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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 U.S. An estimated 570,000 cases of pneumococcal pneumonia occur annually in the U.S., 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 (HMGB1) (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 NF-κB and MAPK 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, in this study, we 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 mice with a targeted deletion of the RAGE-deficient (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. RAGE−/− mice, backcrossed 10 times to a C57BL/6 background, were generated as previously described (25). The Institutional Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam, approved all experiments.
FIGURE 1. Expression of RAGE in lungs during 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 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 ×10.

Design

Pneumonia was induced, as described earlier (26, 27). Mice were lightly anesthetized by inhalation of isoflurane (Abbott Laboratories), and 50 μl containing 5 × 10^3–5 × 10^4 CFUs S. pneumoniae serotype 3 (ATCC 6303; American Type Culture Collection) was inoculated intranasally. Healthy control mice received 50 μl of sterile saline only. In survival studies, mice were monitored for 2 wk.

Preparations of lung homogenates and measurements

At 16 and 48 h after inoculation, mice were anesthetized with ketamine (Eurovet Animal Health) and medetomidine (Pfizer Animal Health). 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). TNF-α, IL-6, MCP-1, and IL-10 levels were determined using an automated counter (Beckham Coulter). Aliquots of sterile isotonic saline. Cell counts were determined using an automated counter (Beckham Coulter).

Bronchoalveolar lavage

In separate mice, not used for pathology or preparation of lung homogenates, BALF was obtained and differential counts were conducted, 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).

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 Abs used were goat anti-mouse RAGE polyclonal Abs (Neuromics), and secondary Abs were biotinylated rabbit anti-goat Abs (Dako-antibody). ABC solution (DakoCytomation) was used as an detection enzyme. Diaminobenzidine peroxidase (Dako) was used as substrate for visualization. Counterstaining was performed with methyl green (Sigma-Aldrich). H&E stainings were performed, as described (29). To score lung inflammation and damage, the lung samples were screened for the following parameters: interstitial inflammation, vasculitis, bronchiitis, edema, and pleuritis. Each parameter was graded on a scale of 0–5, 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 analyzed (blinded) in four non-overlapping areas in each specimen and graded on a scale of 0–5, as described above. The mean neutrophil staining score was expressed as the mean score of four areas. Fibrinogen 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 1-glutamin at a final concentration of 0.2 × 10^7/ml. Cells were then allowed to adhere in 12-well microtiter plates (Greiner Biosciences) overnight at 37°C. Adherent monolayer cells were washed thoroughly with RPMI 1640 with 1-glutamin. S. pneumoniae D39 (provided by P. Hermans, Department of Pediatrics, University Medical Center Rotterdam, Erasmus MC-Sophia, The Netherlands) were added at a multiplicity of infection of 50 and spun onto cells at 2000 rpm for 5 min, after which plates were placed at 37°C for 10 min. Each well was then washed five times with ice-cold PBS to remove extracellular bacteria. To determine bacterial killing after 10 min, cells were lysed with sterile H2O; the number of intracellular bacteria was 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 was 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 S. pneumoniae pneumonia, we performed immunochemical stainings of RAGE of lung tissue from Wt mice after inoculation with 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, whereas 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 S. pneumoniae-infected mice displayed the same pattern of RAGE positivity as lungs from healthy Wt mice, i.e., the interalveolar septae stained positive for RAGE staining with an endothelial pattern; however, pneumonia was associated with an up-regulation 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).

RAGE$^{−/−}$ 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$^{−/−}$ mice were inoculated intranasally with 5 × 10^3 or 5 × 10^4 CFUs of S. pneumoniae and monitored for 14 days (Fig. 2). After inoculation of
spleen harvested from RAGE/H11569/H11569, 8–10 mice per genotype at each time point. RAGE between 2 and 4 days, and all Wt mice were dead shortly after the 5 pneumonia. Additional experiments were performed using S. pneumoniae loads in lung homogenate (A), and spleen (B), blood (C), and BALF (D) were determined in Wt and RAGE−/− mice 16 and 48 h after intranasal inoculation with 5 × 10^5 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.

FIGURE 3. RAGE−/− mice demonstrate a reduced local bacterial outgrowth and dissemination during S. pneumoniae pneumonia. To investigate whether the diminished lethality of RAGE−/− mice was associated with changes in bacterial outgrowth, we examined the bacterial loads in the lungs and distant body sites of Wt and RAGE−/− 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−/− mice had significantly lower bacterial loads in their lung homogenates and BALF compared with Wt mice (p < 0.01; Fig. 3, A and B). In addition, blood and spleen harvested from RAGE−/− 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. 3, C 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−/− macrophages demonstrate an increased capacity to kill S. pneumoniae
To investigate whether the decreased bacterial outgrowth in RAGE−/− mice could be the result of an intrinsic effect in the ability of RAGE−/− macrophages to kill S. pneumoniae, we examined the killing of S. pneumoniae by Wt and RAGE−/− macrophages ex vivo. RAGE−/− 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).

RAGE−/− 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. 5, A–D, upper panels) and 48 h (Fig. 5, E–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. 5, A 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 Materials and Methods) and the percentage of inflamed lung tissue were similar in the two mouse strains at 16 h (Fig. 5, C and D). After 48 h, however, the inflammatory infiltrate became more diffuse and dense in both groups, but significantly less pronounced in RAGE−/− mice (Fig. 5, G and H). In conclusion, RAGE−/− mice showed less lung inflammation in the later phase of the infection.

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

FIGURE 2. RAGE−/− mice demonstrate a reduced mortality during pneumococcal pneumonia. Survival of Wt and RAGE−/− mice after intranasal inoculation with 5 × 10^4 (A) or 5 × 10^5 CFUs (B) S. pneumoniae. Mortality was assessed four times daily for 14 days (n = 12–15 mice per group in each experiment). 5 × 10^4 CFUs, Wt mice started dying after 2 days and all mice had died by day 6. In contrast, the first RAGE−/− 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−/− 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. Additional experiments were performed using the 5 × 10^5 dose.

RAGE deficiency diminishes bacterial outgrowth and dissemination to distant organs during S. pneumoniae pneumonia
To investigate whether the diminished lethality of RAGE−/− mice was associated with changes in bacterial outgrowth, we examined the bacterial loads in the lungs and distant body sites of Wt and RAGE−/− mice 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−/− mice had significantly lower bacterial loads in their lung homogenates and BALF compared with Wt mice (p < 0.01; Fig. 3, A and B). In addition, blood and spleen harvested from RAGE−/− 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. 3, C 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.

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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 with the Wt mice, as reflected by neutrophil stainings of lung tissue (Fig. 6, A, B, D, and E). The granulocyte Ly-6 scores of the lungs (semiquantified according to the scoring system described in Materials and Methods) 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 with Wt mice at both time points, whereas 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. 7, A and B) and more fibrinogen deposition at 48 h (Fig. 7, D vs C), 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. 7, A and B) and by decreased fibrinogen deposition (Fig. 7, E vs D). 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. 8 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

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

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<th>16 h</th>
<th>48 h</th>
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<tr>
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<td>Wt</td>
<td>RAGE−/−</td>
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<tr>
<td><strong>Lung Homogenate</strong></td>
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<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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<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>
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<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>
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<tr>
<td><strong>BALF</strong></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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<td>MCP-1</td>
<td>273 ± 68</td>
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<td>IL-10</td>
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<tr>
<td>KC</td>
<td>461 ± 54</td>
<td>238 ± 51**</td>
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<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 postinoculation of 5 × 10⁵ CFUs *S. pneumoniae*. n = 8–10 mice per group. \( * p < 0.05 \); \( ** p < 0.01 \); \( *** p < 0.001 \) vs Wt mice.
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 is 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.

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. In this study, we 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 up-regulated during pulmonary inflammation. First, rats with acute lung injury induced by intratracheal administration of 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). In this study, we report for the first time that pneumonia caused by S. pneumoniae is associated with an up-regulation 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). Because 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), HMBG1 (7, 8), and some members of the S100 family (9–11). From these ligands, HMBG1 and S100 family members are likely to be released in this pneumonia model. Previously, we showed that HMBG1 levels were higher in BALF from patients with pneumonia at the site of infection compared with 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 (CLP) (24) and a reduction in neutrophil influx in the peritoneal cavity after thioglycolate peritonitis (36). In addition, in vitro studies have suggested that RAGE is an endothelial counterreceptor for the β2 integrin Mac-1 (35, 36), and that a functional interplay between RAGE and Mac-1 on leukocytes is required for HMBG1-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 because 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. In this study, we 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 wk 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 advanced glycation end products 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 CLP, RAGE−/− 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. (54) also found a survival benefit for RAGE−/− mice in a CLP model and in a model of systemic challenge with Listeria monocytogenes. 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, remain 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, and in contrast 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−/− mice subjected to CLP, arguably would impair antibacterial defense against fecal flora (24). Moreover, recent studies have revealed an important role for the NF-κB subunit RelA in the host response to pneumococcal pneumonia: RelA-deficient mice (generated on a TNFα−/− 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, and increased growth of pneumococci upon intratracheal infection (60), and mice deficient in both TNF-α and IL-1Rs 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 (CLP 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 a unique feature of RAGE-mediated cellular activation is the prolonged time course that appears to overwhelm autoregulatory feedback inhibition loops (44).

Pneumonia remains a leading cause of morbidity and mortality, and 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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