RAGE and the innate immune response in infection and inflammation

van Zoelen, M.A.D.

Citation for published version (APA):
Receptor for advanced glycation end products protects against *Klebsiella pneumoniae* induced pneumonia in mice

Marieke A.D. van Zoelen,1,2 Alex F. de Vos,1,2 Sandrine Florquin,3 Angelika Bierhaus,4 Regina de Beer,1,2 Peter P. Nawroth4 and Tom van der Poll1,2

Submitted

1Center for Infection and Immunity Amsterdam (CINIMA), 2Center for Experimental and Molecular Medicine (CEMM), 3Department of Pathology; Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
4Department of Internal Medicine and Clinical Chemistry, University of Heidelberg, Heidelberg, Germany
**ABSTRACT**

*Klebsiella* species is the second most commonly isolated gram-negative organism in sepsis and a frequent causative pathogen in pneumonia. The receptor for advanced glycation end products (RAGE) is expressed on different cell types and plays a key role in diverse inflammatory responses. We here investigated the role of RAGE in the host response to *Klebsiella (K.) pneumoniae* pneumonia and intranasally inoculated RAGE deficient (RAGE-/-) and normal wild-type (Wt) mice with *K. pneumoniae*. *K. pneumoniae* pneumonia was associated with an increased pulmonary expression of RAGE compared to healthy, uninfected mice. RAGE deficiency impaired host defense as reflected by a worsened survival, increased bacterial outgrowth and dissemination in RAGE deficient mice. RAGE deficient mice infected with *K. pneumoniae* showed similar lung inflammation, and slightly elevated - if any - cytokine and chemokine levels and unchanged hepatocellular injury. In addition, RAGE deficient mice displayed an unaltered response to intranasally instilled *Klebsiella* lipopolysaccharide with respect to pulmonary cell recruitment and local release of cytokines and chemokines. These data suggest that RAGE contributes to an effective antibacterial host response during *K. pneumoniae* pneumonia. Furthermore, we established that RAGE plays an insignificant part in the lung inflammatory response to either intact *Klebsiella* or *Klebsiella* LPS.
INTRODUCTION

Gram-negative pneumonia is a common and serious illness that is a major cause of morbidity and mortality in humans. *Klebsiella (K.) pneumoniae* is a frequently isolated causative pathogen in lower respiratory tract infection (1-3). The increasing microbial resistance to antibiotics resulting in therapy failure and higher mortality rates is an issue of major concern (1). Therefore it is important to gain more insight into the pathogenesis of pneumonia.

Triggering of receptor for advanced glycation end products (RAGE) results in sustained receptor-dependent signaling and activation of nuclear factor-κB and mitogen-activated protein kinase pathways. Its known ligands are amongst others advanced glycation end products (4), amyloid (5), β-sheet fibrils (6), high mobility group box 1 (7, 8) and some members of the S100 family, including S100A12 (9), S100B (10) and S100P (11). Inhibition of RAGE signaling has been found to reduce inflammatory responses in animal models of hepatic injury (12-14), diabetic atherosclerosis (15, 16), delayed type hypersensitivity (17, 18), type II collagen-induced arthritis (18) and sepsis (17).

RAGE is expressed in normal, healthy lungs (19-23) and pulmonary RAGE expression is enhanced in patients with pneumonia (21). Earlier, we found that RAGE deficiency protects against pneumonia caused by the gram-positive bacterium *Streptococcus pneumoniae* (*S. pneumoniae*) as reflected by an enhanced survival, diminished outgrowth at the primary site of infection and a decreased spreading of bacteria to other body compartments together with reduced lung damage (24) (chapter 3). Whereas *S. pneumoniae* is the most commonly isolated pathogen in patients with community-acquired pneumonia, *K. pneumoniae* is a causative organism in both community-acquired and nosocomial pneumonia (25, 26). We here sought to determine the role of RAGE in pneumonia caused by *K. pneumoniae*. For this, we first investigated RAGE expression in the lungs during *K. pneumoniae* pneumonia and next, we intranasally infected RAGE deficient mice (RAGE−/− mice) with *K. pneumoniae* and compared the course of the infection in these mice with that in concurrently infected wild-type (Wt) mice.
Materials and Methods

Animals
Ten week old male RAGE deficient (RAGE−/−) mice were generated as previously described (17) and backcrossed ten times to a C57Bl/6 background. Wt type C57Bl/6 mice were obtained from Harlan Sprague Dawley Inc. (Horst, The Netherlands). The Institutional Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam, approved all experiments.

Induction of pneumonia and LPS induced lung inflammation
Pneumonia was induced as described earlier (27-29). *K. pneumoniae* serotype 2 (ATCC 43816; American Type Culture Collection, Manassas, VA) was cultured for 16 h at 37 °C in 5% CO₂ in tryptic soy broth (TSH, Difco, Detroit, MI). This suspension was diluted 1:100 in fresh medium and grown for 3 h to midlogarithmic phase. Bacteria were harvested by centrifugation at 1,500 x g for 15 min., washed twice in sterile 0.9% saline and resuspended in saline. The number of colony forming units (CFUs) was determined by plating 10-fold dilutions of the suspensions on blood agar plates. After preparation of the bacterial inoculum, mice were slightly anesthetized by inhalation of isoflurane (Abott, Queensborough, Kent, UK) and 50 µl of bacterial suspension (1 x 10⁴ CFUs) was inoculated intranasally. In survival studies, mice were monitored for 2 weeks (1 x 10⁴ CFUs). In a separate experiment, 50 µl of lipopolysaccharide (LPS) from *K. pneumoniae* (Sigma, St. Louis, MO) (100 µg) was inoculated intranasally. Uninfected (control) mice and mice inoculated with *K. pneumoniae* or with LPS from *K. pneumoniae* were anesthetized with ketamine (Eurovet Animal Health BV, Bladel, The Netherlands) and medetomidine (Pfizer Animal Health BV, Capelle aan de IJssel, The Netherlands) at indicated time points for sample harvesting.

Preparation of blood samples and organ homogenates
Blood was collected from the *vena cava inferior* in heparin containing tubes and centrifuged at 1500 x g for 10 minutes, after which plasma was collected and frozen at -20 °C until assayed. The lungs were removed and processed as described previously (27, 30, 31). Lungs, liver and spleen were harvested and homogenized at 4 °C in 4 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, OK) which was carefully cleaned and disinfected with 70% ethanol after each homogenization. Serial 10-fold dilutions in sterile saline were made from these homogenates and blood and 50 µl volumes were plated onto sheep-blood agar plates and incubated at 37 °C and 5% CO₂. CFUs were counted after 24 h. For cytokine, chemokine and myeloperoxidase (MPO) measurements, lung homogenates were lysed in 1 volume of lysis buffer (300 mM NaCl, 15 mM Tris [tris(hydroxymethyl)aminomethane], 2 mM MgCl₂, 2 mM Triton X-100,
pepstatin A, leupeptin and aprotinine [20 ng/ml], pH 7.4) on ice for 30 minutes and centrifuged at 1500 x g at 4 °C for 10 minutes. The supernatants were frozen at -20 °C until assayed.

**Bronchoalveolar lavage**
The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott Laboratories, Sligo, Ireland). Bilateral bronchoalveolar lavage (BAL) was performed by instilling two 0.5-ml aliquots of sterile isotonic saline. 0.9-1 ml of lavage fluid was retrieved per mouse.

**Cell counts and differentials**
Cell counts were determined in BALF using an automated counter (Beckham Coulter, Coulter ZF, Mijdrecht, The Netherlands). Subsequently, BALF was centrifuged and supernatant was stored at -20 °C until further assayed; the pellet was suspended in phosphate-buffered saline until a final concentration of 10^5 cells/ml and differential cell counts were performed on cytospin Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland).

**Histological examination**
Lungs for histologic examination were harvested after 24 and 48 h, fixed in 4% formaldehyde and embedded in paraffin. 4-µm thick sections were stained with hematoxylin-eosin and analyzed by a pathologist who had no knowledge of the genotype of the mice. To score lung inflammation and damage, the lung samples were screened for the following parameters: interstitial inflammation, vaculitis, bronchitis, edema and pleuritis. Each parameter was graded on a scale of 0 to 5 (0, absent; 1, very mild; 2, mild; 3, moderate; 4, severe and 5, very severe). The total histology score was expressed as the (mean) sum of the score for all parameters. Immunostaining for RAGE was performed on paraffin slides after deparaffinization and rehydration using standard procedures. Endogenous peroxidase activity was quenched using 1.5% H_2O_2 in PBS. Primary antibodies used were goat anti-mouse RAGE polyclonal antibodies (Neuromics, Edina, MN) and secondary antibodies were biotinylated rabbit anti-goat antibodies (DakoCytomation, Glostrup, Denmark). ABC solution (DakoCytomation, Glostrup, Denmark) was used as the detection enzyme. DAB peroxidase (Sigma, St. Louis, MO) was used as substrate for visualization. Counterstaining was performed with methylgreen (Sigma, St. Louis, MO).

**Assays**
Tumor necrosis factor (TNF)-α, interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1 and IL-10 levels were determined using a cytometric beads array (CBA)
multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturer’s recommendations. Keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP-2) levels were measured by ELISA (R&D Systems, Abingdon, United Kingdom) according to the manufacturer’s instructions. Myeloperoxidase (MPO) was measured by ELISA (Hycult Biotechnology BV, Uden, The Netherlands). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined with commercially available kits (Sigma-Aldrich, St. Louis, MO), using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany).

Statistical analysis
All data are expressed as means ± SEM. Differences between groups were analyzed by Mann-Whitney U test. Values of \( p < 0.05 \) were considered to represent a statistically significant difference.

Results
RAGE expression in the lungs
To obtain constitutive and \( K. \) pneumoniae-induced RAGE expression, we performed immunohistochemical stainings of RAGE of lung tissue from healthy, uninfected Wt mice and from Wt mice after inoculation with \( K. \) pneumoniae. In accordance with the literature (19-23), the normal, healthy mice showed extensive RAGE staining in their lungs (Fig. 1A), being mainly present in the interalveolar septae in an endothelial pattern, while bronchial epithelial cells were negative for RAGE staining (Fig. 1A, arrow). The specificity of the RAGE staining was confirmed by immunohistochemical analysis of lungs obtained from RAGE \(^{-/-} \) mice, used as negative controls (Fig. 1B). Lungs from \( K. \) pneumoniae infected mice displayed the same pattern of RAGE positivity as lungs from

![Figure 1](image). Expression of RAGE in lungs during \( K. \) pneumoniae pneumonia. Representative view of a lung from a normal, uninfected Wt mouse (A) displaying ubiquitous expression of RAGE on the surface of endothelium. (B) Absence of RAGE positivity in the lung of a RAGE \(^{-/-} \) mouse. (C) Lungs from a Wt mouse 48 h after the inoculation of \( K. \) pneumoniae. Arrow indicates bronchial epithelium in healthy lungs (A), being negative for RAGE staining. RAGE staining; original magnification x10.
healthy Wt mice, i.e. the interalveolar septae stained positive for RAGE staining with an endothelial pattern; however, RAGE expression was enhanced following pulmonary infection with *K. pneumoniae* as reflected by more intense staining (Fig. 1C).

**RAGE deficiency enhances lethality due to *K. pneumoniae* pneumonia**

To study the contribution of RAGE to the outcome of *Klebsiella* pneumonia, Wt and RAGE<sup>−/−</sup> mice were intranasally inoculated with *K. pneumoniae* and observed for 14 days (Fig. 2). Although the first deaths occurred after 2 days in both strains, all RAGE<sup>−/−</sup> mice had died after 10 days, while only 50% of the Wt mice had died at the end of the observation period (*p* < 0.05). Thus, RAGE deficiency rendered mice more susceptible to *K. pneumoniae* induced death.

![Figure 2. RAGE<sup>−/−</sup> mice demonstrate an increased mortality during *K. pneumoniae* pneumonia.](image)

**RAGE deficiency facilitates early bacterial outgrowth and dissemination**

To obtain insight in the mechanism underlying the higher mortality of RAGE<sup>−/−</sup> mice, we repeated this experiment and sacrificed mice 24 and 48 h after infection (i.e. directly before the first mice started dying) to enumerate bacterial counts in lungs, blood, liver and spleen. At 24 h after infection, bacterial outgrowth in the lungs were similar in Wt and RAGE<sup>−/−</sup> mice. However, after 48 h, the number of *Klebsiella* CFUs was higher in the lungs of RAGE<sup>−/−</sup> mice when compared to the Wt mice (*p* < 0.05; Fig. 3A). At 24 h, significantly increased CFU counts were recovered from blood, liver and spleen harvested from the RAGE<sup>−/−</sup> mice compared to Wt mice. Liver homogenates from RAGE<sup>−/−</sup> mice showed increased bacterial loads at 48 h as well (all *p* < 0.05; Fig. 3B-D). Thus, RAGE serves to limit the outgrowth of *K. pneumoniae* in the lungs and the ensuing dissemination to the blood stream and distant organs.
Figure 3. RAGE-/- mice demonstrate an enhanced local bacterial outgrowth and dissemination during K. pneumoniae pneumonia. Bacterial loads in lung homogenate (A), blood (B), liver (C) and spleen (D) were determined in Wt and RAGE-/- mice 24 and 48 h after intranasal inoculation 1 x 10^4 CFUs K. pneumoniae. Data are means ± SEM of 8-10 mice per genotype at each time point. *, p < 0.05 vs Wt mice.

Figure 4. Unchanged lung inflammation during Klebsiella pneumonia. Wt and RAGE-/- mice were inoculated intranasally with 1 x 10^4 CFUs K. pneumoniae. Representative HE stainings of lung tissue at 24 (A and B) and 48 (C and D) h post inoculation in Wt (A and C) and RAGE-/- (B and D) mice. Original magnification x20. Graphical representation of the degree of lung inflammation at 24 and 48 h (E) determined according to the scoring system described in the Materials and Methods section. Myeloperoxidase (MPO) levels in lung tissues (F). Data are means ± SEM of 8-10 mice per genotype at each time point.
RAGE deficiency does not impact on lung inflammation during *K. pneumoniae* pneumonia

Considering that RAGE signaling results in sustained cellular activation we were interested to study the role of RAGE in lung inflammation during *Klebsiella* pneumonia. Thus, we analyzed lung tissue slides obtained from Wt and RAGE$^{-/-}$ mice 24 and 48 h after infection. At both time points, both mouse strains displayed interstitial inflammation together with vasculitis, peri-bronchitis, edema and pleuritis (Fig. 4A-D). Importantly, in contrast to our expectation, the extent of lung inflammation, as determined by the semi-quantitative scoring system described in the Materials and Methods section, analyzing the severity of vasculitis, bronchitis, edema and pleuritis, was not different between Wt and RAGE$^{-/-}$ mice (Fig. 4E). In addition, MPO concentrations in lung homogenates of Wt and RAGE$^{-/-}$ mice were similar at both time points (Fig. 4F), indicating that RAGE deficiency did not influence neutrophil recruitment. Together these data suggest that RAGE does not play a significant role in the lung inflammation that accompanies *Klebsiella* pneumonia.

Cytokine and chemokine levels

In pulmonary infection, cytokines and chemokines production is an important factor in the host immune response (32, 33). We determined the influence of RAGE deficiency on pulmonary and systemic cytokine and chemokine concentrations during *Klebsiella* pneumonia. Levels of the cytokines tumor necrosis factor (TNF)-α, IL-6, MCP-1 and IL-10 and of chemokines KC and MIP-2 did not differ between the two mouse strains at 24 h. At 48 h, MCP-1 and KC concentrations were increased in the lungs ($p < 0.05$; Table I). In plasma, TNF-α, IL-6 nd MCP-1 levels were similar between the two mouse strains at both time points, while IL-10 was elevated in the RAGE$^{-/-}$ mice at 48 h ($p < 0.05$; Table I).

Wt and RAGE$^{-/-}$ mice display similar hepatocellular injury

This model of *Klebsiella* pneumonia is associated with hepatocellular injury (29). Considering the enhanced lethality and sustained elevated bacterial loads in liver homogenates in RAGE$^{-/-}$ mice, we were interested to examine the extent of hepatocellular injury in both mouse strains (Fig. 5). At 24 h after infection, neither RAGE$^{-/-}$ nor Wt mice demonstrated elevated plasma concentrations of AST or ALT. At 48 h post infection, both mouse strains had strongly elevated plasma transaminase levels; although these levels tended to be higher in RAGE$^{-/-}$ mice, the differences with Wt mice did not reach statistical significance.
Table I. Cytokine and chemokine levels in Wt and RAGE<sup>−/−</sup> mice<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>Wt 24 h</th>
<th>RAGE&lt;sup&gt;−/−&lt;/sup&gt; 24 h</th>
<th>Wt 48 h</th>
<th>RAGE&lt;sup&gt;−/−&lt;/sup&gt; 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lung homogenate (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>724 ± 187</td>
<td>446 ± 66</td>
<td>1,679 ± 932</td>
<td>5,151 ± 1617</td>
</tr>
<tr>
<td>IL-6</td>
<td>1348 ± 252</td>
<td>723 ± 240</td>
<td>706 ± 301</td>
<td>844 ± 223</td>
</tr>
<tr>
<td>MCP-1</td>
<td>6172 ± 381</td>
<td>6006 ± 444</td>
<td>2,861 ± 282</td>
<td>4,838 ± 623 **</td>
</tr>
<tr>
<td>IL-10</td>
<td>545 ± 99</td>
<td>482 ± 50</td>
<td>26 ± 3</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>KC</td>
<td>13991 ± 1847</td>
<td>13026 ± 1851</td>
<td>11,257 ± 2,251</td>
<td>20,478 ± 3,372 *</td>
</tr>
<tr>
<td>MIP-2</td>
<td>8082 ± 1818</td>
<td>11298 ± 2300</td>
<td>12,085 ± 4,367</td>
<td>7,548 ± 1,467</td>
</tr>
<tr>
<td><strong>Plasma (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>87 ± 30</td>
<td>141 ± 47</td>
<td>62 ± 18</td>
<td>56 ± 26</td>
</tr>
<tr>
<td>IL-6</td>
<td>211 ± 49</td>
<td>211 ± 58</td>
<td>277 ± 159</td>
<td>478 ± 157</td>
</tr>
<tr>
<td>MCP-1</td>
<td>827 ± 188</td>
<td>1,777 ± 444</td>
<td>519 ± 139</td>
<td>526 ± 231</td>
</tr>
<tr>
<td>IL-10</td>
<td>14 ± 3</td>
<td>21 ± 3</td>
<td>1 ± 1</td>
<td>8 ± 4 *</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are means ± SEM at 24 or 48 h post inoculation of 1 x 10⁴ CFUs K. pneumoniae. n = 8-10 mice per group.

*, p < 0.05 vs Wt mice; **, p < 0.01 vs Wt mice.

**Figure 5.** Hepatocellular injury during K. pneumoniae pneumonia. Wt and RAGE<sup>−/−</sup> mice were inoculated intranasally with 1 x 10⁴ CFUs K. pneumoniae and sacrificed after 24 and 48 h. Aspartate aminotransferase (AST, A) and alanine aminotransferase (ALT, B) in plasma of Wt and RAGE<sup>−/−</sup> mice. Data are means ± SEM of 8-10 mice per genotype at each time point.
Table II. Cell counts, cytokine and chemokine levels in bronchoalveolar lavage fluid after *Klebsiella* LPS

<table>
<thead>
<tr>
<th></th>
<th>Wt</th>
<th>RAGE&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells (x 10⁵/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cells</td>
<td>5.8 ± 1.0</td>
<td>8.2 ± 1.4</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>5.5 ± 1.0</td>
<td>7.7 ± 1.3</td>
</tr>
<tr>
<td>Cytokines (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>5,561 ± 819</td>
<td>6,212 ± 568</td>
</tr>
<tr>
<td>IL-6</td>
<td>2,542 ± 973</td>
<td>3,989 ± 583</td>
</tr>
<tr>
<td>KC</td>
<td>1327 ± 209</td>
<td>870 ± 81</td>
</tr>
<tr>
<td>MIP-2</td>
<td>661 ± 79</td>
<td>516 ± 34</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are means ± SEM at 6 h post inoculation of 100 μg LPS from *K. pneumoniae*. *n = 8-10 mice per group.*

**RAGE<sup>−/−</sup>** mice demonstrate an unchanged inflammatory response to *K. pneumoniae* LPS

In light of the strong expression of RAGE in the lung and its reported role as a receptor mediating pro-inflammatory effects, we were surprised to find unaltered lung inflammation and (if anything) higher cytokine levels in RAGE<sup>−/−</sup> mice during *Klebsiella* pneumonia. To obtain further evidence for a modest role of RAGE in the induction of lung inflammation in response to a gram-negative bacterium, we compared the inflammatory response to *Klebsiella* lipopolysaccharide (LPS), administered via the airways, in Wt and RAGE<sup>−/−</sup> mice. In these studies we harvested bronchoalveolar lavage fluid (BALF) 6 h after LPS administration considering that this time point is representative for examining LPS responses in the pulmonary compartment (27, 34, 35). In line with the observations during respiratory tract infection with live *K. pneumoniae*, RAGE<sup>−/−</sup> mice displayed an unaltered response to intranasally instilled *Klebsiella* LPS with respect to pulmonary cell recruitment and local release of cytokines and chemokines (Table II).

**DISCUSSION**

*Klebsiella* species is the second most commonly isolated gram-negative organism in sepsis (36, 37) and a frequent causative pathogen in pneumonia (1). RAGE is a multiligand receptor of the immunoglobulin superfamily that is expressed in all tissues by a wide range of cell types, including cells involved in the innate immune system, *e.g.* neutrophils, monocytes, macrophages and endothelial cells (38). RAGE has the ability to activate...
signaling pathways, resulting in pro-inflammatory gene expression upon interaction with several distinct endogenous proinflammatory ligands. Therefore, RAGE may function as a sensor of danger signals leading to a certain amount of inflammation and hence play a beneficial role in bacterial eradication during infection. However, interaction of RAGE with its ligands and the subsequently induced inflammation can also worsen tissue damage, thereby exerting detrimental effects. Earlier, we found that RAGE impairs host defense in gram-positive pneumonia. We here established that RAGE contributes to an effective antibacterial host response during \textit{K. pneumoniae} pneumonia. Indeed, RAGE\textsuperscript{-/-} mice displayed higher bacterial counts at the primary source of infection and increased spreading of bacteria to blood and distant organs, which was associated with a decreased survival.

We confirmed previous studies showing that normal healthy lungs constitutively express RAGE, especially in the alveolar septae in an endothelial pattern (19-23). Earlier, pulmonary RAGE expression was shown to be enhanced in patients with postobstructive pneumonia (21). Our present finding that \textit{K. pneumoniae} pneumonia is associated with an upregulation of interalveolar RAGE expression in the lungs together with similar observations in pneumococcal pneumonia strongly suggests that enhanced pulmonary RAGE expression is a common response to bacterial pneumonia.

The current data should be considered in the context of several earlier studies on the role of RAGE during bacterial infections. In a model of polymicrobial abdominal sepsis induced by cecal ligation and puncture, RAGE\textsuperscript{-/-} mice had an improved survival together with a reduced NF-κB activation in the peritoneum; bacterial growth and dissemination were not determined in this study (17). We previously investigated the role of RAGE during abdominal sepsis induced by the gram-negative bacterium \textit{Escherichia coli} (\textit{E. coli}), showing that RAGE deficiency was associated with an enhanced outgrowth of \textit{E. coli} locally and in distant organs together with more severe liver injury (chapter 2). In contrast, we found that in a model of pneumonia by the gram-positive bacterium \textit{S. pneumoniae}, RAGE deficiency was associated with a reduced bacterial outgrowth and dissemination and less severe lung damage (24) (chapter 3). RAGE deficiency or inhibition did not impact on the growth of \textit{Listeria monocytogenes} after intraperitoneal injection (39). These data suggest that RAGE plays differential roles in antibacterial defense during experimental infections with different pathogens.

The recruitment of neutrophils is an important part of host defense against pneumonia (40, 41). RAGE has been implicated as a mediator of cell trafficking. Indeed, RAGE\textsuperscript{-/-} mice had a lower number of adherent inflammatory cells on the peritoneum after cecal
ligation and puncture (17) and a reduction in neutrophil influx in the peritoneal cavity during thioglycollate peritonitis (42). Furthermore, in vivo studies have suggested that RAGE is an endothelial counter receptor for the β2 integrin Mac-1 (42, 43). During pneumococcal pneumonia RAGE-/- mice showed an attenuated influx of neutrophils into the lungs (24) (chapter 3). In contrast, we did not find an effect of RAGE deficiency on cell influx during K. pneumoniae pneumonia, as indicated by histopathology and pulmonary MPO concentrations. Moreover, leukocyte counts and differentials in BALF harvested after intrapulmonary delivery of Klebsiella LPS were similar in Wt and RAGE-/- mice. Together these data suggest that RAGE does not play a role of importance in leukocyte recruitment to the lungs during gram-negative infection, and that the impact of RAGE on cell trafficking may depend on the inflammatory stimulus and the organ involved. Of note, the model of (sterile) lung inflammation induced by Klebsiella LPS was also used to study the role of RAGE while avoiding potential bias due to differences in the pro-inflammatory stimulus caused by differences in pulmonary bacterial loads between Wt and RAGE-/- mice. These experiments confirmed the results obtained after infection with viable Klebsiella, i.e. that RAGE plays an insignificant part in the lung inflammatory response to either intact Klebsiella or Klebsiella LPS.

Pneumonia represents a major cause of morbidity and mortality. We here show that RAGE plays a protective role during respiratory tract infection by a common gram-negative causative pathogen, K. pneumoniae, by improving antibacterial defense in lungs and reducing bacterial dissemination.

Acknowledgements

We thank Regina de Beer, Joost Daalhuisen and Marieke S. ten Brink for expert technical assistance.
REFERENCES


34. Leemans, J. C., M. J. Vervoordeldonk, S. Florquin, K. P. van Kessel, and T. van der Poll.


