lavage fluid (BALF) obtained from the site of infection than in lavage fluid from healthy controls. Summarized, in severe sepsis the kinetics of HMGB1 release may differ depending on the primary source of infection. In patients with severe infection, HMGB1 release may predominantly occur at the site of infection.

Since HMGB1 is a pro-inflammatory mediator that contributes to acute lung injury, we determined HMGB1 levels in BALF of patients during mechanical ventilation and ventilator–associated pneumonia (VAP) in chapter 9. Although HMGB1 levels were elevated after “short–term” mechanical ventilation, differences were not statistically
Summary, general discussion and future perspectives for research and therapy

Significant compared with healthy volunteers. HMGB1 levels, however, were significantly higher in “long–term” mechanical ventilation patients. With unilateral VAP, HMGB1 levels from the infected lung were not different from those measured in the contralateral non–infected lung. We here conclude that “long–term” mechanical ventilation is associated with increased HMGB1 levels, in contrast to “short–term” mechanical ventilation. In addition, HMGB1 levels during VAP are increased compared to healthy volunteers, however; they are not different from those found in patients intubated and mechanically ventilated for a similar period of time.

In vitro data have shown that HMGB1 can induce activation of intracellular signaling pathways via interaction with at least three pattern recognition receptors: TLR2, TLR4 and RAGE. The objective of chapter 10 was to investigate the role of these receptors in the in vivo response to HMGB1. We therefore administered HMGB1 to Wt, TLR2–/–, TLR4–/– and RAGE–/– mice. All genotypes showed similar plasma levels of tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-10 and thrombin-anti-thrombin complexes after intraperitoneal injection of HMGB1. Compared to Wt mice, both TLR4–/– and RAGE–/– mice displayed lower TNF-α and IL-6 concentrations and lower neutrophil numbers in their peritoneal lavage fluid. In contrast, TLR2–/– mice showed increased levels of TNF-α and IL-6 in their peritoneal cavity relative to Wt mice. Together, these data indicate that HMGB1 induces release of cytokines, activation of coagulation and neutrophil recruitment in vivo via a mechanism that at least in part depends on TLR4 and RAGE.

MRP8 and 14 can form heterodimers that elicit a variety of inflammatory responses. We recently showed that MRP8/14 is a ligand for TLR4, and that mice deficient in MRP8/14 are protected against endotoxic shock-induced lethality. Chapter 11 describes the expression and role of MRP14 in clinical and experimental sepsis. In that chapter we demonstrated for the first time that sepsis patients display elevated circulating MRP8/14 concentrations and that LPS injection results in systemic MRP8/14 release in healthy humans. In patients with peritonitis, MRP8/14 levels in abdominal fluid were >15-fold higher than in plasma. MRP14–/– (and thereby MRP8/14 deficient) mice displayed an improved defense against *E. coli* abdominal sepsis in an early phase, as indicated by a transient diminished dissemination of the bacteria. In addition, MRP14–/– mice demonstrated decreased systemic inflammation, as reflected by lower cytokine plasma concentrations and less severe liver damage. We here conclude that human sepsis and endotoxemia are associated with enhanced release of MRP8/14. In abdominal sepsis, MRP8/14 release likely primarily occurs at the site of the infection, facilitating bacterial dissemination in an early phase and liver injury.

Knowledge about sRAGE involvement in host defense during sepsis or infection is limited. It has previously been shown that sRAGE plasma levels are increased in patients with sepsis and that non-survivors had higher circulating sRAGE concentrations than survivors,
suggesting that sRAGE is related to severity and outcome of sepsis. Other clinical studies have shown that higher plasma levels of sRAGE are associated with a reduced risk of coronary artery disease, hypertension, the metabolic syndrome, arthritis and Alzheimer’s disease. **Part III** focuses on sRAGE expression and its high-affinity ligand - S100A12 - in severe infection and inflammation.

S100A12 (or MRP6) is highly expressed and serum levels correlate with individual disease activity in patients with inflammatory diseases. **Chapter 12** reports on the extent of S100A12 and its soluble high-affinity receptor sRAGE in patients with severe sepsis stratified to the three most common infectious sources and in healthy human volunteers intravenously challenged with endotoxin. Patients with severe sepsis displayed increased circulating S100A12 concentrations. All severe sepsis subgroups (sepsis due to pneumonia, peritonitis and urinary tract infection) had elevated serum S100A12. Remarkably, patients with sepsis due to pneumonia had the highest S100A12 levels. Intravenous LPS injection in healthy humans elevated systemic S100A12 levels. In contrast to S100A12, sRAGE concentrations did not change during severe sepsis or human endotoxemia. Together, in severe sepsis, S100A12 is released systemically irrespective of the primary source of infection. Human endotoxemia induces S100A12 as well and concentrations of its high-affinity soluble receptor sRAGE do not change during sepsis or human endotoxemia.

We next analyzed the expression of S100A12 and RAGE in patients suffering from acute respiratory distress syndrome (ARDS) in **chapter 13**. Patients with ARDS had significantly enhanced pulmonary S100A12 expression and higher S100A12 protein concentrations in BALF than healthy controls. Extensive RAGE staining was found in non-inflamed tissue from healthy controls and this staining resembled the broad RAGE expression pattern seen in inflamed lung tissue. Levels of sRAGE were not significantly elevated in ARDS. S100A12 concentrations decreased with time from disease onset. In healthy volunteers, S100A12 was elevated in BALF after LPS inhalation. **In vitro** experiments confirmed strong pro-inflammatory effects of human S100A12. Concluding, S100A12 and its receptor RAGE are found at high concentrations in pulmonary tissue and BALF in acute lung injury. S100A12 expression may reflect neutrophil activation during lung inflammation and contribute to pulmonary inflammation and endothelial activation via binding to RAGE.

Patients with respiratory failure often require supplemental oxygen therapy and mechanical ventilation. Although both supportive measures are necessary to guarantee an adequate oxygen uptake, they can also cause or worsen lung inflammation and injury. Hyperoxia induced lung injury is characterized by infiltration of neutrophils into the lungs. In **chapter 14**, we tried to establish whether lung injury induced by hyperoxia is associated with increased levels of sRAGE in the bronchoalveolar space. In addition, in this chapter we investigated the expression and function of urokinase plasminogen activator receptor (uPAR), an important mediator for leukocyte trafficking. sRAGE was increased in BALF
from mice exposed to hyperoxia. In addition, hyperoxia induced migration of uPAR positive granulocytes into the lungs from Wt mice compared to healthy control Wt mice (exposed to room air). uPAR deficiency was associated with a diminished neutrophil influx into lung tissue and bronchoalveolar space, which was accompanied by a strong reduction in lung injury. Furthermore, in uPAR−/− mice, activation of coagulation was diminished. These data show that hyperoxia induced lung injury is associated with enhanced sRAGE in the lungs and that uPAR deficiency is associated with a diminished neutrophil influx into lung tissue and bronchoalveolar space and decreased pulmonary injury.

Finally, we found in chapter 15 that intranasal LPS induced bronchoalveolar sRAGE levels in mice. Additionally, we examined the role of monocyte chemoattractant protein-1 (MCP-1) in lung inflammation induced by LPS or lipoteichoic acid (LTA), constituents of the gram-negative and gram-positive bacterial cell wall, respectively. Healthy humans demonstrated elevated MCP-1 concentrations in their BALF after inhalation of LPS. Similarly, intranasal administration of LPS or LTA to mice resulted in a rise in BALF MCP-1 levels. Murine alveolar macrophage-like cells released significant amounts of MCP-1 upon stimulation with LPS or LTA in vitro. Compared to Wt mice, MCP-1−/− mice demonstrated lower TNF-α levels and a diminished neutrophil influx into their bronchoalveolar space after either LPS or LTA instillation. After intrapulmonary delivery of LPS, MCP-1−/− mice had decreased IL-6 and KC concentrations and less severe lung inflammation upon histopathological examination. Remarkably, MCP-1 deficiency was associated with an early enhancement of IL-10 release in BALF after both LPS and LTA instillation. Hence, MCP-1 is a pro-inflammatory mediator during pulmonary inflammation induced by either LPS or LTA.

**GENERAL DISCUSSION AND FUTURE PERSPECTIVES FOR RESEARCH AND THERAPY**

The innate immune response is the crucial first line of defense against pathogens. With the experimental studies described in this thesis, we intended to obtain further insight into the role of RAGE, its ligands and other alarmins in host defense during clinically important infections with the eventual goal to optimize therapies against specific pathogens. While interpreting the results, one has to keep in mind that a careful balance between the inflammatory and anti-inflammatory response is vital in order to survive or recover from a severe infection.

In the first part of this thesis we focused on RAGE involvement during respiratory tract infections caused by the diverse pathogens *S. pneumoniae, K. pneumoniae, M. tuberculosis* and influenza A virus. Our finding that RAGE deficiency is beneficial in one pneumonia
model and detrimental in the other, clearly adds to the notion that RAGE mediated host defense against different pathogens, although all of them cause pneumonia, relies on distinct mechanisms. While RAGE-/- mice had a better survival during pneumonia induced by the gram-positive bacterium *S. pneumoniae*, the same mouse strain showed a worsened survival during pneumonia induced by the gram-negative pathogen *K. pneumoniae*. Since RAGE recognizes tertiary structures rather than amino acid sequences, RAGE has the ability to engage classes of molecules rather than individual ligands. To date, RAGE has been found to interact mainly with alarmins. It would be highly interesting to investigate whether RAGE can directly bind, become activated and mount a first immune reaction after ligation with specific PAMPs as well, similarly to TLRs. If so, this could be part of the explanation of our observation that RAGE involvement during pneumonia has such opposite effects on mortality. In addition, RAGE mediated effects on other first-line defense mechanisms such as chemotaxis, killing, phagocytosis and respiratory bursting could depend on the pathogen as discussed in the separate chapters.

We encountered some unexpected findings while studying the *in vivo* role of RAGE in tuberculosis. *A priori*, we hypothesized that RAGE would have a marked influence on the chronic lung inflammation that accompanies tuberculosis, considering its strong expression within the pulmonary compartment and its established role as a pro-inflammatory receptor in multiple disease models. Much to our surprise, RAGE deficiency resulted in an enhanced inflammatory response in the lungs of mice infected with *M. tuberculosis* via the airways, which was associated with an adverse long-term outcome as reflected by accelerated weight loss and an increased mortality. So, whereas in many other models of chronic inflammation, RAGE inhibition is of benefit, we found detrimental effects in chronic inflammation induced by *M. tuberculosis*. Of interest, we found that influenza A virus pneumonia, a disease in which T-cell mediated immune response is also crucial for the host, has a better outcome in a host lacking RAGE.

In the second and third part, we describe enhanced expression of the alarmins HMGB1, MRP8/14 and S100A12 in patients with sepsis. During sepsis, the innate immune response will likely be induced by (exogenous) PAMPs (predominantly via TLRs) and these (endogenous) alarmins (via RAGE, TLRs and other receptors). It has been suggested that alarmins can serve the host to warn for eminent danger (mechanical trauma, chemical insults (strong acids or bases, poisons), heat, cold, etc.). The function(s) and/or effects of alarmins during infection still have to be established. We and others demonstrated that inhibition of HMGB1 or MRP14 decreases mortality in septic mice. Until now, not much research has been done on the potential role of other alarmins in sepsis, leaving an open and interesting field to be explored.
Clearly, the host response is complex and the result of millions of years of evolution. Knowledge derived from this research may pave the way for innovative and better therapies for patients with life threatening infections or severe inflammation.