RAGE and the innate immune response in infection and inflammation

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Chapter 3

The receptor for advanced glycation end products impairs host defense in pneumococcal pneumonia

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Abstract

Streptococcus (S.) pneumoniae is the most common cause of community-acquired pneumonia. The receptor for advanced glycation end products (RAGE) is a multiligand receptor that is expressed ubiquitously in the lungs. Engagement of RAGE leads to activation of multiple intracellular signaling pathways, including NF-κB and subsequent transcription of several pro-inflammatory mediators. To determine the role of RAGE in the innate immune response to S. pneumoniae pneumonia, RAGE deficient (RAGE-/-) and wild-type (Wt) mice were intranasally inoculated with S. pneumoniae. S. pneumoniae pneumonia resulted in an upregulation of constitutively present RAGE expression in lung tissue, especially in the interalveolar septae. RAGE-/- mice showed an improved survival, which was accompanied by a lower bacterial load in the lungs at 16 h and a decreased dissemination of the bacteria to blood and spleen at 16 and 48 h after inoculation. RAGE-/- macrophages showed an improved killing capacity of S. pneumoniae in vitro. Lung inflammation was attenuated in RAGE-/- mice at 48 h after inoculation, 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 (6 h) inflammatory response to S. pneumoniae did not reveal an early accelerated or enhanced immune response. These data suggest 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.
INTRODUCTION

*Streptococcus pneumoniae* is the leading causative pathogen in community-acquired pneumonia and a major cause of morbidity and mortality in humans (1, 2). Pneumococci account for up to 36% of adult community-acquired pneumonia in the United States. An estimated 570,000 cases of pneumococcal pneumonia occur annually in the United States, resulting in 175,000 hospitalizations. Worldwide *S. pneumoniae* is responsible for an estimated ten million deaths annually, making pneumococcal pneumonia a major health threat (2). With the increasing incidence of antibiotic resistance in this pathogen, there is an urgent need to expand our knowledge of the host defense mechanisms that influence the outcome in *S. pneumoniae* pneumonia (3).

The receptor for advanced glycation end products (RAGE) interacts with diverse ligands such as 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). RAGE was first identified in lung tissue (12, 13). Recent studies have confirmed the expression of RAGE in normal, healthy lungs (14-18), mainly on endothelial and respiratory epithelial cells. Moreover, pulmonary RAGE expression is enhanced in patients with pneumonia and tuberculosis (16).

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 several models, including models of hepatic injury (19-21), diabetic atherosclerosis (22, 23), delayed type hypersensitivity (24, 25), type II collagen-induced arthritis (25), and sepsis (24). Given the ubiquitous expression of RAGE in the lungs, it is likely that this receptor plays a role in the regulation of lung inflammation. Therefore, we here sought to determine the role of RAGE in pneumonia caused by *S. pneumoniae*. For this, we first investigated RAGE expression in the lungs during *S. pneumoniae* pneumonia and next, we intranasally infected RAGE deficient mice (RAGE−/− mice) with *S. pneumoniae* and compared the course of the infection in these mice with that in concurrently infected wild-type (Wt) mice.
Materials and Methods

Animals
C57Bl/6 Wt mice were obtained from Harlan Sprague Dawley Inc. (Horst, The Netherlands). RAGE−/− mice, backcrossed ten times to a C57Bl/6 background, were generated as previously described (24). The Institutional Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam, approved all experiments.

Design
Pneumonia was induced as described earlier (26, 27). Mice were lightly anesthetized by inhalation of isoflurane (Abbott Laboratories, Queensborough, Kent, UK) and 50 µl containing 5 x 10⁴ - 5 x 10⁵ CFUs S. pneumoniae serotype 3 (ATCC 6303; American Type Culture Collection, Rockville, MD) was inoculated intranasally. Healthy control mice received 50 µl sterile saline only. In survival studies, mice were monitored for 2 weeks.

Preparations of lung homogenates and measurements
At 16 and 48 h after inoculation, mice were anesthetized with ketamine (Eurovet Animal Health BV, Bladel, The Netherlands) and medetomidine (Pfizer Animal Health BV, Capelle aan de IJssel, The Netherlands). In one experiment, the 6 h time point was used. Whole lungs, spleens, bronchoalveolar lavage fluid (BALF, see below) and blood were collected for CFU determination and additional assays (see below) as described before (26, 27). Tumor necrosis factor (TNF)-α, 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). Cytokine-induced neutrophil chemoattractant (KC) and macrophage inflammatory protein 2 (MIP-2) levels were measured by ELISA (R&D Systems, Abingdon, United Kingdom). Myeloperoxidase (MPO) was measured by ELISA (Hycult Biotechnology BV, Uden, The Netherlands). Thrombin-antithrombin complexes (TATc) were measured by ELISA (Enzygnost TAT Micro, Dade Behring, Marburg, Germany).

Bronchoalveolar lavage
In separate mice, not used for pathology or preparation of lung homogenates, BALF was obtained and differential counts were carried out as described earlier (28). Briefly, the trachea was exposed through a midline incision and BALF was harvested by instilling and retrieving two 0.5-ml aliquots of sterile isotonic saline. Cell counts were determined using an automated counter (Beckham Coulter, Coulter ZF, Mijdrecht, The Netherlands).
Histology
Lungs for histology were prepared and analyzed as described previously (26). Lungs were harvested, fixed in 4% formaldehyde, embedded in paraffin and cut in 4-µm thick sections for staining procedures. Immunostaining for RAGE was performed on paraffin slides after deparaffinization and rehydration using standard procedures. Endogenous peroxidase activity was quenched using 1.5% H2O2 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 Aldrich, St. Louis, MO). Hematoxylin-eosin stainings were performed as described (29). To score lung inflammation and damage, the lung samples were screened for the following parameters: interstitial inflammation, vasculitis, bronchitis, edema and pleuritis. Each parameter was graded on a scale of 0 to 5 with 0 as “absent” and 5 as “very severe”. The total “lung inflammation score” was expressed as the (mean) sum of the score for all parameters. Neutrophil stainings were performed as described previously (29). Neutrophil infiltration was analysed (blinded) in four non overlapping areas in each specimen and graded on a scale of 0 to 5, as described above. The mean neutrophil staining score was expressed as the mean score of four areas. Fibrin(ogen) stainings were performed as earlier described (30, 31).

Killing of *S. pneumoniae* by macrophages
Killing of *S. pneumoniae* was determined according to a protocol published recently (32, 33) with minor modifications. Peritoneal lavage was performed in Wt and RAGE−/− mice (n = 6-7 per strain) using 5 ml of sterile saline. Lavage fluid was collected in sterile tubes and put on ice. Peritoneal macrophages were washed, counted and resuspended in RPMI 1640 with L-glutamin at a final concentration of 0.2 x 10⁶/ml. Cells were then allowed to adhere in 12-well microtiter plates (Greiner, Alphen aan de Rijn, The Netherlands) overnight at 37 °C. Adherent monolayer cells were washed thoroughly with RPMI 1640 with L-glutamin. *S. pneumoniae* D39 delta (kindly provided by Dr. P.W. Hermans, Department of Pediatrics, University Medical Center Rotterdam, Erasmus MC-Sophia, The Netherlands) were added at a multiplicity of infection (MOI) of 50 and spun onto cells at 2,000 r.p.m. for 5 min., after which plates were placed at 37 °C for 10 min. Each well was then washed 5 times with ice-cold PBS to remove extracellular bacteria. To determine bacterial killing after 10 min., cells were lysed with sterile H2O and the number of intracellular bacteria were determined by plating serial 10-fold dilutions onto sheep-blood agar plates and bacterial counts were enumerated after 16 h.
Statistical analysis
All data are expressed as means ± SEM. Differences between groups were analyzed by Mann-Whitney \( U \) test. Kaplan-Meier analysis were performed by log rank test. A \( p \) value less than 0.05 was considered statistically significant.

RESULTS

RAGE expression in the lungs
Previous studies showed that normal, healthy lungs express RAGE (14-18) and that pulmonary RAGE expression is enhanced in patients with interstitial and postobstructive pneumonia (16); data about causative pathogens were not reported in this latter study. To determine whether RAGE expression changes during \textit{S. pneumoniae} pneumonia, we performed immunhistochemical stainings of RAGE of lung tissue from Wt mice after inoculation with \textit{S. pneumoniae}. In line with the literature (14-18), we found that normal, healthy mice show extensive RAGE staining in their lungs (Fig. 1A). RAGE was mainly present in the interalveolar septae in an endothelial pattern, while bronchial epithelial cells were negative for RAGE staining (Fig. 1A, arrow). Immunohistochemical analysis of lungs obtained from RAGE\(^{+/−}\) mice, used as negative controls, confirmed the specificity of the RAGE staining (Fig. 1B). Lungs from \textit{S. pneumoniae} infected mice displayed the same pattern of RAGE positivity as lungs from healthy Wt mice, \textit{i.e.} the interalveolar septae stained positive for RAGE staining with an endothelial pattern; however, pneumonia was associated with an upregulation of RAGE expression as reflected by more intense staining (Fig. 1C). Strikingly, neutrophils recruited to an area with confluent pneumonia did not express or hardly expressed RAGE (Fig. 1D, asterisk).

Figure 1. Expression of RAGE in lungs during \textit{S. 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) and (D) Lungs from a Wt mouse 48 h after the inoculation of \textit{S. pneumoniae}. Arrow indicates bronchial epithelium in healthy lungs (A), asterisk indicates neutrophils in an area with confluent pneumonia (D), both being negative for RAGE staining. RAGE staining; original magnification x10.
RAGE-deficient mice are protected against lethality during pneumococcal pneumonia

In a first attempt to determine the role of RAGE in the outcome of pneumococcal pneumonia, Wt and RAGE-deficient mice were inoculated intranasally with 5 x 10^4 or 5 x 10^5 CFUs of *S. pneumoniae* and monitored for 14 days (Fig. 2A and 2B). After inoculation of 5 x 10^4 CFUs, Wt mice started dying after 2 days and all mice had died by day 6. In contrast, the first RAGE-deficient mice died after 3 days and only 81% had died at the end of the observation period (p < 0.05 vs Wt mice). After inoculation with the higher dose, the survival curve showed a more steep decrease between 2 and 4 days and all Wt mice were dead shortly after day 4; RAGE-deficient mice displayed a delayed mortality and 13% survived (p < 0.01 for the difference between groups). These data suggest that RAGE contributes to lethality during *S. pneumoniae* pneumonia. Further experiments were performed using the 5 x 10^5 dose.

![Figure 2.](image)

**Figure 2.** RAGE-deficient mice demonstrate a reduced mortality during pneumococcal pneumonia. Survival of Wt and RAGE-deficient mice after intranasal inoculation with 5 x 10^4 (A) or 5 x 10^5 CFUs (B) *S. pneumoniae*. Mortality was assessed four times daily for 14 days (n = 12-15 mice per group in each experiment).

RAGE deficiency diminishes bacterial outgrowth and dissemination to distant organs during *S. pneumoniae* pneumonia

To investigate whether the diminished lethality of RAGE-deficient mice was associated with changes in bacterial outgrowth, we examined the bacterial loads in the lungs and distant body sites of Wt and RAGE-deficient mice at 16 and 48 h after induction of pneumonia (*i.e.* the latter time point is directly before the first mice started dying). At 16 h after inoculation, RAGE-deficient mice had significantly lower bacterial loads in their lung homogenates and
Chapter 3

**Figure 3.** RAGE<sup>−/−</sup> mice demonstrate a reduced local bacterial outgrowth and dissemination during *S. pneumoniae* pneumonia. Bacterial loads in lung homogenate (A), bronchoalveolar lavage fluid (BALF, B), blood (C) and spleen (D) were determined in Wt and RAGE<sup>−/−</sup> mice 16 and 48 h after intranasal inoculation 5 x 10<sup>5</sup> CFUs *S. pneumoniae*. Data are means ± SEM of 8-10 mice per genotype at each time point. *p < 0.05 vs Wt mice; **, p < 0.01 vs Wt mice; ***, p < 0.005 vs Wt mice.

BALF compared to Wt mice (p < 0.01; Fig. 3A and B). In addition, blood and spleen harvested from RAGE<sup>−/−</sup> mice also contained fewer bacteria than the corresponding body samples from Wt mice at 16 h as well as at 48 h (both p < 0.05; Fig. 3C and D). These data indicate that endogenous RAGE promotes the outgrowth of bacteria at the primary site of infection and facilitates the dissemination to distant sites.

**RAGE<sup>−/−</sup> macrophages demonstrate an increased capacity to kill *S. pneumoniae***

To investigate whether the decreased bacterial outgrowth in RAGE<sup>−/−</sup> mice could be the result of an intrinsic effect in the ability of RAGE<sup>−/−</sup> macrophages to kill *S. pneumoniae*, we examined the killing of *S. pneumoniae* by Wt and RAGE<sup>−/−</sup> macrophages *ex vivo*. RAGE<sup>−/−</sup> macrophages had an improved capacity to rapidly kill *S. pneumoniae* as depicted in Fig. 4 by a decreased number of intracellular bacteria after 10 min. of incubation (p < 0.05).

**Figure 4.** RAGE<sup>−/−</sup> macrophages show an increased capacity to rapidly kill *S. pneumoniae*. Wt and RAGE<sup>−/−</sup> macrophages were incubated with viable *S. pneumoniae* (MOI 50) and the number of intracellular bacteria after 10 min. was assessed as described in the Materials and Methods section (means ± SEM of 7 mice per genotype). *, p < 0.05 vs Wt macrophages.

**RAGE<sup>−/−</sup> mice show reduced lung inflammation during *S. pneumoniae* pneumonia***

To evaluate the role of endogenous RAGE in lung inflammation and injury during
S. pneumoniae pneumonia, we analyzed lung tissue slides obtained from Wt and RAGE−/− mice 16 (Fig. 5A-D, upper panels) and 48 h (Fig. 5E-H, lower panels) after inoculation. Upon histopathologic examination, the lungs of Wt mice showed interstitial inflammation together with vasculitis, bronchitis, edema and pleuritis at both 16 and 48 h (Fig. 5A and E, respectively). Whereas the extent of lung inflammation (e.g., interstitial inflammation and edema) in RAGE−/− mice at 16 h after inoculation (Fig. 5B) did not differ from that of Wt mice, pulmonary inflammation at 48 h was less profound in these animals (Fig. 5F) compared with that in Wt mice. The mean total histology score of the lungs (determined using the scoring system described in the Materials and Methods section) and the percentage of inflamed lung tissue were similar in the two mouse strains at 16 h (Fig. 5C and 5D). After 48 h, however, the inflammatory infiltrate became more diffuse and dense in both groups, but significantly less pronounced in RAGE−/− mice (Fig. 5G and 5H). In conclusion, RAGE−/− mice showed less lung inflammation in the later phase of the infection.

Figure 5. RAGE−/− mice display reduced lung inflammation. Wt and RAGE−/− mice were inoculated intranasally with 5 x 10^5 CFUs S. pneumoniae. Representative HE stainings of lung tissue at 16 (A and B) and 48 (E and F) h post inoculation in Wt (A and E) and RAGE−/− (B and F) mice. Original magnification x200. Graphical representation of the degree of lung inflammation at 16 and 48 h (C and G) and percentage of inflamed area (D and H), determined according to the scoring system described in the Materials and Methods section. Data are means ± SEM of 8-12 mice per genotype at each time point. *, p < 0.05 vs Wt mice.

RAGE−/− mice show a reduced neutrophil migration to the lungs
RAGE has been implicated to play a role in neutrophil migration possibly mediated by the β2 integrin CD11b/CD18 (34-36). Given that leukocyte recruitment to the site of infection is an important part of host defense during pneumonia (37, 38), we next
investigated neutrophil influx in the lungs of Wt and RAGE–/– mice at 16 and 48 h after inoculation with S. pneumoniae. At 48 h, but not at 16 h, RAGE–/– mice had a decreased influx of neutrophils in lung tissue compared to the Wt mice, as reflected by neutrophil stainings of lung tissue (Fig. 6A, B and D, E). The granulocyte Ly-6 scores of the lungs (semi-quantified according to the scoring system described in the Materials and Methods section) were
significantly lower at 48 h in RAGE−/− mice than in Wt mice (p < 0.05; Fig. 6F). In line, RAGE−/− mice had lower MPO levels in their lung homogenates at both time points, although at 16 h this difference was borderline significant (p = 0.05 at 16 h and p < 0.01 at 48 h vs Wt mice; Fig. 6G). Additionally, RAGE deficiency also resulted in a diminished influx of neutrophils in the bronchoalveolar space at both time points as reflected by a reduced number of neutrophils in BALF (p < 0.001; Fig. 6H) and lower MPO concentrations in BALF (p < 0.05; Fig. 6I). These data indicate that RAGE deficiency is associated with diminished neutrophil recruitment to both the lung interstitium and the bronchoalveolar space.

RAGE−/− mice have decreased cytokine and chemokine levels in their lungs and blood

Cytokines and chemokines play an important role in host defense against bacterial pneumonia (38, 39). Thus, we determined the concentrations of TNF-α, IL-6, MCP-1, IL-10, KC and MIP-2 in lung homogenates and BALF obtained 16 and 48 h after infection. At 48 h, TNF-α, IL-6 and MCP-1 concentrations were reduced in lung homogenates as well as in BALF from RAGE−/− mice (all p < 0.05 with the exception of TNF-α in lung homogenates, p = 0.05; Table I). KC and MIP-2 concentrations in BALF from RAGE−/− mice were lower compared to Wt mice at both time points, while in lung homogenates, MIP-2 was diminished in the RAGE mice at 16 h only. IL-10 concentrations did not differ between the two mouse strains in either the bronchoalveolar space or lung homogenates. Plasma cytokine levels did not differ between the two mouse strains at both time points, except for IL-6 levels which were lower in the RAGE−/− mice 16 h after inoculation (data not shown).

RAGE−/− mice demonstrate decreased coagulation activation during S. pneumoniae pneumonia

Pulmonary coagulopathy is an important feature of pneumonia (40, 41). Earlier, we demonstrated that our S. pneumoniae model is associated with enhanced pulmonary fibrinogen deposition (30). To investigate whether RAGE deficiency influences the activation of the coagulation system, we measured TATc levels in BALF and plasma and performed fibrinogen stainings on lung tissue slides and 16 and 48 h after intranasal inoculation of S. pneumoniae. Wt mice displayed evidence for local and systemic activation of coagulation: relative to uninfected mice, their lungs revealed elevated TATc concentrations in BALF and plasma at both time points (Fig. 7A and 7B) and more fibrinogen deposition at 48 h (Fig. 7D vs 7C), but not at 16 h (data not shown). Importantly, coagulation activation was less profound in RAGE−/− mice, as reflected by lower BALF and plasma TATc levels (p < 0.005 at 48 h in BALF and p < 0.01 at 16 h in plasma vs Wt mice; Fig. 7A and 7B) and by decreased fibrinogen deposition (Fig. 7E vs 7D). Together, these data indicate that RAGE deficiency diminishes the activation of coagulation both locally and systemically during S. pneumoniae pneumonia.
Impact of RAGE deficiency on the early immune response

To determine the impact of RAGE deficiency on the immediate host response to pneumococci in the airways, we infected Wt and RAGE-/- mice with *S. pneumoniae* by intranasal inoculation and killed them 6 h thereafter. At this early time point the bacterial loads did not differ between the mouse strains (Fig. 8A and B for lung homogenate and BALF; bacteria were not or barely disseminated at this early phase), indicating that the accelerated bacterial clearance observed in RAGE-/- mice only occurs after this early phase of the infection. Responses accountable for an appropriate innate immune response during pneumonia include the neutrophil influx in the lungs. We did not find a difference in the pulmonary cell influx (table II). In addition, the local production of proinflammatory cytokines at the site of infection are a critical component of the innate immune response. To address this issue we measured local TNF-α, IL-6, MCP-1 and IL-10. TNF-α concentrations were lower in the lung homogenates from the RAGE-/- mice after 6 h (*p* = 0.05; Table II), the other cytokine levels did not differ between the two mouse strains at this time point.

Table I. RAGE-/- mice display reduced cytokine and chemokine levels in their lungs

<table>
<thead>
<tr>
<th></th>
<th>16 h</th>
<th>48 h</th>
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<tr>
<td></td>
<td>Wt</td>
<td>RAGE-/-</td>
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<tr>
<td>Lung homogenate (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>29 ± 2.4</td>
<td>26 ± 2.6</td>
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<tr>
<td>IL-6</td>
<td>36 ± 3.7</td>
<td>33 ± 8.3</td>
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<tr>
<td>MCP-1</td>
<td>479 ± 74.9</td>
<td>373 ± 53.6</td>
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<tr>
<td>IL-10</td>
<td>989 ± 229.4</td>
<td>770 ± 195.0</td>
</tr>
<tr>
<td>KC</td>
<td>21,417 ± 1,824</td>
<td>21,333 ± 1,745</td>
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<tr>
<td>MIP-2</td>
<td>11,108 ± 2,234</td>
<td>6,445 ± 751 *</td>
</tr>
<tr>
<td>BALF (pg/ml)</td>
<td></td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>3,328 ± 546</td>
<td>4,471 ± 1,048</td>
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<tr>
<td>IL-6</td>
<td>951 ± 174</td>
<td>376 ± 96 *</td>
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<tr>
<td>MCP-1</td>
<td>273 ± 68</td>
<td>156 ± 57</td>
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<tr>
<td>IL-10</td>
<td>20 ± 4.7</td>
<td>12 ± 3.5</td>
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<tr>
<td>KC</td>
<td>461 ± 54</td>
<td>238 ± 51 **</td>
</tr>
<tr>
<td>MIP-2</td>
<td>190 ± 22</td>
<td>121 ± 33 *</td>
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*a Data are means ± SEM at 16 or 48 h post inoculation of 5 x 10^5 CFUs *S. pneumoniae.*  

n = 8-10 mice per group.  

*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001 vs Wt mice.
Figure 7. RAGE−/− mice demonstrate decreased local and systemic activation of coagulation. Wt and RAGE−/− mice were inoculated intranasally with 5 x 10⁵ CFUs S. pneumoniae. Thrombin-antithromin complex (TATc, A and B) concentrations were measured locally (bronchoalveolar lavage fluid (BALF, A) and systemically (plasma, B). Dotted lines represent the mean values obtained from normal (uninfected) mice. Data are means ± SEM of 8-11 mice per genotype at each time point. *, p < 0.05 vs Wt mice; ***, p < 0.005 vs Wt mice. Representative fibrin(ogen) immunostaining of lung tissue of uninfected Wt (C) and of Wt and RAGE−/− mice 16 and 48 h after inoculation of S. pneumoniae (D and E, respectively). Original magnification x10.

Figure 8. Effect of RAGE deficiency on the early bacterial outgrowth during pneumococcal pneumonia. Bacterial loads in lung homogenate (A) and bronchoalveolar lavage fluid (BALF, B) were determined in Wt and RAGE−/− mice 6 h after intranasal inoculation 5 x 10⁶ CFUs S. pneumoniae. Data are means ± SEM of 8 mice per genotype at each time point.
Table II. Early neutrophil influx and cytokine levels in lungs

<table>
<thead>
<tr>
<th></th>
<th>Wt</th>
<th>RAGE&lt;−/−</th>
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<tbody>
<tr>
<td><strong>BALF</strong></td>
<td></td>
<td></td>
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<tr>
<td>Cell count (x 10⁶/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.53 ± 0.32</td>
<td>1.46 ± 0.18</td>
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<tr>
<td>Neutrophils</td>
<td>1.13 ± 0.34</td>
<td>0.93 ± 0.19</td>
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<tr>
<td><strong>Lung homogenate</strong></td>
<td></td>
<td></td>
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<tr>
<td>Cytokines (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>166 ± 22.4</td>
<td>84 ± 20.7 *</td>
</tr>
<tr>
<td>IL-6</td>
<td>771 ± 125.4</td>
<td>691 ± 209.9</td>
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<tr>
<td>MCP-1</td>
<td>1,673 ± 215.5</td>
<td>1,563 ± 327.0</td>
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<tr>
<td>IL-10</td>
<td>207 ± 49.4</td>
<td>186 ± 48.7</td>
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* Data are means ± SEM at 6 h post inoculation of 5 x 10⁵ CFUs S. pneumoniae. n = 8 mice per group. *, p < 0.05 vs Wt mice.

**Discussion**

*S. pneumoniae* is the most frequently isolated pathogen in community-acquired pneumonia and a major health threat worldwide (1, 2). RAGE has been associated with diverse inflammatory processes. Although RAGE expression has been documented in normal healthy lungs (14-18) and (to a larger extent) in lungs from patients with pneumonia (16), the impact of RAGE on the host response to respiratory tract infection has not been investigated before. We here used a model of gram-positive lung infection to determine the role of RAGE in the host response to severe *S. pneumoniae* pneumonia. We found that RAGE deficiency protects against pneumococcal pneumonia 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. This increased resistance against *S. pneumoniae* in RAGE<−/− mice could at least in part be explained by an enhanced killing capacity of RAGE<−/− macrophages.

Our results on pulmonary RAGE expression extend previous investigations in finding extensive RAGE expression in normal, healthy lungs (14-18) and an increase in RAGE expression during interstitial and postobstructive pneumonia (16); the latter study left unclear whether patients with bacterial pneumonia were included in the analysis. Of note, two other studies showed that constitutively present RAGE is not upregulated during
pulmonary inflammation. First, rats with acute lung injury induced by intratracheally administered LPS displayed no change in the distribution of RAGE-expressing cells (17). Secondly, patients with the acute respiratory distress syndrome did not have increased pulmonary expression of RAGE (18). We here report for the first time that pneumonia caused by *S. pneumoniae* is associated with an upregulation of interalveolar RAGE expression in the lungs.

RAGE−/− mice demonstrated less lung inflammation at 48 h after inoculation, as reflected by histopathology and cytokine and chemokine levels. This is in line with other reports in which inhibition of the interaction of RAGE with its ligands led to a reduced inflammation in models of hepatic injury (19-21), diabetic atherosclerosis (22, 23), delayed type hypersensitivity (24, 25), type II collagen-induced arthritis (25) and sepsis (24). In addition, in a recent study RAGE was found to be important for the development of lung fibrosis upon intratracheal administration of bleomycin (42). Since activation of RAGE triggers multiple intracellular signaling pathways, including NF-κB, resulting in the transcription of proinflammatory factors (43, 44), these findings could at least in part be attributed to the blockade of RAGE interaction with its ligands via diminished activation of NF-κB, resulting in attenuated tissue injury/damage and/or inflammation (24, 45). This blockade of the ligand-receptor interaction and prevention of the subsequent proinflammatory stimulus might therefore be an explanation for the less severe pulmonary damage in the mice lacking RAGE in our model. RAGE can interact with several different ligands such as advanced glycation end products (4), amyloid (5), β-sheet fibrils (6), high mobility group box 1 (HMGB1) (7, 8) and some members of the S100 family (9-11). From these ligands, HMGB1 and S100 family members are likely to be released in this pneumonia model. Previously, we showed that HMGB1 levels were higher in bronchoalveolar lavage fluid (BALF) from patients with pneumonia at the site of infection compared to BALF from healthy controls (46). From the S100 family members, definitive evidence for binding to RAGE has only been deduced for S100A12, S100B and S100P (9-11). From these, S100A12 levels are increased in BALF from patients with acute lung injury and from healthy volunteers after LPS inhalation (18), but evidence that a functional S100A12 gene is not present in the murine genome (47) implies that RAGE-S100A12 ligation does not attribute to the host response to pneumonia in mice. Until now, there are no data that suggest that S100B and S100P are likely to play an important role in pneumonia. By far the brain is the richest source of S100B and astrocytes represent the cell type with the highest expression. S100P, initially identified in placenta, is expressed in a number of cells and tissues and is significantly upregulated in highly metastatic cancer cells suggesting an involvement in tumor cell migration. Nevertheless, future research is warranted to investigate whether RAGE-S100B and/or RAGE-S100P
ligation play a role during pneumonia and other infectious diseases. Furthermore, the decreased proinflammatory stimulus provided by the lower bacterial loads could have further contributed to the reduced pulmonary injury.

Pneumonia caused by *S. pneumoniae* is characterized by the recruitment of neutrophils to the site of infection (37, 38). Earlier RAGE has been shown to be involved in cell recruitment; RAGE deficient mice displayed a diminished number of adherent inflammatory cells on the peritoneum after cecal ligation and puncture (24) and a reduction in neutrophil influx in the peritoneal cavity after thioglycollate peritonitis (36). In addition, *in vitro* studies have suggested that RAGE is an endothelial counter receptor for the β2 integrin Mac-1 (35, 36) and that a functional interplay between RAGE and Mac-1 on leukocytes is required for high mobility group box 1 mediated inflammatory cell recruitment (34). Our findings that RAGE−/− mice have a diminished neutrophil recruitment to the lungs during *S. pneumoniae*, are in line with these data.

Peritoneal macrophages harvested from RAGE−/− mice showed an increased capacity to kill *S. pneumoniae*. A limitation of our study is that we did not study alveolar macrophages or neutrophils due to the fact that *S. pneumoniae* killing assays with these cell types do not yield reliable results in our hands. Further studies are warranted to address this issue. In accordance with our earlier study, *S. pneumoniae* pneumonia was associated with activation of the coagulation system (30). Pulmonary coagulopathy now gains more and more interest as a new target in therapeutic studies of acute lung injury of pneumonia (41, 48). Therefore, and since RAGE has been implicated as a mediator of coagulation (49-51), we were interested to study the role of RAGE in pulmonary coagulopathy in pneumococcal pneumonia. We here found that the RAGE−/− mice displayed less activation of coagulation, as reflected by decreased TATc BALF and plasma concentrations and reduced fibrin deposition in lung tissue. This could at least in part be explained by the lower bacterial loads in these animals. In addition, another explanation might be that RAGE signaling contributes to coagulation activation via a more direct way. Administration of soluble RAGE *in vivo* for 6 weeks in a model of chronic vascular inflammation in diabetic apolipoprotein E deficient mice suppressed levels of tissue factor, the main initiator of coagulation in sepsis in general (52) and in this model of pneumococcal pneumonia in particular (53), in the aorta (51). Furthermore, soluble RAGE, anti-RAGE or antisense RAGE have been reported to inhibit tissue factor expression by monocytes or endothelial cells stimulated with AGEs or serum amyloid A *in vitro* (49, 50).

Knowledge of the role of RAGE in host defense against bacterial infection is highly limited. In a model of polymicrobial abdominal sepsis induced by cecal ligation and
puncture (CLP) RAGE<sup>−/−</sup> mice were reported to have an improved survival together with a reduced NF-κB activation in the peritoneum; bacterial growth and dissemination were not determined in this study (24). Furthermore, Lutterloh et al. also found a survival benefit for RAGE<sup>−/−</sup> mice in a CLP model and in a model of systemic challenge with Listeria (L.) monocytogenes (54). Moreover, they reported that RAGE deficiency – either genetically or pharmacologically induced – did not influence bacterial loads after CLP or during systemic L. monocytogenes challenge. To the best of our knowledge other investigations on the role of RAGE in bacterial infection have not been reported. The exact mechanisms by which RAGE impairs the outcome of polymicrobial peritonitis (24) and L. monocytogenes infection (54) – in combination with unchanged bacterial loads - and of S. pneumoniae pneumonia (the current study) – in combination with decreased bacterial outgrowth and dissemination - remains to be determined. In general the innate immune response to severe bacterial infection can act as a double-edged sword, on the one hand protecting the host against invading pathogens, on the other hand potentially destroying cells and tissues. In particular in our model of pneumococcal pneumonia a reduced early inflammatory response in the lung may facilitate the growth and subsequent dissemination of bacteria (38, 55, 56), as most clearly demonstrated by mice in which the action of endogenously produced TNF-α is blocked (28, 57, 58) or in which both TNF-α and IL-1 signaling pathways are blocked (55). In the same line of thinking, a diminished neutrophil influx to the lungs is expected to impair antibacterial defense against S. pneumoniae pneumonia (38, 56, 59). In addition, the reduced number of inflammatory cells, relative to Wt mice, in the peritoneum of RAGE<sup>−/−</sup> mice subjected to cecal ligation and puncture arguably would impair antibacterial defense against faecal flora (24). Moreover, recent studies have revealed an important role for the the NF-κB subunit RelA in the host response to pneumococcal pneumonia: RelA-deficient mice (generated on a TNF receptor type 1-deficient background) infected with S. pneumoniae displayed decreased cytokine expression, alveolar neutrophil emigration and lung bacterial killing (60), inhibition of NF-κB nuclear translocation in airway epithelial cells increased growth of pneumococci upon intratracheal infection (60) and mice deficient in both both TNF-α and IL-1 receptors show decreased lung NF-κB activation, neutrophil recruitment and bacterial clearance during pneumococcal pneumonia (55). Nonetheless, RAGE deficiency confers a net benefit to the host in both models of severe bacterial infection (cecal ligation and puncture and pneumococcal pneumonia), suggesting that the delicate balance between benefit and harm resulting from the inflammatory response to infection can be disturbed by intact RAGE signaling. In this respect it should be noted that an unique feature of RAGE-mediated cellular activation is the prolonged time course which appears to overwhelm autoregulatory feedback inhibition loops (44).
Pneumonia remains a leading cause of morbidity and mortality and \textit{S. pneumoniae} is the most frequently isolated pathogen in community-acquired pneumonia. Using a well-established model of murine pneumococcal pneumonia, the current study is the first to establish that RAGE plays a detrimental role in the host defense during pneumonia caused by gram-positive bacteria.

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REFERENCES


