Endogenous danger signals in infectious diseases

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Chapter 5

High-mobility group box 1 and the Receptor for Advanced Glycation Endproducts contribute to lung injury during *Staphylococcus aureus* pneumonia.

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ABSTRACT

**Introduction:** Staphylococcus (S.) aureus has emerged as an important cause of necrotizing pneumonia. Lung injury during *S. aureus* pneumonia may be enhanced by local release of Damage Associated Molecular Patterns such as High-mobility Group Box 1(HMGB1). In the current study we sought to determine the functional role of HMGB1 and its receptors Toll-like Receptor 4 (TLR4) and the Receptor for Advanced Glycation End Products (RAGE) in the injurious host response to *S. aureus* pneumonia.

**Methods:** Pneumonia was induced in wild type (Wt), TLR4 deficient (*tlr4*-/-) and RAGE deficient (*rage*-/-) mice by intranasal inoculation of 1x10^7 colony-forming units (CFU) of a USA300 *S. aureus*. In a separate set of experiments, Wt mice were injected intraperitoneally with a monoclonal anti-HMGB1 antibody or an isotype matched control antibody immediately before and every 24 hours after intranasal infection of *S. aureus*. Mice were sacrificed at 6, 24, 48 or 72 hours after infection for harvesting of blood and organs.

**Results:** *S. aureus* pneumonia was associated with HMGB1 release in the bronchoalveolar compartment peaking after 24 hours. Anti-HMGB1 attenuated lung pathology and protein leak and reduced interleukin-1β release 6 hours after infection, but not at later time points. RAGE deficiency more modestly attenuated lung pathology without influencing protein leak, while TLR4 deficiency did not impact on lung injury.

**Conclusion:** These data suggest that HMGB1 and RAGE, but not TLR4, contribute to lung injury accompanying the early phase of *S. aureus* pneumonia.
The Gram-positive bacterium *Staphylococcus (S.) aureus* is a frequent colonizer of the human body, and when the opportunity arises, able to cause a wide array of clinical syndromes [1]. Infections caused by this pathogen impose a high burden on healthcare, largely due to the increasing incidence of antibiotic resistance [2]. Over the past few years, highly virulent methicillin resistant *S. aureus* strains, in particular USA300, have become prevalent in the community as well[2] and have emerged as an important cause of (necrotizing) pneumonia[3]. Pneumonia caused by these strains have a fulminant onset determined by staphylococcal virulence factors and the nature of the immune response[3,4]. More insight in pathogenic mechanisms that influence the outcome of lower airway infection by *S. aureus* could help in the development of new (immuno-modulating) therapies.

Staphylococcal pneumonia is associated with a massive influx of neutrophils and release of cytotoxic granular proteins[5-7]. Together with invasive infection, intense host defense mechanisms likely contribute to lung tissue damage and release of damage associated molecular patterns (DAMPs)[4,7,8]. Pattern recognition receptors that engage with these self-derived proteins may contribute to the severity of pneumonia as they perpetuate (excessive) inflammation. High-mobility Group Box 1 (HMGB1) is a DAMP that may be of particular interest since it is associated with delayed and sustained release during infection[9]. HMGB1 is a highly conserved non-histone nuclear protein which is either released passively during cell injury or secreted actively upon inflammatory stimuli[9]. Depending on specific posttranslational redox modifications HMGB1 can act as a cytokine via receptors such as the receptor for advanced glycation end products (RAGE) and Toll-like receptor (TLR)4 or as a chemotactic factor by forming a heterocomplex with the chemokine CXCL12 via the chemokine receptor CXCR4[10].

In this study we investigated the role of HMGB1 in experimentally induced *S. aureus* pneumonia. This newly developed mouse model of pneumonia is associated with severe pulmonary inflammation and massive influx of neutrophils. In order to study the role of HMGB1 in the pathogenesis of *S. aureus* lung infection we inoculated wildtype (Wt) mice with a USA300 strain of *S. aureus* and treated animals with a control or an anti-HMGB1 antibody. In addition, we investigated Wt mice and mice deficient for TLR4 or RAGE, the receptors implicated in mediating the proinflammatory effects of HMGB1, after induction of *S. aureus* pneumonia.
METHODS

Ethics statement:
Experiments were carried out in accordance with the Dutch Experiment on Animals Act and approved by the Animal Care and Use Committee of the University of Amsterdam (Permit number: DIX100121).

Mice
C57Bl/6 wild type (Wt) mice were purchased from Charles River Laboratories Inc. (Maarstricht, the Netherlands). RAGE deficient (rage−/−)[11] and tlr4−/− mice[12], backcrossed > 10 times to a C57BL/6 background were generated as described and bred in the animal facility of the Academic Medical Center (Amsterdam, the Netherlands). Experiments were carried out in accordance with the Dutch Experiments on Animals Act and approved by the Animal Care and Use Committee of the University of Amsterdam (Permit number: DIX100121)

Design
Wt, Rage−/− and tlr4−/− mice were lightly anesthetized by inhalation of isoflurane (Abbot Laboratories, Queensborough, Kent, UK) and intranasally inoculated with a sublethal dose of 1x10⁷ S. aureus USA300 (BK 11540) in a 50 μl saline solution (n=7-8 per strain). This sublethal dose was determined in a pilot study: Mice intranasally inoculated with 1x10⁸ S. aureus already died after 24 hours, whereas mice that were infected with 1x10⁷ S. aureus were clinically ill, but remained alive until 72 hours after infection (data not shown). In separate experiments, Wt mice were injected intraperitoneally with either 150 μg monoclonal anti-HMGB1 (2G7, IgG2b; Cornerstone Therapeutics, Cary, NC) or an isotype matched control antibody (MOPC-195, IgG2b; Sigma, St. Louis, MO) in 100 μl phosphate-buffered saline (PBS) immediately before and every 24 hours after intranasal infection of S. aureus. The dose of this anti-HMGB1 monoclonal antibody is selected according to a previous study and is able to effectively inhibit HMGB1 induced pathology in a mouse model of infectious lung injury [13]. Mice were sacrificed at 6, 24, 48 or 72 hours after infection.

Bacterial cultures
Measurements of bacterial loads were done as described previously[14]. In brief, blood was drawn into heparinized tubes, bronchoalveolar lavage (BAL) fluid was obtained as described below and organs were removed aseptically and homogenised in 4 volumes of sterile isotonic saline using a tissue homogenizer (Biospec Products, Bartlesville, UK). To determine bacterial loads, ten-fold dilutions were plated on blood agar plates and incubated at 37°C for 16 h.
**Bronchoalveolar lavage**

The trachea and bronchi were exposed through a midline incision. The left main bronchus was ligated and the trachea was cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott Laboratories, Sligo, Ireland). Unilateral, right-sided BAL was performed by instilling three 0.3 ml aliquots of sterile PBS. 0.7-0.9 ml of BAL fluid was retrieved per mouse. Total cell numbers in BAL were counted using a Z2 Coulter particle count and size analyzer (Beckman-Coulter, Inc., Miami, FL). BAL fluid differential cell counts were determined on cytopsin preparations stained with a modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, IL).

**Assays**

Cytokines and chemokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10, Keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2 (all R&D systems, Minneapolis, MN) were measured in BAL fluid using specific ELISAs according to manufacturer’s recommendations. Total protein concentrations were measured using a DC protein assay (Bio-Rad Laboratories, Veenendaal, the Netherlands).

**HMGB1 Western blot**

For Western blotting of HMGB1, non-reduced BAL fluid samples from Wt mice were diluted with 3x Laemmlisi buffer (n=5 per time point). After heating, samples were run on 15% polyacrylamide SDS gels and subsequently transferred to blotting membrane (polyvinylidene difluoride membranes; Immobilon P, Pharmacia, Piscataway, NJ). Following blocking with 5% nonfat dry milk proteins (Protifar; Nutricia, Zoetermeer, the Netherlands) in 0.1% Tween 20 PBS (PBS-T), membranes were washed and incubated overnight in 1 µg/ml primary rabbit anti-HMGB1 polyclonal antibody (Abcam, Cambridge, United Kingdom) in 1% nonfat dry milk proteins in PBS-T at 4 °C. After washing with PBS-T, membranes were probed with anti-rabbit-HRP-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA) for 1 h at room temperature in 1% bovine serum albumin in PBS-T. After washing with PBS-T, membranes were incubated with Lumi-Light Plus Western Blotting Substrate (Roche, Mijdrecht, The Netherlands) and positive bands were detected using a LAS3000 Luminescent image Analyzer dark box (Fujifilm, Tokyo, Japan). Band densities were determined using AIDA Image analyzer software (Raytest, Straubenhardt, Germany), and expressed as a percentage of band densities of naive control samples.

**Histology**

Lung pathology scores were determined as described before [14]. In brief, the left lung lobe was harvested at the indicated time points, fixed in 10% buffered formalin, and embedded in paraffin. 4 µm sections were stained with haematoxylin and eosin (HE)
and analyzed by a pathologist blinded for groups. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: bronchitis, edema, interstitial inflammation, intra-alveolar inflammation, pleuritis, endothelialitis and percentage of the lung surface demonstrating confluent inflammatory infiltrate. Each parameter was graded 0–4, with 0 being ‘absent’ and 4 being ‘severe’. The total pathology score was expressed as the sum of the score for all parameters.

**Statistical analysis**

Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation unless indicated otherwise. Differences between groups of mice were analyzed by Mann-Whitney U test. Analyses were done using GraphPad Prism version 5.0, Graphpad Software (San Diego, CA). Values of p <0.05 were considered significantly different.

**RESULTS**

**HMGB1 is released in bronchoalveolar lavage fluid during staphylococcal pneumonia**

To examine HMGB1 release in murine *S. aureus* pneumonia we performed a Western blot by running non-reduced BAL fluid samples harvested at various time points in Wt mice after intranasal inoculation of a sublethal dose of *S. aureus*. Uninfected mice displayed

![Figure 1. HMGB1 concentrations in mouse BAL fluid during S. aureus pneumonia.](image)

(A) Wt mice were intranasally infected with $1 \times 10^7$ colony forming units (CFU) *S. aureus* and euthanized after 6, 24, 48 and 72 hours. HMGB1 concentrations in BAL fluid were quantified by densometric analysis of HMGB1 Western blots and expressed as a percentage of the mean density of control BAL fluid samples (0h). Data represent the means ± standard error of the mean (n = 5 mice per time point). *p*<0.05 versus naïve mice (0h).
almost no detectable levels of HMGB1. HMGB1 bands were detected as early as 6 hours post infection. HMGB1 concentrations peaked after 24 hours and decreased thereafter (Figure 1).

**HMGB1 and RAGE, but not TLR4, contribute to lung pathology**

To evaluate the role of HMGB1, RAGE and TLR4 in the severity of lung pathology, we analyzed pulmonary inflammation and injury in lung tissue slides obtained from anti-HMGB1 treated, \textit{rage}^/- and \textit{tlr4}^/- mice and their respective control animals at 6, 24, 48 and 72 hours after infection with \textit{S. aureus} (Figures 2 and 3). Total pathology scores were

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**Figure 2. Anti-HMGB1 treated mice show reduced lung pathology early after induction of \textit{S. aureus} pneumonia.**

Representative slides of lung haematoxylin and eosin (HE) staining of control treated (A) and anti-HMGB1 treated mice (C), original magnification x2. The boxed areas are also shown at a higher magnification for controls (B) and anti-HMGB1-treated mice (D), original magnification x10. Scale bars indicate 200 µm. Total pathology scores were determined at the indicated time points post infection in control treated (grey) and anti-HMGB1 treated mice (white) according to the scoring system described in the Methods section (E). Total protein was measured in BAL fluid from control (grey) and anti-HMGB1 treated (white) mice (F). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (7-8 mice per group at each time point). **p<0.01 versus control treated mice at the same time point.
Chapter 5

...quantified according to the scoring system described in the Methods section. Mice displayed signs of acute lung injury including interstitial inflammation, endothelialitis, bronchitis and pleuritis already after 6 hours; the extent of lung pathology upon histological examination remained relatively stable thereafter. At 6 hours post infection, anti-HMGB1 treatment reduced total pathology scores (P < 0.01 versus Wt mice), which was mostly caused by a reduction of lung edema compared to control antibody treatment (Figure 2A-E). In accordance, anti-HMGB1 attenuated protein leak into the bronchoalveolar space at this early time point, as reflected by lower total protein concentrations in BAL fluid (P < 0.01 versus Wt mice). At later time points anti-HMGB1 did not impact on the extent of lung pathology or protein leak.

TLR4 deficiency did not influence lung pathology (Figure 3). Rage−/− mice, however, displayed modestly attenuated lung pathology at 6 hours post infection (P < 0.05 versus Wt mice), which was primarily caused by reduced pleuritis. Although total lung pathol-
ogy scores were similar in rage−/− and Wt mice at later time points, the former mouse strain did show significantly smaller areas of confluent inflammatory infiltrates at 48 hours after infection (17±5 % of their lung surface versus 31±2 % in Wt mice, mean ± standard error of the mean, P <0.05, Supplemental Figure 1). Remarkably, while RAGE deficiency did not impact on total protein levels in BAL fluid, tlr4−/− mice displayed an increase of BAL fluid total protein content 6 hours after infection (P < 0.01 versus Wt mice). In conclusion, these data indicate that RAGE and HMGB1 contribute to the extent of lung pathology in S. aureus pneumonia, but, considering their differential impact on histological features, most likely via different mechanisms.

**HMGB1 or RAGE do not influence neutrophil influx during staphylococcal pneumonia**

One of the hallmarks in S. aureus pneumonia is a massive influx of neutrophils into the lung parenchyma [5,6]. Anti-HMGB1 did not influence neutrophil counts in BAL fluid at any time after infection with S. aureus (Table 1). Similarly, RAGE or TLR4 deficiency did not impact on neutrophil influx, except for modestly lower neutrophil numbers in BAL fluid of tlr4−/− mice harvested 72 hours after infection (Table 2, P < 0.05 versus Wt mice).

<table>
<thead>
<tr>
<th></th>
<th>Control IgG2b</th>
<th>Anti-HMGB1</th>
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<tbody>
<tr>
<td>t= 6h</td>
<td>508 (390-1169)</td>
<td>520 (207-736)</td>
</tr>
<tr>
<td>t= 24h</td>
<td>434 (391-580)</td>
<td>665 (450-934)</td>
</tr>
<tr>
<td>t= 48h</td>
<td>1376(1054-1796)</td>
<td>1339 (1147-1770)</td>
</tr>
<tr>
<td>t= 72h</td>
<td>600 (516-891)</td>
<td>519(425-841)</td>
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</tbody>
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**Table 1. Influx of neutrophils in BAL fluid of Control or anti-HMGB1 treated mice.** Neutrophil numbers (x1000/ml) in BAL fluid at 6, 24, 48 and 72 hours after intranasal S. aureus infection in control and anti-HMGB1 treated mice. Data are medians (interquartile ranges); n=7-8 mice per group per time point.

<table>
<thead>
<tr>
<th></th>
<th>Wt</th>
<th>Tlr4−/−</th>
<th>Rage−/−</th>
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<tbody>
<tr>
<td>t= 6h</td>
<td>239 (217-310)</td>
<td>397 (221-535)</td>
<td>526 (350-647)</td>
</tr>
<tr>
<td>t= 24h</td>
<td>298 (250-380)</td>
<td>236 (196-275)</td>
<td>342 (262-404)</td>
</tr>
<tr>
<td>t= 48h</td>
<td>429 (218-652)</td>
<td>522 (421-756)</td>
<td>395 (314-541)</td>
</tr>
<tr>
<td>t= 72h</td>
<td>146 (99-190)</td>
<td>90 (81-132)</td>
<td>76 (47-104)</td>
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**Table 2. Influx of neutrophils in BAL fluid of Wt, tlr4−/− or Rage−/− mice.** Neutrophil numbers (x1000/ml) in BAL fluid at 6, 24, 48 and 72 hours after intranasal S. aureus infection in Wt, tlr4−/− and rage−/− mice. Data are medians (interquartile ranges); n=7-8 mice per group per time point. * p<0.05 versus Wt mice at the same time point.
RAGE and HMGB1, but not TLR4, contribute to pulmonary cytokine and chemokine release early after infection with *S. aureus*

To further investigate the role of HMGB1 in lung inflammation during *S. aureus* pneumonia we measured cytokines (TNF-α, IL-1β and IL-6) and chemokines (KC and MIP-2) in BAL fluid from mice treated with anti-HMGB1 or control antibody (Figure 4). All cytokines and chemokines reached peak concentrations at 6 hours after infection. At this time point anti-HMGB1 treatment decreased IL-1β concentrations in BAL fluid ($P < 0.05$ versus mice treated with control antibody), without influencing the levels of other proinflammatory mediators. At later time points, cytokine and chemokine levels were low in all mice and not different between groups with the exception of lower KC levels in anti-HMGB1 treated mice at 48 hours. Cytokine levels in BAL fluid of *tlr4*−/− mice were similar to those in Wt mice at all time points; *tlr4*−/− mice showed lower KC concentrations at 48 hours relative to Wt mice (Figure 5). *Rage*−/− mice had lower TNF-α and IL-6 levels at 24 hours of infection and lower KC concentrations at 24 and 48 hours when compared with Wt mice.

**Figure 4.** Anti-HMGB1 treatment reduces IL-1β and KC levels in BAL fluid after intranasal infection with *S. aureus*.

Cytokine (TNF-α, IL-6 and IL-1β) (A-C) and chemokine (KC and MIP-2) (D-E) levels in BAL fluid at different timepoint after intranasal infection of $1\times 10^7$ CFU *S. aureus* in mice treated with control (grey) and anti-HMGB1 antibodies (white). Data are expressed as box-and-whisker diagrams depicting the median, the smallest observation, lower quartile, median, upper quartile and largest observation (7-8 mice per group at each time point). * $p<0.05$, ** $p<0.01$ versus Wt mice at the same time point.
HMGB1 and RAGE in staphylococcal pneumonia

HMGB1 has been shown to impair bacterial clearance during pneumonia caused by Pseudomonas (P.) aeruginosa[13,15]. To investigate the role of HMGB1 in clearance of S. aureus, we quantified bacterial loads in BAL fluid, liver and blood after intranasal inoculation with 10^7 S. aureus of mice treated with anti-HMGB1 or control antibody. Although HMGB1 was highly present at the primary site of infection during S. aureus pneumonia, anti-HMGB1 treatment did not influence bacterial loads at any time point (Figure 6A).

We next investigated the role of TLR4 and RAGE in bacterial clearance. In analogy to the results obtained after anti-HMGB1 treatment, tlr4^−/− mice showed similar bacterial loads as Wt mice at all time points (Figure 6B). Rage^−/− mice displayed 10 times lower bacteria in BAL fluid after 6 hours (P < 0.01 versus Wt mice), but not at later time points (Figure 6B). Bacteria that disseminated into the circulation were almost immediately cleared in all mice. No differences in bacterial loads in blood or livers were observed between experimental groups (data not shown). Together these data indicate that RAGE impairs clearance of S. aureus in the bronchoalveolar compartment early after infection and that HMGB1 has no role herein. Moreover, TLR4 does not contribute antibacterial defense during S. aureus pneumonia.

Figure 5. Rage^−/− mice show lower levels of TNF-α and IL-6 in BAL fluid during S. aureus pneumonia
Cytokine (TNF-α, IL-6 and IL-1β) (A–C) and chemokine (KC and MIP-2) (D-E) levels 6, 24, 48 and 72 hours after intranasal S. aureus infection in Wt (dark grey) and tlr4^−/− (light grey) and rage^−/− mice (white). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (7-8 mice per group at each time point). * p<0.05, ** p<0.01 versus Wt mice.
S. aureus is a major bacterial pathogen responsible for both healthcare and community associated infections[1]. Pulmonary infection by S. aureus may develop into necrotizing pneumonia and can be very severe as a consequence of both virulence factors and an intense immune response[4]. Lung injury may be further aggravated due to enhancement of inflammation caused by the release of DAMPs such as HMGB1[4,7,8]. In the current study we intranasally challenged mice with staphylococci to determine the functional role of HMGB1 and two of its proinflammatory receptors in S. aureus pneumonia[9]. We show that S. aureus pneumonia is associated with HMGB1 release in the bronchoalveolar compartment peaking after 24 hours. Although relatively little HMGB1 was released at 6 hours, anti-HMGB1 treatment was able to attenuate lung pathology and protein leak, accompanied by lower IL-1β concentrations in BAL fluid at this time point. TLR4, which has been identified as the dominant proinflammatory receptor for HMGB1[10], had no or very limited impact on the injurious host response during S. aureus pneumonia. RAGE deficiency, however, was associated with reduced lung pathology although rage−/− mice did not phenocopy anti-HMGB1 treated mice, suggesting that different mechanisms are involved. Our results suggest a harmful role for both HMGB1 and RAGE in the development of lung injury during the early phase of severe pneumonia caused by a clinical relevant Gram-positive pathogen.

Previous studies investigated the role of HMGB1 in lung injury associated with Gram-negative pneumonia in mice with various comorbid conditions[13,15]. Anti-HMGB1 treatment attenuated pulmonary neutrophil recruitment and lung injury upon airway
infection with *P. aeruginosa* of mice deficient for the cystic fibrosis transmembrane conductance regulator[13] and mice exposed to hyperoxia[15]. In accordance, anti-HMGB1 decreased neutrophil accumulation and lung edema in mice treated with intratracheal endotoxin[16], the proinflammatory component of the Gram-negative bacterial cell wall. Moreover, HMGB1 has been reported to contribute to hemorrhage- and ventilator-induced lung injury in mice[17,18]. To the best of our knowledge, our investigation is the first to study the role of HMGB1 in lung injury elicited by a Gram-positive pathogen. For this we administered a monoclonal anti-HMGB1 antibody previously shown to inhibit HMGB1-induced acute and chronic inflammation in mice[13,19], including pneumonia[13]. Although HMGB1 levels only tended to increase 6 hours after induction of *S. aureus* pneumonia, anti-HMGB1 reduced protein leak and lung edema at this time point, which is in accordance with results obtained during Gram-negative pneumonia[13,15,16]. In contrast, we did not find a role for HMGB1 in neutrophil recruitment, which at least in part may be explained by differential pattern recognition receptors and integrins involved in attraction of neutrophils to the lungs by Gram-negative and Gram-positive stimuli[6].

The current model of *S. aureus* pneumonia is associated with a strong early and transient cytokine and chemokine response in the bronchoalveolar space peaking after 6 hours. Although HMGB1 was not statistically significantly increased at 6 hours, antagonizing this relatively little HMGB1 diminished the greatly enhanced IL-1β levels in BAL fluid, which may have contributed to reduced protein leak and pathology at this time point. In accordance, anti-HMGB1 was previously shown to inhibit IL-1β release after airway endotoxin exposure and in pulmonary injury induced by hemorrhage[16,17]. Interestingly enough we could not find differences in other cytokines at this time point. These seemingly discrepant results are not easily explained and require further investigation. As cytokine production was considerably reduced at time points later than 6 hours in all groups, blockade of HMGB1 from 24 hours onward had only little effect. As such, the brisk induction of inflammatory mediators after intrapulmonary delivery of *S. aureus* is most likely initiated via a TLR2-MyD88 dependent mechanism[20], while the subsequent release of HMGB1 apparently does not sustain this response, although anti-HMGB1 reduced the already low KC levels in BAL fluid at 48 hours.

Several receptors have been implicated in mediating cellular effects of HMGB1[9]. Purified HMGB1 binds specifically to TLR4 to induce proinflammatory cytokine release by an interaction that requires a cysteine in position 106[10]. In addition, HMGB1 can mediate proinflammatory effects by binding of partner molecules such as bacterial ligands, extracellular cell free DNA, nucleosomes and IL-1β, through which other pattern recognition receptors can be activated[9]. Our group previously showed that intraperitoneal injection of recombinant HMGB1 induces inflammation by mechanisms that partially depend on TLR4 and RAGE[21]. We here show that TLR4 is not involved
in the lung pathology induced by *S. aureus*. We did not expect a direct role for TLR4 in the initiation of lung inflammation induced by *S. aureus*, considering that this pathogen does not express known TLR4 ligands[20]. Of note, however, TLR4 not only can function as a receptor for HMGB1, but also for other DAMPs released upon acute lung injury[22]. As such, our results argue against an important role for TLR4 as a DAMP receptor contributing to lung injury during *S. aureus* pneumonia. In contrast, earlier investigations reported a clear role for HMGB1–TLR4 signalling in sterile injury models induced by ischemia-reperfusion[23] and trauma[24]. Remarkably, TLR4 seemed to protect against epithelial barrier disruption, as indicated by increased protein levels in BAL fluid of *tlr4*<sup>−/−</sup> mice early after infection. Previous studies reported reduced epithelial barrier integrity in hyperoxia-induced lung injury in *tlr4*<sup>−/−</sup> and C3H/HeJ mice by a mechanism that involved a diminished capacity to upregulate anti-apoptotic proteins[25,26]. Further studies are warranted to establish the mechanism by which TLR4 protects against alveolar protein leak during *S. aureus* pneumonia.

RAGE has been implicated in lung injury induced by hyperoxia[27] or bleomycin[28]. In addition, in pneumonia caused by *Streptococcus pneumoniae*, *rage*<sup>−/−</sup> mice showed mitigated lung injury and neutrophil migration[29]. Nonetheless, *Rage*<sup>−/−</sup> mice showed only discreetly attenuated lung pathology at 6 hours after infection with *S. aureus* without alterations in neutrophil recruitment or protein leak. *Rage*<sup>−/−</sup> mice did demonstrate reduced TNFα, IL-6 and KC concentrations in BAL fluid 24 hours post infection. Thus, although RAGE contributed to lung inflammation during experimental *S. aureus* pneumonia, its role was modest and unlikely mediated by HMGB1. RAGE can interact with several ligands besides, including advanced glycation end products, amyloid, β-sheet fibrils and members of the S100 protein family[9]. Which RAGE ligand(s) contribute to its effects in *S. aureus* pneumonia remains to be established. The current finding that *rage*<sup>−/−</sup> mice displayed increased clearance of *S. aureus* from the bronchoalveolar compartment is in line with an earlier study from our laboratory reporting attenuated bacterial growth and dissemination in *rage*<sup>−/−</sup> mice after induction of pneumonia by *Streptococcus pneumoniae*[29].

**CONCLUSIONS**

In conclusion, we here describe distinct roles for HMGB1 and RAGE in lung injury accompanying the early phase of *S. aureus* pneumonia. HMGB1 is a proinflammatory mediator which is released in the bronchoalveolar compartment and contributes to protein leak and lung edema, while it does not influence neutrophil recruitment or bacterial clearance. Although TLR4 has been implicated as the dominant proinflammatory receptor for HMGB1[10], this receptor had only limited impact on the injurious host response during
S. aureus pneumonia. RAGE deficiency, however, was associated with reduced lung pathology and inflammation as well suggesting that both HMGB1 and RAGE are harmful in the development of lung injury during the early phase of severe S. aureus pneumonia.

The current finding that neither anti-HMGB1 nor deficiency for the major DAMP receptors RAGE or TLR4 impact on the sustained lung pathology accompanying S. aureus pneumonia suggest that other DAMPs and receptors are involved. Candidates meriting further investigation include TLR3 (extracellular RNA), TLR9 (extracellular DNA) and the inflammasomes (multiple DAMPs).

**KEY MESSAGES**

- *S. aureus* pneumonia is associated with early (6 hours after infection) bronchoalveolar HMGB1 release, which contributes to inflammation, protein leak and lung edema.
- TLR4 has only limited impact on the injurious host response during *S. aureus* pneumonia
- RAGE enhances pulmonary inflammation and pathology early after intranasal *S. aureus* infection.
REFERENCES


Figure S1. *Rage*−/− show smaller areas of confluent inflammatory infiltrates at 48 hours after *S. aureus* infection.

Representative slides of lung HE staining of Wt (A), *tlr4*−/− (B) and *rage*−/− mice (C) at 48 hours after infection, original magnification x2. Scale bars indicate 200 µm. The percentage of the lung surface demonstrating confluent inflammatory infiltrate was determined in Wt (dark grey), *tlr4*−/− (light grey) and *rage*−/− mice (white) (D). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (7-8 mice per group at each time point). * p<0.05 versus Wt mice at the same time point.