Endogenous danger signals in infectious diseases

Achouiti, A.
Chapter 7

The receptor for advanced glycation end products promotes bacterial growth at distant body sites in *Staphylococcus aureus* skin infection

Ahmed Achouiti\(^1,2\), Cornelis van ‘t Veer\(^1,2\), Alex F. de Vos\(^1,2\), Tom van der Poll\(^1,2,3\)

\(^1\)Center for Experimental and Molecular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands. \(^2\)Center for Infection and Immunity Amsterdam, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands. \(^3\)Division of Infectious Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands.

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ABSTRACT

The receptor for advanced glycation endproducts (RAGE) has been implicated in the regulation of skin inflammation. We here sought to study the role of RAGE in host defense during skin infection caused by Staphylococcus (S.) aureus, the most common pathogen in this condition. Wild-type (Wt) and RAGE deficient (rage⁻/⁻) mice were infected subcutaneously with S. aureus and bacterial loads and local inflammation were quantified at regular intervals up to 8 days after infection. While bacterial burdens were similar in both mouse strains at the primary site of infection, rage⁻/⁻ mice had lower bacterial counts in lungs and liver. Skin cytokine and chemokine levels did not differ between groups. In accordance with the skin model, direct intravenous infection with S. aureus was associated with lower bacterial loads in lungs and liver of rage⁻/⁻ mice. Together these data suggest that RAGE does not impact local host defense during S. aureus skin infection, but facilitates bacterial growth at distant body sites.
1. INTRODUCTION

*Staphylococcus (S.) aureus* is both a common commensal bacterium and an important pathogen responsible for a wide array of human infectious diseases [1]. The vast majority of staphylococcal infections involve skin and soft tissue [2]. In recent years, the incidence of staphylococcal skin infection has remarkably increased due to the emergence of more virulent and antibiotic resistant strains [3,4]. This urges the need to gain more understanding of protective immune responses during *S. aureus* infections, which could help in the development of new therapeutic strategies.

The cutaneous immune response against invading staphylococci encompasses a variety of mechanisms, which are essential for bacterial clearance, including the production of proinflammatory cytokines, recruitment of innate and adaptive immune cells and formation of neutrophil abscesses [5-7]. A protein that may aid in the control of bacterial skin infection is the Receptor for Advanced Glycation End products (RAGE). RAGE is expressed on various cell types [8-11] and binds several damage associated molecular patterns (DAMPs) such as high mobility group box (HMGB)-1 and S100 proteins, which are released during invasive diseases [8,12]. Engagement of RAGE activates the NF-κB pathway, which in turn upregulates expression of RAGE, perpetuating the inflammatory response [8,12]. Previous studies have shown that RAGE contributes to the establishment and maintenance of sterile cutaneous inflammation [9,10]. In addition, RAGE contributes to infiltration of neutrophils, as it upregulates adhesive molecules [12] and acts as an adhesive molecule itself by binding to β2-integrins on neutrophils [8,13,14]. Previous investigations have pointed to a complex role for RAGE in the outcome of infectious diseases, depending on the pathogen or site of infection [15-17]. In the current study we sought to investigate the role of RAGE during *S. aureus* skin infection.

2. METHODS

2.1. Mice

C57Bl/6 wild-type (Wt) mice were purchased from Charles River Laboratories Inc. (Maastricht, the Netherlands). RAGE deficient (*Rage*-/-) mice, backcrossed > 10 times to a C57Bl/6 background were generated as described [18] and bred in the animal facility of the Academic Medical Center (Amsterdam, the Netherlands). 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: DIX101223 and DIX102335).
2.2. Design

Abscess formation in mice was induced as previously described [19]. In short, mice were lightly anesthetized by inhalation of isoflurane (Abbott Laboratories, Queensborough, Kent, UK), shaved at the right flank and subcutaneously injected with a suspension of $1 \times 10^5$ colony forming units (CFU) of *S. aureus* (Newman strain) in phosphate buffered saline (PBS) that was mixed with an equal volume of autoclaved dextran beads in PBS (Cytodex-1 microcarrier beads; Sigma, St. Louis, Missouri) which was prepared according to the manufacturer’s instructions, in a total volume of 100 μl (n=7-8 per strain). Abscesses were serially measured with a digital calliper for 8 days. In addition, mice were sacrificed at 6 hours or 1, 2, 4 or 8 days post infection. After euthanization, blood was drawn into heparinized tubes and livers and lungs were removed aseptically and homogenised in 4 volumes of sterile isotonic saline using a tissue homogenizer (Biospec Products, Bartlesville, UK). Abscesses were excised using 8 mm punch biopsies (Stiefel, Wächtersbarg, Germany) and homogenised in 4 ml sterile isotonic saline. In separate experiments, bacteremia was induced by intravenously injecting $1 \times 10^6$ of *S. aureus* (Newman strain) in a 200 μl saline solution in the tail vein (n=7-8 per strain). Mice were sacrificed 6, 24 or 48 hours thereafter. Blood was obtained and organs were collected and homogenised as described above. To determine bacterial loads, ten-fold dilutions were plated on blood agar plates and incubated at 37°C for 16 h.

2.3. Assays

Homogenates were processed for cytokine measurements as described [20]. Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2 (all R&D systems, Minneapolis, MN) and myeloperoxidase (MPO, Hycult Biotechnology BV, Uden, the Netherlands) concentrations were measured in skin homogenates using ELISAs according to manufacturer’s recommendations. Plasma TNF-α and IL-6 were measured by cytometric bead array flex set assay (BD Biosciences, San Jose, CA) in accordance to the manufacturer’s instructions.

2.4. Statistical analysis

Data are expressed as medians and interquartile ranges. Differences between *rage*<sup>−/−</sup> and Wt 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 statistically significant different.
3. RESULTS

3.1. RAGE does not influence local growth of *S. aureus* in the skin or abscess size, but facilitates bacterial growth at distant body sites

To study the role of RAGE in *S. aureus* skin infection we used an abscess model in which staphylococci ($10^5$ CFU) were injected subcutaneously together with dextran beads [19] in Wt and *rage*<sup>−/−</sup> mice [18]. We determined bacterial loads in standardized punch biopsies taken from the infection site at 6 hours or 1, 2, 4 or 8 days after infection. Bacterial loads were similar in both mouse strains at all time points (Figure 1A). In accordance, while the infection caused abscesses in all mice, the abscess size was similar in Wt and *rage*<sup>−/−</sup> mice at all time points (Figure 1B). This model of skin infection resulted in bacterial dissemination to distant sites. Remarkably, *rage*<sup>−/−</sup> mice displayed reduced bacterial burdens in liver (at 4 days; p<0.05) and lung (8 days; p<0.05) (Figure 1C-D). Bacteria were hardly found in blood (data not shown). These data suggest that RAGE does not impact on local host defense in the skin but enhances growth of *S. aureus* at distant sites in localized skin infection.

![Figure 1. RAGE does not influence local host defense, but promotes bacterial outgrowth at distant sites.](image)

Bacterial loads in abscesses of euthanized mice (A) and (serially measured) abscess areas (B) were similar in wildtype (Wt; closed dots) and RAGE deficient (*rage*<sup>−/−</sup>; open dots) mice until 8 days after subcutaneous infection with $1\times10^5$ colony forming units. *Rage*<sup>−/−</sup> mice show reduced bacterial loads in lungs (C) and livers (D). Data represent the medians ± interquartile range (n = 7-8 mice per time point). *p<0.05, versus Wt mice at the same time point.
Table 1. RAGE has a limited role in neutrophil emigration and cytokine response in *S. aureus* skin infection. MPO, cytokine (TNF-α, IL-1β, IL-6) and chemokine (KC and MIP-2) levels (pg/ml) in skin homogenates at different time points after subcutaneous *S. aureus* infection in Wt and rage−/− mice. Data are expressed as medians and interquartile ranges (7-8 mice per group at each time point). ** p<0.01 versus Wt mice at the same time point.

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<td>87 (10-135)</td>
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Table 1. RAGE has a limited role in neutrophil emigration and cytokine response in *S. aureus* skin infection. MPO, cytokine (TNF-α, IL-1β, IL-6) and chemokine (KC and MIP-2) levels (pg/ml) in skin homogenates at different time points after subcutaneous *S. aureus* infection in Wt and rage−/− mice. Data are expressed as medians and interquartile ranges (7-8 mice per group at each time point). ** p<0.01 versus Wt mice at the same time point.
3.2 Impact of RAGE on neutrophil influx and cytokine responses in cutaneous skin infection

Previous studies have shown that RAGE promotes neutrophil emigration [8,12-14], a hallmark for abscess formation in staphylococcal skin disease [5]. To determine local influx of neutrophils, we measured MPO concentrations, which correlate with the degree of infiltrated neutrophils [21], in skin biopsies taken at the infection site. Wt and rage−/− mice displayed similar MPO levels at all time points, except at 2 days after infection when rage−/− mice had lower values (Table 1, p<0.01). RAGE activation leads to a sustained NF-κB activation thereby perpetuating inflammatory responses [8,12]. To determine the role of RAGE in cytokine and chemokine responses we measured the concentrations of these inflammatory mediators in skin biopsies obtained at different time points. We found no differences in cytokine or chemokine concentrations between Wt and rage−/− mice (Table 1). Together these data suggest a limited role for RAGE in cutaneous inflammation during S. aureus infection.

3.3. RAGE deficiency facilitates bacterial outgrowth during S. aureus bacteremia

To further investigate the role of RAGE in bacterial dissemination, we directly infected Wt and rage−/− mice via the tail vein with 10⁶ S. aureus and determined bacterial loads in blood and organs at 6, 24 or 48 hours after infection. In accordance with the skin infection model, rage−/− mice showed slightly, but significantly lower bacterial loads in lungs and livers compared to Wt mice (both p<0.05) at 6 hours (Figure 2). This effect was temporary as bacterial loads were similar in all body compartments after 24 and 48 hours. Together these results suggest that RAGE transiently inhibits bacterial clearance at distant sites after dissemination from local S. aureus infection.
3.4. The impact of RAGE on systemic cytokine production in *S. aureus* bacteremia

Previous studies have shown that RAGE is able to enhance the systemic inflammatory response by ligation of several endogenous molecules such as HMGB1 [8,12]. To determine whether RAGE would influence the inflammatory response in *S. aureus* bacteremia we measured plasma TNF-α and IL-6 at different time points post infection. At 6 hours after infection, TNF-α levels were reduced in *rage*<sup>−/−</sup> mice. As TNF-α levels decreased over time, no differences were observed between the two mouse strains thereafter (Table 2). *Rage*<sup>−/−</sup> mice displayed higher IL-6 levels after 48 hours (Table 2).

**Figure 2. RAGE promotes bacterial outgrowth after systemic *S. aureus* infection.** Wt (closed dots) and *rage*<sup>−/−</sup> (open dots) mice were intravenously infected with 1x10<sup>6</sup> colony forming units *S. aureus*. Bacterial loads were then determined in blood (A), spleen (B), lungs (C) and liver (D) in euthanized mice after 6, 24 or 48 hours. Data are expressed as medians ± interquartile range. (7-8 mice per group at each time point).

* p<0.05, versus Wt mice at the same time point.

**Table 2. Systemic cytokine release after intravenous *S. aureus* infection.** Cytokine (TNF-α, IL-6) levels (pg/ml) in plasma at different time points after intravenous *S. aureus* infection in Wt and *rage*<sup>−/−</sup> mice. Data are expressed as medians and interquartile ranges (7-8 mice per group at each time point). * p<0.05, versus Wt mice at the same time point, ** p<0.01, versus Wt mice at the same time point.
4. DISCUSSION

RAGE has been implicated in a diverse array of proinflammatory mechanisms in different infectious diseases [16,22]. Although RAGE is highly expressed on skin cells [9-11], its impact on the host response during cutaneous infections has not been investigated before. In the current study, we subcutaneously infected Wt and rage⁻/⁻ mice with S. aureus, the most commonly isolated microorganism in skin and soft tissue infections [2]. We demonstrate that the presence of RAGE hardly impacts local disease, but enhances bacterial outgrowth in distant organs. Bypassing the skin, by directly injecting bacteria into the circulation, revealed a similar but transient effect of RAGE on bacterial outgrowth in distant body compartments. Our results suggest that RAGE has little if any role in local defense, but promotes bacterial outgrowth at distant sites during staphylococcal skin infection.

Previous investigations have pointed to a complex role for RAGE in the outcome of infectious diseases depending on the pathogen and site of infection [15-17]. While RAGE promoted lethality in sepsis induced by cecal ligation and puncture [22] and bacterial outgrowth in pneumonia caused by Gram-positive organisms, such as Streptococcus pneumoniae [17] and S. aureus [23], it reduced bacterial outgrowth in Gram-negative models, such as Escherichia coli induced peritoneal sepsis [24] and pneumonia [25]. In the current study, RAGE did not influence host defense at the site of cutaneous S. aureus infection, as bacterial loads and abscess sizes were similar in Wt and rage⁻/⁻ mice. Despite this limited role in local disease, RAGE promoted bacterial outgrowth in distant organs upon dissemination from 4 days onward. Of note, the overall level of dissemination was low and did not seem to change much over the course of infection. Nonetheless, differences between mouse strains became apparent at different time points after infection, i.e., at day 4 in livers and at day 8 in lungs, which could be related in differential expression of RAGE in different organs. Direct systemic infection showed that RAGE similarly enhanced bacterial outgrowth in lungs and liver at 6 hours. These data suggest that RAGE does not enhance dissemination itself, but that bacterial multiplication is transiently influenced at distant sites, after spread from the primary site of infection. From 24 hours onward, RAGE deficiency seemed to accelerate bacterial outgrowth compared to Wt mice, which may have led to a trend towards higher bacterial loads at the 48 hour time point. It would be of interest to determine bacterial loads at later time points to determine whether RAGE deficiency would significantly impair host defense. The results on bacterial outgrowth in this manuscript are similar to previous findings showing higher bacterial numbers in Gram-positive infection models [17,23]. Naïve rage⁻/⁻ macrophages display enhanced expression of TLR2 [26], which could contribute to a better recognition of lipoteichoic acid on the Gram-positive wall, and promotion of bacterial clearance [27]. Conversely, RAGE is recently identified as a receptor for lipopolysaccharide [26],
expressed in the cell wall of Gram-negative bacteria, which could explain impaired host defense of rage<sup>−/−</sup> mice in Gram-negative infection models [24,25].

RAGE binds several DAMPs, which triggers multiple intracellular signaling pathways, resulting in the translocation of NF-κB and the transcription of proinflammatory proteins [12]. Activation of the NF-κB pathway upregulates expression of RAGE, leading to sustained inflammation [8,12]. Nonetheless, we show that RAGE does not impact cutaneous inflammation as determined by similar cytokine levels after S. aureus infection. In systemic models of infection, RAGE promotes the harmful effects of the systemic inflammatory response syndrome [22], likely via DAMPs such as HMGB-1 [28]. The absence of RAGE reduced the early TNFα response in this model, which is in accordance with observations during pneumococcal bacteremia [15]. IL-6 concentrations, however, were increased in rage<sup>−/−</sup> mice at the latest time point. In this intravenous infection model TNFα release is transient (Table 2), even after administration of higher doses of S. aureus (data not shown), while IL-6 release is sustained, suggesting that especially at late time points plasma IL-6, but not TNFα levels are mainly driven by the extent of bacteremia. Possibly, the higher IL-6 levels in rage<sup>−/−</sup> mice at 48 hours post infection are related to the somewhat higher bacterial loads in blood of these mice. Next to inflammation, RAGE has been implicated in neutrophil attraction [12-14,17,22], a hallmark for host defense in S. aureus skin infection [5]. Activation of RAGE upregulates adhesive molecules [12] and RAGE itself has been identified as a counterreceptor for β<sub>2</sub>-integrins [13,14]. In the current study RAGE reduced MPO levels in the skin only at 2 days, correlating to fewer neutrophils in the skin[21]. However, since there were no differences in bacterial outgrowth at any time point, this difference may be irrelevant. The limited effect of RAGE deficiency on neutrophil infiltration may be due to compensation of other adhesive molecules, such as Intercellular Adhesion Molecule (ICAM)-1. A recent study has demonstrated that RAGE and ICAM-1 work in concert in mediating β<sub>2</sub>-integrin-dependent neutrophil adhesion during acute trauma-induced inflammation [14]. It would be of interest to determine whether mice deficient in both RAGE and ICAM-1 show impaired neutrophil attraction and host defense in S. aureus skin disease.

In conclusion, our results show that RAGE has a limited role in local host defense during S. aureus skin infection as determined by similar abscess size, bacterial loads, cytokine release and minimally altered neutrophil numbers in the skin. RAGE promoted bacterial outgrowth at distant organs during localized skin disease and after direct intravenous infection in different body compartments. Taken together, these data suggest that RAGE directly impacts on bacterial outgrowth at distant sites, rather than on dissemination, during S. aureus skin infection.
REFERENCES


