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RAGE during infectious diseases

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1. ABSTRACT

The receptor for advanced glycation end products (RAGE) is a multiligand receptor that is expressed at high levels in the lungs. 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). Infection is associated with the release of these endogenous proteins, such as high-mobility group box-1 (HMGB1) and S100A12. Engagement of RAGE by its diverse ligands results in receptor-dependent signaling and activation of NF-κB. Furthermore, RAGE acts as an endothelial adhesion receptor for leukocyte integrins and promotes leukocyte recruitment. Inhibition of RAGE signaling reduces inflammatory responses in several (non-infectious) models as well as in infectious models of cecal ligation and puncture and *S. pneumoniae* pneumonia. Importantly, RAGE signaling inhibition increased bacterial outgrowth and dissemination in an *E. coli* abdominal sepsis model. This review describes experimental studies that provide further insight into the role of RAGE and its ligands in host defense during clinically important infections, which eventually may contribute to better therapies against specific pathogens.

2. INTRODUCTION

Multicellular animals have evolved defense and repair mechanisms to counteract threats such as infection and trauma-correlated tissue injury. To start such an inflammatory response, the immune system first must detect the potential life-threatening event and it does so by recognizing so-called danger signals. These signal molecules have been classically divided in i) exogenous, pathogen-associated molecular patterns (PAMPs) (1) which are conserved motifs on pathogens that are not found in higher eukaryocytes and ii) endogenous innate danger molecules, also named damage-associated molecular patterns (DAMPs) or alarmins, which are structurally diverse proteins rapidly released by the host itself during infection or (sterile) tissue damage (2).

Examples of PAMPs are lipopolysaccharide (LPS) from the outer membrane of gram-negative bacteria, peptidoglycan present in most bacteria, lipoteichoic acid in many gram-positive bacteria, bacterial DNA, viral DNA/RNA and mannans in the yeast cell wall. PAMPs are recognised by pattern recognition receptors (PRRs), in particular Toll-like receptors (TLRs) and Nod-like receptors (NLRs), leading to an inflammatory response via several signaling pathways amongst which nuclear factor-

κappa B (NF-κB) activation and subsequent tumour necrosis factor (TNF)-α production.

Putative DAMPs, the endogenous equivalents of PAMPs, include heat-shock proteins, nucleosomes, interleukins such as IL-1α, high-mobility group box 1 (HMGB1) and some S100 proteins (S100A8/A9, S100A12) (3). DAMPs can be released either actively or passively following non-programmed cell death (necrosis) but are not released by apoptotic cells (4) and they have activating effects on receptor-expressing cells engaged in host defense.

TLRs and NLRs can also recognize DAMPs, and their interaction also leads to NF-κB activation, suggesting that PAMPs and DAMPs at least in part use the same receptors and signaling pathways. Interestingly, however, Liu *et al.* propose that DAMPs and PAMPs are treated differently by the immune system; they suggest that DAMPs - but not PAMPs - bring CD24-Siglec G/10 into the proximity of TLRs/NLRs, leading to a repressed DAMP initiated TLR/NLR signaling (5).

During infection, host defense systems encounter PAMPs from pathogens and DAMPs - that are released from tissues - to warn the host for eminent danger. Besides TLRs and NLRs, the multiligand receptor RAGE or receptor for advanced glycation end products is regarded as a prototypic DAMP receptor that can bind several DAMPs, including HMGB1 (6, 7) and S100A12 (7-9). Other known RAGE ligands include S100B (10), S100P (11), amyloid (12), β-sheet fibrils (13); in addition, RAGE can interact with β2 integrins (14). Ligand binding to RAGE leads to receptor-dependent signaling and activation of NF-κB and mitogen-activated protein kinase pathways (15, 16). RAGE activation is involved in various experimentally induced infectious and sterile inflammatory diseases, including cecal ligation and puncture (CLP) induced abdominal sepsis (17), hepatic injury (18-20), diabetic atherosclerosis (21, 22), type II collagen induced arthritis (23), diabetic nephropathy, and delayed type hypersensitivity (17, 23). The focus of this review is the role of RAGE in infectious diseases.

3. RAGE: EXPRESSION AND PUTATIVE FUNCTIONS

RAGE is expressed at high levels in the lungs and at low levels 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 (24-26).

During embryonic development, RAGE is highly expressed. One possible function of RAGE during embryonic life is the promotion of neurite outgrowth of neurons in the developing nervous system (27). However, $RAGE^{-/-}$ mice demonstrate neither obvious neuronal deficits nor overt behaviour abnormalities (28), indicating that RAGE may contribute to neuronal development, but that there are redundant systems that substitute for this receptor in its absence. Upon aging, RAGE expression increases again, although it is not known whether this is due to accumulation of RAGE ligands which upregulate the receptor's expression or whether this represents a compensatory mechanism protecting aging cells.

Considering the extensive RAGE expression in healthy, adult lung tissue relative to other tissues, pulmonary expression of RAGE is most widely investigated. Current studies point to an important role of RAGE in the pulmonary compartment in both physiological and pathological states. First of all, under physiological circumstances, RAGE is expressed at high basal levels in the lungs (on varying cell types: alveolar type I and II cells, endothelial cells and macrophages) relative to other tissues (29-35), suggesting that RAGE may have lung specific functions distinct from the role of RAGE in other adult tissues. In particular, although the kidney is dramatically affected by microangiopathy and fibrosis - which is substantially attributed to RAGE - in patients with diabetes, the lungs, with its significantly higher baseline RAGE expression than the kidney, remain unaffected. In addition, RAGE has been found to be specifically localised near the basal cell membrane within alveolar pneumocytes (31, 36-40). These two findings raised the question whether RAGE has a function in normal healthy lungs. Indeed, Englert *et al.* reported that aged $RAGE^{-/-}$ mice spontaneously develop pulmonary fibrosis-like alterations; lungs from 19 to 24 month-old $RAGE^{-/-}$ mice displayed increased staining for collagen and had elevated levels of hydroxyproline compared to wild type mice (30). *In vitro* data show that RAGE knockdown in pulmonary fibroblasts increases their proliferation and migration, suggesting an important protective function of RAGE in the lungs and that loss of RAGE may be related to functional changes of pulmonary cell types resulting in fibrotic disease (35). Furthermore, RAGE on epithelial cells promotes their adherence on human collagen (a major component of the alveolar basal lamina) and spreading morphology, which may facilitate gas exchange and alveolar stability *in vivo* (35, 36). Altogether, these data suggest that RAGE plays a role in maintaining lung homeostasis in normal, healthy lungs. More studies are needed to unreveal the function(s) of pulmonary RAGE in physiology more precisely.

This putative functional role of RAGE in healthy lungs might account for the observation that inhibition of RAGE signaling attenuates pathological sterile inflammatory responses in diverse non-pulmonary experimental studies (17-23), while in pulmonary pathological non-infectious inflammatory conditions, somewhat conflicting results emerge. Lung injury induced by either bleomycine or hyperoxia is diminished in $RAGE^{-/-}$ mice (41, 42), indicating a detrimental role of RAGE. In contrast, Englert *et al.* found that $RAGE^{-/-}$ mice show more severe lung fibrosis after asbestos administration as measured by histological scoring and total lung hydroxyproline quantification (30). Notably, in all these studies, mice were much younger at the time of sacrification than the aged (19-24 months-old) $RAGE^{-/-}$ mice that developed pulmonary fibrosis spontaneously in the experiment of Englert *et al.* (30). Interestingly, broncho-alveolar lavage fluid (BALF) and lung

homogenates from patients with idiopathic pulmonary fibrosis demonstrate lower membrane bound (and soluble) RAGE protein levels relative to healthy donor samples (30, 35).

4. RAGE: A RECEPTOR WITH MULTIPLE AND DIVERSE LIGANDS

RAGE is composed of three immunoglobulin-like regions, a transmembrane domain and a highly charged short cytosolic tail that is essential for intracellular signaling (43). The V domain of 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 (44). Originally, RAGE was identified as a receptor for advanced glycation end products (AGEs), 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 (45). Ongoing studies revealed that RAGE is able to recognize a wide range of endogenous molecules that alert the immune system and trigger a defensive immune response, the DAMPs. The DAMPs and RAGE ligands shown to be released during infection, HMGB1 and S100A12 (Figure 1), are discussed below, as well as its counter-receptors, the β2 integrins.

4.1. Putative RAGE ligands during infection

4.1.1. HMGB1

HMGB1 is a 215 amino acid protein that is highly conserved among species. In the nucleus, HMGB1 is a nonhistone DNA-binding protein that acts as a structural component to facilitate the assembly of nucleoprotein complexes (46). Extracellularly, HMGB1 acts as a cytokine. When cells die in a non-programmed way (necrosis), HMGB1 is passively released in the extracellular milieu, whereas apoptotic cells modify their chromatin so that HMGB1 binds irreversibly and consequently is not released (4). HMGB1 can also actively be released into the extracellular environment from a variety of cells including monocytes, macrophages, endothelial cells, enterocytes, pituicytes, dendritic cells and natural killer cells, in response to inflammatory stimuli, including PAMPs (47). During infection, elevated HMGB1 levels could be due to passive as well as active release. Currently used (and published) HMGB1 detection methods do not distinguish between these (and possible other) different forms of HMGB1. Further experiments are needed to 1) investigate the biological activity of (different forms of) HMGB1 and 2) develop HMGB1 ELISA assays that distinguish between these (possibly also functionally) different forms of HMGB1. Most studies on HMGB1 and infection involve sepsis, the second leading cause of death in noncoronary intensive care units and the 10th leading cause of death overall (48, 49). HMGB1 serum concentrations are elevated in patients with severe sepsis (50-53) and its release may predominantly occur at the site of infection; both patients with peritonitis and with pneumonia displayed elevated levels in fluid obtained from the abdomen and bronchoalveolar space, respectively (53). In a(n expermental) model of CLP induced sepsis, the kinetics of HMGB1 release *in vivo* was delayed and more sustained when compared with the secretion of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 (54, 55). In line, various interventions that inhibit HMGB1 activity or production, such as anti-HMGB1 antibodies, the A-box segment of HMGB1, ethyl pyruvate and nicotine reduced CLP induced sepsis and/or LPS lethality even if treatment was postponed for many hours up to one day after the challenge (54, 56-58). Apoptosis of immune cells has been implicated as a crucial event in the pathogenesis of sepsis, playing a major role in immunosuppression and lethality (59). Interestingly, prevention of lymphocyte apoptosis improved survival after CLP (60), whereas anti-HMGB1 treatment reduced lethality in the same model without influencing apoptosis (55), indicating that HMGB1 is downstream of apoptosis in the final common pathway to organ damage in severe sepsis. These findings suggest that the release of HMGB1 is a relatively late event in sepsis that significantly contributes to an adverse outcome. Furthermore, highly pure HMGB1 has been reported not to not have cytokine inducing capacity itself, but to activate cells indirectly by first acquiring immune stimulating CpG DNA (61) which is released in the circulation during bacterial sepsis. A very recent study, however, showed that HMGB1-mediated induction of macrophage cytokine production requires binding to TLR4, and that binding and signaling are dependent upon a molecular mechanism that requires cysteine in position 106 within the B box (62). Together these data suggest that HMGB1 may exert pro-inflammatory activities in a direct TLR4-dependent way and an indirect way via binding of DAMPs and other agonistic molecules.

4.1.2. S100A12

S100A12, alsow known as EN-RAGE (extracellular newly identified ligand of RAGE) or myeloid-related protein (MRP)-6, is a calcium binding protein expressed in the cytoplasm of neutrophils (63), where it comprises 5% of the total protein content (64). Besides neutrophils, S100A12 is found in monocytes (63) and lymphocytes (65) and provokes pro-inflammatory responses in endothelial cells (33). Although many RAGE ligands are promiscuous with regard to receptor use, S100A12 has only been shown to bind to RAGE. S100A12 is strongly expressed in inflammatory diseases such as Crohn's disease, cystic fibrosis, atherosclerosis, rheumatoid arthritis and Kawaski disease (66-72). Furthermore, in the lungs, S100A12 and RAGE are found at high concentrations during acute lung injury (ALI) (33). S100A12 expression may reflect neutrophil activation during and contribute to pulmonary inflammation and endothelial activation via binding to RAGE (33).

4.1.3. β2 integrins

During infectious diseases, leukocyte migration to the site of infection is a primary process of host defense to help combat invading pathogens. RAGE has been implicated in the regulation of cell migration. RAGE is a counter-receptor for leukocyte integrins; in particular, RAGE has been identified as a binding partner for the β2

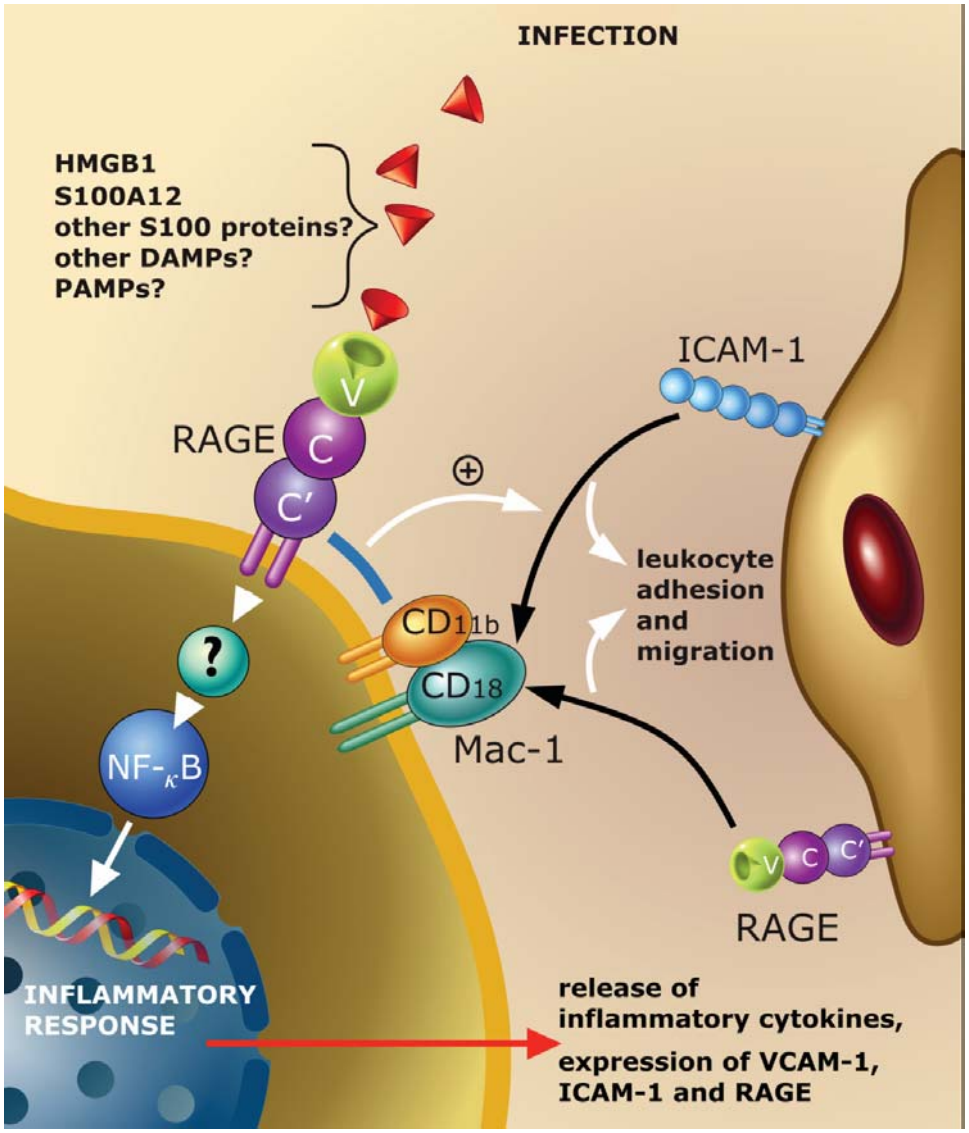

Figure 1. Putative RAGE involvement during infection. The DAMPs HMGB1 and S100A12 are shown to be released during infection (50-53) (and unpublished data) and to bind to and activate RAGE (6-9). It has to be determined whether other S100 proteins and other DAMPs are RAGE ligands (indicated as red shapes) that are released during infection. 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. Engagement of RAGE by its ligands results in receptor-dependent signaling and activation of NF-κB leading to a pro-inflammatory response; the signaling pathway is largely unknown. In addition, RAGE interacts as an endothelial (and epithelial) adhesion receptor with the leukocyte integrin CD11b/CD18 (Mac-1) (lower, right section) (14, 73). Furthermore, lateral (*in cis*) RAGE - Mac-1 interaction on the leukocyte surface is mediated by HMGB1 and activates Mac-1 - ICAM-1 dependent adhesion and migration and augments leukocyte recruitment as well (74) (indicated by the blue line and "+").

integrins Mac-1 and p150, 95 (14, 73). *In vivo* studies showed that the interaction of RAGE with β2 integrins mediated leukocyte recruitment: $RAGE^{-/-}$ mice showed a diminished number of adherent inflammatory cells on the peritoneum after CLP (17) and less intraperitoneal neutrophil influx during after thioglycollate peritonitis (14). Interestingly, HMGB1 can activate lateral (*in cis*) RAGE - Mac-1 interactions on the leukocyte cell surface, enhancing Mac-1 - ICAM-1-dependent adhesion and migration (74) (Figure 1). Additionally, a recent report shows that endothelial expressed RAGE acts in concert with ICAM-1 in mediating β_2 integrin–dependent leukocyte adhesion during acute trauma–induced inflammation (75).

5. SOLUBLE RAGE (sRAGE)

Soluble RAGE (sRAGE) 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) and circulates in plasma.

sRAGE has been suggested to be involved in inflammatory processes in several ways. First, circulating levels of sRAGE are associated with various inflammatory diseases in patients (32, 76, 77) and in rat models of experimental ALI (32). Secondly, it is thought that sRAGE can compete with full length cell-surface RAGE for ligand binding, preventing these ligands to bind to their receptors (including RAGE), and/or to exert effects otherwise. Indeed, the exogenous administration of sRAGE reduced inflammatory responses in several animal models, including models of hepatic injury (18-20), diabetic atherosclerosis (21, 22), delayed type hypersensitivity (17, 23), type II collagen-induced arthritis (23) and experimental auto-immune encephalomyelitis (78). The role of sRAGE in infectious diseases is unknown. Based on experimental studies in rats and in patients with ALI, sRAGE has been described as a marker of lung injury (32).

6. RAGE SIGNALING

RAGE engagement by its myriad ligands induces NF-κB activation. The signaling cascade(s) of RAGE ultimately activating NF-κB remains to be elucidated. The cytosolic portion of RAGE is estimated to consist of 43 amino acids (79) which is - compared to other PRRs, the TLRs and IL-1 receptors – short. This raises questions as to whether RAGE is a signal transducer. The RAGE mutant lacking this intracellular tail fails to activate NF-κB and behaves like a dominant negative, extinquishing the release of pro-inflammatory cytokines from macrophages (80, 81). These findings suggest critical involvement of the RAGE cytosolic portion in transducing the signal from the cell surface to the nucleus. This small intracellular part of RAGE does not contain a known signaling domain or motif. One of the possibilities is that RAGE uses yet unknown adaptors framing a whole "new" signaling pathway to NF-κB. Another possibility is that the RAGE tail interacts with a TIR-containing protein which then recruits the downstream TIR-containing proteins in a way similar to TLR-mediated signaling cascades. Furthermore, RAGE could transduce signals from the cell surface to the nucleus by bypassing the TIR-containing adaptor, directly interacting with member(s) of the signaling cascade. Besides triggering NF-κB activation, RAGE engagement by its diverse ligands is linked to a diversity of signaling pathways, including mitogen-activated protein kinase (MAPK) family members such as Jun-N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) (82, 83), PI3K/Akt (84), Jak/STAT (85) and Src family kinases (86).

Several papers have been published on putative direct binding partners using the cytoplasmic tail of RAGE as bait, transducing the signal to more downstream pathways. Ishihara *et al.* reported both ERK 1 and 2 as direct binding partners (87). Further truncation of the membrane-proximal part of the cytoplasmic portion of RAGE disclosed a possible ERK docking site. In addition, Hudson *et al.* showed that RAGE-ligand ligation induced activation of Rho GTPases Rac-1 and Cdc42 and subsequent cellular migration is mediated by binding of the cytosolic portion of RAGE to Diaphanous-1 (88).

Furthermore, it is suggested that AGEs induce RAGE dependent signals leading to cell cycle progression by converging on p21/ Ras expression (89). Another set of *in vitro* experiments showed that dominant negative Ras mutants are able to inhibit both AGE and HMGB1 induced activation of NF-κB-dependent transcription, whereas inhibition of Rho, Rac, or Cdc42 had no significant effect (90). These data indicate that Ras but not the Rho family members are involved in the RAGE-mediated activation and apparent nuclear translocation of NF-κB.

Also, a previously unknown codependence between signals derived from RAGE and from bone morphogenetic protein (BMP) receptor (BMPR) II has been suggested (91). In this study it was shown that a single ligand of either RAGE or BMPRII (S100A4 or BMR-2, respectively) can recruit each other's cell surface receptor to relay signals necessary to orchestrate a functional response, in this case to coordinate cytoskeletal changes with matrix metalloproteinase activity and Rho/Rac activation required for cell motility.

The broad expression of RAGE, its diversity of ligands and possible contaminating elements in the preparations used in experiments may account for this rather extraordinary variety of observed assortment of signals.

7. RAGE AND INFECTIOUS DISEASES

A rapidly increasing amount of literature implicates RAGE in the pathogenesis of (abdominal) sepsis and pneumonia, suggesting that RAGE (ligand) directed therapies might offer new treatment opportunities for human disease in the future.

7.1. RAGE and abdominal sepsis

The clinical syndrome of sepsis is the result of a systemic response of the host to a severe infection, characterized by the concurrent activation of various mediator systems. The involvement of RAGE in abdominal sepsis has thus far been examined in a limited number of studies. In two publications, $RAGE^{-/-}$ mice demonstrated a diminished lethality after induction of polymicrobial sepsis induced by CLP (17, 92). Moreover, anti-RAGE therapy yielded an enhanced survival even when the anti-RAGE antibodies were administered 24 hours after CLP in mice receiving antibiotic treatment (92). The avantage provided by the lack of RAGE was associated with a strongly reduced activation of NF-κB, suggesting that the lower mortality was at least in part due to the absence of excessive NF-κB activation in $RAGE^{-/-}$ mice (17). Furthermore, RAGE deficiency resulted in a decreased accumulation of inflammatory cells in the peritoneum (17), which is in line with an earlier investigation by the same group of authors identifying RAGE as a counter-receptor for the β2 integrin Mac-1 (CD11b/CD18) and thereby as a mediator of leukocyte recruitment and adhesion (14). Also, it is likely that the beneficial effect of RAGE inhibition in this CLP model at least in part is due to inhibition of one of its ligands HMGB1. Indeed, HMGB1 is released in the

circulation after CLP and anti-HMGB1 antibody protected against CLP induced lethality (50, 54).

RAGE deficiency and anti-RAGE therapy were reported not to influence bacterial loads in the peritoneum, liver or spleen in this surgical model of CLP induced sepsis (92). However, the possible role of RAGE in antibacterial defense can not be easily determined from this investigation since host defense against CLP at least in part relies on the extent of intestinal necrosis and the formation of a local abcess (93). Furthermore, all mice in this experiment were treated with broad spectrum antibiotics and bacterial loads were only determined in mice that survived (*i.e.* not at predefined time points after CLP). Therefore, we used our model of abdominal sepsis induced by intraperitoneal injection of the gram-negative bacterium *Escherichia coli* (*E. coli*) (94-96) to evaluate whether RAGE affects antibacterial defense. This model is a relevant tool to study the role of receptors/mediators in limiting the growth and dissemination of bacteria after a primary intra-abdominal infection and to determine the contribution of these proteins to specific immune responses (94-96). *E. coli* induced sepsis was associated with upregulated RAGE expression (95). RAGE deficiency (either genetically using $RAGE^{-/-}$ mice or pharmacologically using anti-RAGE IgG antibodies) was associated with an enhanced bacterial outgrowth and dissemination (95). As such, we provided evidence that RAGE signaling contributes to an effective antibacterial response during abdominal sepsis. Most likely, RAGE exerts this effect indirectly and not via direct interation with *E. coli*, considering that $RAGE^{-/-}$ leukocytes demonstrated an unaltered capacity to phagocytose and kill *E. coli in vitro*. In addition, the observation that RAGE deficiency in general was associated with an exaggerated host response during *E. coli* sepsis (95) on one hand and with a reduced inflammatory response and better survival in (other) sterile models of intraperitoneal injection of LPS derived from *E. coli* (95, 97, 98) on the other hand, suggests that although RAGE is involved in the immune reaction to *E. coli*, this function can be compensated for by other receptors in the presence of a growing bacterial load. The high-affinity RAGE ligand HMGB1 is released systemically during clinical sepsis (50, 51, 53, 54) as well as in our experimental sepsis model of *E. coli* (96). Importantly, HMGB1 has been reported to transduce cellular signals *in vitro* and *in vivo* by interacting with at least three other receptors, *i.e.* TLR2, TLR4 and TLR9 when HMGB1 is complexed with CPG DNA (61, 97-101). Therefore, one possible explanation for the enhanced inflammation in the $RAGE^{-/-}$ mice during *E. coli* sepsis could be that the absence of RAGE facilitates the interaction between HMGB1 and TLR2, TLR4 and/or TLR9.

We recently published on involvement of ligands of RAGE and HMGB1 in host defense in *E. coli* abdominal sepsis (96). Inhibition of multiple RAGE ligands (by the administration of sRAGE) as well as inhibition of HMGB1 (by the administration of anti-HMGB1 antibodies) resulted in an enhanced bacterial dissemination of *E. coli,* identifying a beneficial role of RAGE ligands, amongst which HMGB1, in the antibacterial response during gram-negative sepsis.

Interestingly, another high-affinity ligand of RAGE, S100A12, is released in patients in the circulation during (abdominal) sepsis and also locally during peritonitis (unpublished data, our laboratory). Furthermore, intravenous LPS injection in healthy humans elevated systemic S100A12 levels, implicating that LPS might be in part responsible for this upregulation during gram-negative infection. Payen *et al.* found that mRNA S100A12 expression by circulating leukocytes in patients with septic shock is diminished during the recovery phase (102). One possible function in host defense of S100A12 during infection and sepsis is its role as a DAMP. Indeed, NF-κB mediated expression of pro-inflammatory cytokines and upregulation of ICAM-1 and VCAM-1 on endothelium has been documented *in vitro* after S100A12 stimulation (33, 103). Furthermore, S100A12 could be beneficial for the host during infection and sepsis due to its (more direct) antibacterial activity. Previously, it was shown that S100A12 has activity primarily against gram-negative bacteria, including *E. coli* (104). Since S100A12 is not present in rodents (105), the functional role of S100A12 during sepsis cannot be easily investigated by inhibiting/deleting S100A12 in animals. Taken together, the role of S100A12 during sepsis has still to be investigated using non-murine models.

Circulating sRAGE concentrations in septic patients are increased and are higher in non-survivors than in survivors as reported by Bopp *et al.*, suggesting that sRAGE is related to severity and clinical outcome in sepsis (106). Knowledge on the role of endogenous sRAGE in sepsis is lacking. Humpert *et al.* demonstrated that sRAGE levels might represent an early marker of microvascular dysfunction, a phenomenon also present in sepsis (107). Additionally, increased sRAGE concentrations in sepsis might indicate an acute inflammation status as splice variants of RAGE or as split off variants of the cell surface RAGE, the latter analogously to ICAM-1, another member of the immunoglobulin superfamily, being a marker of cellular damage during sepsis (108). Furthermore, circulating sRAGE levels might parallel those of HMGB1/S100A12 as a counter-system against HMGB1/S100A12 elicited tissue effects. Further studies are needed to clarify a potential functional role of sRAGE in sepsis and its putative role as a new sepsis marker.

7.2. RAGE and pneumonia

7.2.1. RAGE expression during pneumonia

Infections of the respiratory tract are the 7th leading cause of mortality in the US (109, 110). According to the acquisition of pneumonia and the pathogens involved, community-acquired pneumonia (CAP) can be distinguished from hospital-acquired pneumonia (HAP). While the gram-positive bacterium *Streptococcus* (*S.*) *pneumoniae* is the single most frequent pathogen causing CAP, responsible for up to 60% of cases, *Klebsiella* (*K.*) *pneumoniae*, *Haemophilus* (*H.*) *influenzae, Staphylococcus* (*S.*) *aureus* and viruses are isolated in about 10% each, while *Mycobacterium* (*M.*) *tuberculosis* is more prevalent

in developing countries (111-113). Knowledge of the expression and role of RAGE in host defense during pneumonia is limited. Morbini *et al.* observed more RAGE expression in patients with interstitial and postobstructive pneumonia (31); this study left unclear whether patients with bacterial pneumonia were included in the analysis. Of note, two other reports indicated that constitutively present RAGE is not upregulated during ALI or acute respiratory distress syndrome (ARDS) associated lung inflammation. First, rats with ALI induced by intratracheally administered LPS displayed no change in the distribution of RAGE-expressing cells (32). Additonally, patients with ARDS did not have increased pulmonary expression of RAGE (33).

sRAGE has been described as a marker of lung injury based on studies in patients with ALI and on experimental studies in rats (32). sRAGE was increased in pulmonary edema fluid and serum from patients with either ALI or ARDS and with hydrostatic pulmonary edema, and in BALF from rats with either LPS or hydrochloric acid induced ALI (32).

Given the ubiquitous expression of RAGE in the lungs, its putative role in the regulation of lung inflammation and the somewhat inconsistent findings which currently exist in the literature, we aimed to investigate its role during pulmonary infections, a major cause of morbidity and mortality world-wide. We recently reported that pneumonia in mice induced by the gram-positive bacterium *S. pneumoniae* and by influenza A virus is associated with an upregulation of intra-alveolar (membrane bound) RAGE expression (114, 115). In addition, pulmonary tissue of mice intranasally infected with the gram-negative bacterium *K. pneumoniae* or with *M. tuberculosis* show increased RAGE expression as well (unpublished data). These clinically very different types of lung infections and the role of RAGE therein will be assessed here.

7.2.2. RAGE and gram-positive pneumonia

HMGB1 concentrations were higher in BALF from patients with pneumonia at the site of infection compared to BALF from healthy controls as shown previously (53). During experimental pneumococcal pneumonia, RAGE was found to be detrimental: $RAGE^{-/-}$ mice had a better survival rate in combination with a lower bacterial counts in the lungs and decreased dissemination of *S. pneumoniae* to blood and spleen relative to wild-type mice (114). The difference was possibly in part due to an enhanced killing capacity of $RAGE^{-/-}$ alveolar macrophages. Furthermore, lung injury and neutrophil migration were attenuated in the $RAGE^{-/-}$ mice, which is in line with data on RAGE as an endothelial counter receptor for the β2 integrin Mac-1 (14, 73) and the interplay between RAGE and Mac-1 on leukocytes, required for HMGB1 mediated inflammatory cell recruitment (74). In addition, the blockade of the RAGE - HMGB1 interaction and prevention of the subsequent pro-inflammatory stimulus might be an explanation for the less severe pulmonary damage in the $RAGE^{-/-}$ mice during *S. pneumoniae* pneumonia.

7.2.3. RAGE and gram-negative pneumonia

In contrast to the gram-positive pneumonia results, preliminary data from our laboratory reveal that RAGE plays a beneficial role in mice during the host response to gram-negative pneumonia (unpublished data). Indeed, RAGE deficiency was associated with a worsened mortality, increased bacterial outgrowth and dissemination after *K. pneumoniae* inoculation (unpublished data). Compared to wild type mice, lung inflammation was unaltered and cytokine and chemokine levels were slightly - if at all - elevated. Also, $RAGE^{-/-}$ mice displayed a similar response to intranasally instilled *Klebsiella* LPS with respect to pulmonary cell recruitment and local release of cytokines and chemokines. Together, these results suggest that RAGE contributes to an effective antibacterial host response during *K. pneumoniae* pneumonia, whereas RAGE plays an insignificant part in the lung inflammatory response to either intact *Klebsiella* or *Klebsiella* LPS.

So far, it is not clear whether RAGE can also interact with ligands from pathogens (116). If so, this could be part of the explanation of the opposite effects on mortality of RAGE during gram-positive and –negative pneumonia. Furthermore, RAGE induced effects on other first-line defense mechanisms such as chemotaxis, phagocytosis, killing (including respiratory burst) could depend on the pathogen and may account for the observed effects in gram-positive and -negative pneumonia models. However, this remains speculative until investigations have been performed analysing this interesting issue.

7.2.4. RAGE and viral pneumonia

Seasonal influenza A virus (IAV) infection causes over 200,000 hospitalizations and approximately 41,000 deaths in the United States annually, being the 7th leading cause of mortality (117), besides its potential to cause pandemics. We reported that RAGE deficiency is associated with a better outcome in pulmonary IAV as indicated by a relative protection from IAV induced mortality in mice (115). This was accompanied by an improved viral clearance and enhanced cellular T cell response and activation of neutrophils, implicating that endogenous RAGE impairs the cellular immunity against respiratory tract infection with IAV. The high affinity RAGE ligand HMGB1, as well as sRAGE were upregulated in BALF during IAV pneumonia. So, similar to pneumonia induced by the gram-positive bacterium *S. pneumoniae*, RAGE is detrimental during pneumonia caused by IAV. This is of particular interest, since it is suggested that the greatest proportion of mortality caused by IAV infection is due to secondary bacterial pneumonia, with *S. pneumoniae* as the most frequent pathogen of the superinfection. Therefore, RAGE is a potential treatment target in postinfluenza pneumococcal pneumonia and further research is warranted to investigate this.

7.2.5. RAGE and pulmonary tuberculosis

Tuberculosis remains a major health burden world-wide, responsible for eight million new cases and two million deaths each year (118, 119). Multidrug-resistant strains are on the rise and the frequent occurrence of co-infection with human immunodeficiency virus makes

the treatment and outcome of tuberculosis even more worrisome. Our preliminary data show that RAGE deficiency results in an enhanced inflammatory response in the lungs of mice infected with *M. tuberculosis* via the airways, accompanied by an adverse long-term outcome as reflected by accelerated weight loss and increased mortality. Lung lymphocyte and neutrophil numbers were increased in the $RAGE^{-/-}$ mice (unpublished data). Since bacterial outgrowth did not differ between the two mouse strains, this supports the idea that in this model, mortality is due to the overwhelming inflammation and damage in the lungs and not to the bacterial load. In addition, these findings not only suggest that RAGE is not important for induction of sustained pulmonary inflammation after infection with *M. tuberculosis*, but also that the delicate balance between benefit and harm resulting from the inflammatory response during tuberculosis can be disturbed by the absence of intact RAGE signaling. Of note, this infectious model has the latest end points and longest survival (6 and 28 weeks, respectively) compared with other, previously mentioned pneumonia models in which the mice are sacrificed or dead in maximal 2 weeks. However, mice were still much younger at the time of sacrification or death than the aged (19-24 months old) $RAGE^{-/-}$ mice that developed pulmonary fibrosis spontaneously in the experiment of Englert *et al.* (30). However, pulmonary fibrosis-like alterations in the $RAGE^{-/-}$ mice during experimental tuberculosis as a contribution to their enhanced pulmonary inflammatory response and mortality can not be ruled out, since Englert el al. showed that $RAGE^{-/-}$ mice of 24 (but not 12) weeks old show an increase in collagen content of the lungs with more hydroxyproline content as well (30).

8. CONCLUSIONS AND FUTURE PERSPECTIVES

The innate immune response is the first line of defense against pathogens. The studies discussed here provide further insight into the role of RAGE and its ligands in host defense during clinically important infections, which eventually may contribute to better therapies against specific pathogens. While interpreting the results from preclinical investigations, one has to consider that a careful balance between the inflammatory and anti-inflammatory response is essential in order to survive or recover from a severe infection.

The finding that RAGE deficiency is beneficial in one pneumonia model and detrimental in the other, clearly adds to the notion that the way in which RAGE mediates host defense against different pathogens relies on distinct mechanisms. It would be highly interesting to investigate whether RAGE can directly bind to, become activated and mount a first immune reaction after ligation with specific PAMPs. In addition, RAGE mediated effects on other first-line defense mechanisms such as chemotaxis, killing, phagocytosis and respiratory burst could depend on the pathogen. As such RAGE targeting may be either ineffective or even harmful in some infectious conditions. Therefore, more research is required to warrant clinical trials targeting RAGE in patients with severe infections. In this respect one could think of studies on RAGE inhibition in pneumococcal and IAV pneumonia. In addition, experiments in which RAGE targeting is delayed until after bacterial/viral infection and combined with antibiotic/antiviral therapy should be considered. Moreover, more studies need to be conducted on the role of RAGE in critical organ derangements implicated in the pathogenesis of severe infection, including activation of the coagulation system and the complement system. Until then, RAGE remains a potential yet promising therapeutic target that awaits further research.

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Abbreviations: RAGE; receptor for advanced glycation end products, DAMPs; damage-associated molecular patterns, HMGB1; high-mobility group box 1, PAMPs; pathogen-associated molecular patterns, NF-κB; nuclear factor-κappa B, Mac-1; macrophage-1 antigen, ICAM-1; intercellular adhesion molecule

Key Words: RAGE, infection, sepsis, pneumonia, HMGB1, S100A12, host defense, DAMPs, PAMPs, Review

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