General introduction and outline of the thesis

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Introduction

1. Receptor for advanced glycation end products (RAGE) and alarmins

1.1 Receptor for advanced glycation end products (RAGE)
The receptor for advanced glycation end products (RAGE) is a multiligand receptor of the immunoglobulin superfamily that is expressed at a high level in the lungs and at a low level in normal adult tissues, including on cells involved in the innate immune system, e.g. neutrophils, T and B lymphocytes, monocytes, macrophages, dendritic cells and endothelial cells (1-3). RAGE is composed of 3 immunoglobulin-like regions, a transmembrane domain, and a highly charged short cytosolic tail that is essential for intracellular signaling (Figure 1). The V domain in the extracellular part of the receptor is critical for ligand binding and interacts with a diverse class of ligands due to its ability to recognize 3-dimensional structures rather than specific amino acid sequences (4). Originally, RAGE was identified as a receptor for advanced glycation end products (AGEs), explaining the choice of its name. AGEs are products of non-enzymatic glycation and oxidation of proteins, lipids and other macromolecules that appear, in particular, under conditions of increased availability of reducing sugars and/or enhanced oxidative stress, especially when molecules turn over slowly and aldose levels are elevated (5, 6).

Figure 1. Schematic depiction of RAGE-ligand interaction showing domains of the receptor. The V-type domain is critical for binding of RAGE ligands. Deletion of the cytosolic tail results in a modified form of RAGE that binds ligands, remains firmly embedded in the cell membrane, but does not transmit RAGE-mediated cellular activation (from (7)).
Ongoing studies revealed, however, that RAGE is able to recognize a wide range of endogenous molecules that are released during various conditions of inflammation and/or injury. 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) damage-associated molecular patterns (DAMPs) (8, 9). This “novel” group of molecules has been introduced as important pro-inflammatory factors of innate immunity. Because of their release by activated or damaged cells under conditions of cell stress, the terms “endokines” or “alarmins” are used (10). 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) (11, 12) and some members of the S100 family (e.g. S100A12 (13)). Other examples of alarmins of the S100 family are S100A8 (myeloid-related protein 8, MRP8) and S100A9 (MRP14).

Ligand binding to RAGE leads to sustained receptor-dependent signaling and activation of nuclear factor-κB and mitogen-activated protein kinase pathways. Inhibition of RAGE signaling has been found to reduce inflammatory responses in an infection model of cecal ligation and puncture (CLP (14)) and several other (non-infectious) models of hepatic injury (15-17), diabetic atherosclerosis (18, 19), delayed type hypersensitivity (14, 20) and type II collagen induced arthritis (20). In chapter 2, we investigated the role of RAGE during abdominal sepsis induced by one of the major causative pathogens, *Escherichia coli* (*E. coli*), focusing on the outgrowth of bacteria at the primary site of infection and the subsequent dissemination, and the accompanying systemic inflammatory response syndrome. Given the ubiquitous expression of RAGE in the lungs, it is likely that this receptor plays a role in the regulation of lung inflammation. In chapter 3-6, we studied the role of RAGE during pneumonia caused by (myco)bacteria or influenza A.

### 1.2 High mobility group box 1 (HMGB1)

HMGB1 is a nuclear protein that for a long time was studied for its intranuclear functions only. During the last few years HMGB1 has been discovered to have multiple extracellular functions and to be a damage-associated molecular pattern (DAMP). This re-discovery of HMGB1 as an extracellular protein with cytokine-like activity (Figure 2) and its pivotal role as a late mediator of experimental sepsis have prompted clinical studies to characterize the release of HMGB1 in patients with sepsis. RAGE ligands in general, and HMGB1 in particular, have been implicated in the pathogenesis of abdominal sepsis. RAGE deficiency or administration of anti-HMGB1 antibodies improved survival in a model of abdominal polymicrobial sepsis induced by CLP (14, 21-23). Chapter 7 describes the expression and role of HMGB1 in experimental abdominal *E. coli* sepsis; furthermore, we showed the effects of soluble RAGE on the host defense during abdominal sepsis in that chapter. In chapter 8 we sought to determine the extent of systemic HMGB1 release in patients with
severe sepsis stratified to the three most common primary sources of infection (lungs, abdomen and urinary tract) and to examine local HMGB1 levels, at the site of infection, in patients with peritonitis and pneumonia. In chapter 9 we investigated the local release of HMGB1 in the pulmonary compartment during mechanical ventilation and ventilator-associated pneumonia (VAP). HMGB1 has been reported to transduce cellular signals \textit{in vitro} by interacting with at least three receptors: TLR2, TLR4 and the RAGE (Figure 3). Chapter 10 describes a study in which we tried to determine the role of these pattern recognition receptors in the \textit{in vivo} responses to HMGB1.
1.3 S100 family/ Myeloid-related proteins
S100 proteins comprise a family with more than 20 members, from which at least three have been linked to innate immune functions by their expression by myeloid cells: S100A8 (also called calgranulin A or myeloid-related protein (MRP) 8), S100A9 (calgranulin B or MRP14) and S100A12 (calgranulin C or MRP6). S100A12 has been shown to exhibit its pro-inflammatory activities via interaction with RAGE (13). It has not been experimentally proven that all S100 proteins do so. MRP8 and -14 form heterodimers, which are the biologically relevant forms of these proteins (26-28). MRP8/14 complexes induce a variety of inflammatory reactions and the extent of MRP8/14 expression correlates with disease activity in several inflammatory disorders (29, 30). In chapter 11 we aimed to determine the extent of systemic MRP8/14 release in patients with severe sepsis and to examine local MRP8/14 levels at the site of infection in patients with peritonitis. In addition, we determined the role of MRP8/14 in specific host responses to murine E. coli abdominal sepsis in this chapter.

S100A12 (or MRP6), a high-affinity RAGE ligand, has been described as a biomarker of neutrophil activation in inflammatory diseases, including pulmonary infections and sepsis (31, 32). In chapter 12, circulating S100A12 and sRAGE levels during sepsis and human endotoxemia are described. Because neutrophil activation is a hallmark of acute lung injury (ALI), we hypothesized that S100A12 may trigger innate immune processes during

Figure 3. Signaling pathways downstream of RAGE, TLR4 and TLR2 that mediate the effects of HMGB1 (from (25)).
the acute respiratory distress syndrome (ARDS). We therefore analyzed pulmonary S100A12 and RAGE in patients with ARDS and in subjects instilled with endotoxin in chapter 13.

1.4 Soluble RAGE
Soluble RAGE (sRAGE), a truncated form of full length RAGE, is composed of only the extracellular ligand-binding domain (V-C-C’) lacking the cytosolic and transmembrane domains (i.e., the parts that transfer a signal into the cell, Figure 1) and therefore circulates in plasma. By competing with cell-surface RAGE for ligand binding, sRAGE may contribute to the removal/neutralization of circulating ligands, thus functioning as a decoy. For this reason, recombinant sRAGE has been extensively (and successfully) tested in several animal models for the treatment of RAGE-mediated diseases, including type II collagen induced arthritis (20), delayed-immune hypersensitivity (DTH) (14) and diabetic atherosclerosis (18, 19). Others suggested that sRAGE, besides being a decoy receptor, also has immunomodulating activity of its own. Clinical studies have recently 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. In chapter 12 we described sRAGE levels in patients with sepsis and in healthy volunteers intravenously challenged with lipopolysaccharide (LPS). In addition, we showed local sRAGE levels in ARDS patients and in healthy volunteers after LPS inhalation in chapter 13.

sRAGE had been recently described as a marker of lung injury based on experimental studies in rats and in patients with acute lung injury (33). In chapter 14 and 15 respectively, we tried to establish whether lung injury induced by hyperoxia and LPS was associated with increased levels of sRAGE in the bronchoalveolar space. In chapter 14, we furthermore investigated the effects of urokinase plasminogen activator receptor deficiency during hyperoxia induced lung injury. Chapter 15 describes the role of endogenous monocyte chemoattractant protein-1 during LPS and lipoteichoic acid induced lung inflammation.

2. Infectious and inflammatory diseases studied in this thesis

2.1 Sepsis
Severe sepsis remains a major challenge in the care of critically ill patients. The outcome is poor and mortality rates remain up to 30-40%. Recent insights into the molecular mechanisms responsible for the pathogenesis of the sepsis syndrome shows that sepsis...
is associated with the activation of multiple inflammatory pathways, including the cytokine network and the coagulation system. Pro- and anti-inflammatory pathways are simultaneously activated. Subsequently, cellular activation involving leukocyte-endothelial cell interactions occurs leading to expression of membrane surface molecules such as TLRs, adhesion molecules and cytokine receptors. Furthermore, sepsis leads to a hemostatic imbalance which can result in disseminated intravascular coagulation. While any bacterial infection can theoretically progress and cause systemic inflammation, the respiratory tract and the abdominal cavity are the most frequent sources of sepsis (Figure 4) (34, 35). In chapter 8, 11 and 12 we determined the extent of systemic HMGB1, MRP8/14, S100A12 and sRAGE release in patients with severe sepsis and their subgroups peritonitis, pneumonia and urinary tract infection as primary infections. We also examined local levels of these parameters at the site of infection in patients with peritonitis in these chapters.

2.2 Peritonitis
Peritonitis is most often caused by the presence of bacteria in the otherwise germ-free peritoneal cavity (Figure 5) and is caused predominantly by the perforation of intestines. Acute bacterial peritonitis is a potentially life-threatening disease with a mortality rate ranging between 30% and 50%. Furthermore, peritonitis is the second most common cause of sepsis and the mortality of peritonitis-induced sepsis can be as high as 80%. Therefore we investigated the role of RAGE and the DAMPs HMGB1 and MRP8/14 in the inflammatory response to abdominal sepsis (chapter 2, 7 and 11). The most common causative organisms are enteric gram-negative bacteria. Since *Escherichia (E.) coli* is found in 60% of the cases (36) (Table 1), we used *E. coli* to induce abdominal sepsis in mice.

2.3 Endotoxemia
Systemic inflammation and sepsis are leading causes of mortality worldwide. The systemic spread of endotoxin (LPS) derived from gram-negative bacteria is considered one of the most important correlates of sepsis. Intravenous injection of LPS results in activation of several inflammatory pathways, thereby mimicking – in a qualitative way – the systemic
host response seen in sepsis. As such, this model allows for a close examination of the dynamic change in mediators, receptors and cells during systemic inflammation in humans. Therefore, we used this human model in chapters 8, 11 and 12.

2.4 Respiratory tract infections
Infections of the respiratory tract are the 7th leading cause of mortality in the United States and bacterial pneumonia is the most frequent source of sepsis (34, 38-40). According to the acquisition of pneumonia and the pathogens involved, community-acquired pneumonia (CAP) can be distinguished from hospital-acquired pneumonia (HAP, also called nosocomial pneumonia). While the gram-positive bacterium *Streptococcus pneumoniae* is the single most frequent pathogen causing CAP, responsible for up to 60% of cases, *Klebsiella pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* are isolated in about 10% each (41-43). Pneumococcal pneumonia causes more than 150,000 hospitalizations in the United States annually and *Streptococcus pneumoniae* is the most frequent cause of lethal CAP (44, 45). Due to this high clinical importance and worldwide significance, we investigated the role of RAGE and the local release of HMGB1 during this particular disease in chapter 3 and 8, respectively. *Klebsiella pneumoniae* is a gram-negative opportunistic bacterium, which is an important cause of HAP and sepsis. We used this bacterium to study the role of RAGE in gram-negative pneumonia in mice in chapter 4. One of the most dramatic manifestations of chronic lung inflammation is tuberculosis. Chapter 5 describes the impact of RAGE deficiency on the immune response to instillation of live virulent *Mycobacterium tuberculosis* in mice. Finally, we studied the role of RAGE in pneumonia caused by influenza A virus in chapter 6. Recent outbreaks of highly pathogenic influenza A virus infections have had important economic consequences and the notion that a new influenza pandemic might occur in

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<th>Pathogen</th>
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<tr>
<td><em>E. coli</em></td>
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</tr>
<tr>
<td>Enterobacter/Klebsiella</td>
<td>26</td>
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<tr>
<td><em>Proteus</em></td>
<td>22</td>
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<td><em>Pseudomonas</em></td>
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Table 1. Most frequent gram-negative bacteria isolated from intra-abdominal infections (from (37)).
the near future has triggered renewed interest in influenza infection. Furthermore, we
induced sterile lung inflammation by instilling LPS, a constituent of the gram-negative
bacterial cell wall, or LTA, a component of the gram-positive cell wall in mice (chapter 4
and 15) and in humans (chapter 13).

2.5 Mechanical ventilation
The majority of patients at the intensive care unit are intubated and mechanically
ventilated. Although mechanical ventilation certainly is a cornerstone of intensive care
management, mechanical ventilation may also aggravate pre–existing lung injury or even
initiate pulmonary damage in patients without lung injury at the start of mechanical
ventilation (a phenomenon referred to as ventilator–associated lung injury (46, 47)). In
addition, mechanical ventilation puts patients at risk for a nosocomial infection, so called
ventilator-associated pneumonia (VAP) (48). Similar to ALI/ARDS and pneumonia,
ventilator–associated lung injury is associated with local production of pro-inflammatory
mediators. In chapter 9 we investigated the local release of HMGB1 in the pulmonary
compartment during mechanical ventilation and VAP.

2.6 Hyperoxia induced lung injury
Patients with ALI or ARDS commonly receive supportive care consisting of low
tidal volume protective mechanical ventilation (see above) and the administration of
high concentrations of inspired oxygen (49, 50). Prolonged exposure to high oxygen
concentrations, however, can worsen or induce lung damage in already injured or
previously healthy lungs (51). Hyperoxia induced lung injury is characterized by
infiltration of neutrophils in the lungs. In chapter 14 we determined the expression and
role of urokinase plasminogen activator receptor (uPAR) during hyperoxia induced lung
injury. In this study, we determined local sRAGE levels in bronchoalveolar lavage fluid.

AIM AND OUTLINE OF THIS THESIS

The general aim of this thesis is to enhance our knowledge and insights into the specific role
of RAGE and RAGE ligands, as well as of other damage-associated molecular patterns,
in the innate immune response during severe infection in order to identify potential new
treatment targets for these diseases.

In chapter 2 we investigated the role of RAGE in the inflammatory responses and host
defense against E. coli peritonitis. We studied the role of RAGE in pneumonia caused by
Streptococcus pneumoniae, Klebsiella pneumoniae, Mycoplasma tuberculosis and influenza
A virus in chapters 3-6. Chapter 7 reports on the effects of treatment of sRAGE and anti-
HMGB1 antibodies on the immune response during E. coli peritonitis. In chapter 8 we
sought to determine the extent of systemic HMGB1 release in patients with severe sepsis
stratified to the three most common primary sources of infection (lungs, abdomen and urinary tract) and to examine local HMGB1 levels, at the site of infection, in patients with peritonitis and pneumonia. Chapter 9 focuses on the local release of HMGB1 in the pulmonary compartment during mechanical ventilation and VAP. Chapter 10 addresses the role of TLRs 2 and 4 and RAGE in the in vivo response to HMGB1. In chapter 11 we studied the role of MRP8/14 (or S100A8/A9) during E. coli induced abdominal sepsis. Chapter 12 characterizes systemic S100A12 and sRAGE concentrations during severe infection and human endotoxemia and in chapter 13 these parameters were analyzed in patients with ARDS and in healthy subjects after inhalation of LPS. Chapter 14 reports on sRAGE levels and the role of uPAR during hyperoxia induced lung injury. Lastly, chapter 15 addresses sRAGE levels and the role of endogenous MCP-1 during LPS and LTA induced lung inflammation.
References


