Endogenous danger signals in infectious diseases

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Chapter 11
-Short Communication-

Myeloid-related protein 8/14 reduces *Staphylococcus aureus* growth in a subcutaneous infection model

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Submitted
**ABSTRACT**

*Staphylococcus (S.) aureus* is the most common cause of skin and soft tissue infections. Staphylococcal skin infections often result in the formation of neutrophil abscesses which contain high quantities of antimicrobial proteins such as the myeloid-related protein (MRP)8-MRP14 complex (MRP8/14). MRP8/14 is the most abundant cytosolic protein in neutrophils, which is actively secreted at infectious sites and has been shown to exert antimicrobial, proinflammatory and chemotactic activity. In the current study we sought to determine the role of MRP8/14 in the host response during *S. aureus* skin infection. Wild-type and MRP8/14 deficient mice were infected subcutaneously with *S. aureus* and bacterial loads and local inflammation were quantified at regular intervals up to 12 days after infection. While MRP8/14 did not influence the formation of abscesses, it facilitated the elimination of *S. aureus* and promoted neutrophil transmigration and the cutaneous inflammatory response. Together our data suggest that MRP8/14 is a protective mediator in *S. aureus* skin disease.
INTRODUCTION

Staphylococcus (S.) aureus is an important causative pathogen for both hospital-acquired and community-onset infections [1]. This gram-positive bacterium is primarily involved in skin and soft tissue infections [2,3]. Given the emergence of virulent and antibiotic resistant strains [4-6], more understanding of immune responses is needed, which could help to identify new therapies for staphylococcal disease.

Staphylococci that invade the skin induce an early cutaneous immune response to attract neutrophils that form an abscess at the infected site [7-9]. Neutrophil abscesses contain high quantities of antimicrobial peptides, including the heterodimer of myeloid-related protein (MRP)8 and MRP14 (MRP8/14 or calprotectin) [7,10,11]. MRP8/14 is the most abundant cytoplasmic protein in the neutrophil cytosol and can be actively secreted at infectious sites [12,13]. Previous studies have shown that MRP8/14 is a strong inhibitor of staphylococcal growth and virulence in vitro, which is mediated by the chelation of divalent cations zinc and manganese [11,14,15]. Accordingly, MRP8/14 improves host defense in vivo when staphylococci are injected into the mouse tail vein [11,14]. Next to its antimicrobial effects, MRP8/14 induces a variety of proinflammatory immune responses including the enhancement of cytokine release via Toll-like receptor 4 [16] and the recruitment of neutrophils [17-20].

In the current study, we aimed to determine the role of MRP8/14 in S. aureus induced skin infection in mice. We demonstrate that MRP8/14 reduces staphylococcal outgrowth in skin abscesses. In addition, MRP8/14 contributed to the cutaneous cytokine response and to neutrophil infiltration. Our data suggest a protective role for MRP8/14 in staphylococcal skin disease.

METHODS

Mice

C57Bl/6 Wild type (Wt) mice were purchased from Charles River Laboratories Inc. (Maastricht, the Netherlands). MRP14 deficient (Mrp14−/−) mice, backcrossed > 10 times to a C57Bl/6 background were generated as described [21] 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: DIX102552).
Design

Abscess formation in mice was induced as previously described [22]. 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 12 days. In addition, mice were sacrificed at 6 hours or 1, 2, 4, 8 or 12 days post infection. After euthanization abscesses were excised using 8 mm punch biopsies (Stiefel, Wächtersbarg, Germany) and homogenised in 4 ml sterile isotonic saline. To determine bacterial loads, ten-fold dilutions were plated on blood agar plates and incubated at 37°C for 16 h.

Assays

Homogenates were processed for cytokine measurements as described [23]. Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, 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 and expressed per mg tissue.

Statistical analysis

Data are expressed as mean ± SEM, as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation or as medians and interquartile ranges. Differences between mrp14−/− 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.

RESULTS AND DISCUSSION

MRP8/14 reduces staphylococcal outgrowth in skin abscesses

Staphylococcal skin infections often develop into neutrophil abscesses that contain an abundance of neutrophil-released MRP8/14[10,11]. MRP8/14 is involved in a variety of innate immune responses [15-19]. To study the role of MRP8/14 in *S. aureus* skin infection we used an abscess model in which staphylococci ($10^5$ CFU) were injected subcutaneously together with dextran beads [22] in Wt and mrp14−/− mice. *Mrp14−/−* mice lack MRP8 at a protein level, despite normal MRP8 mRNA levels, and are therefore considered...
deficient for MRP8/14 [16,21,24]. Serial measurements of lesional sizes in Wt and *mrp14*^-/-^ mice showed no statistical differences for up to 12 days (Figure 1A). Interestingly, however, *mrp14*^-/-^ mice showed a trend towards larger abscess areas when compared to Wt mice in the first 4 days. At day 4, 7 out 8 *mrp14*^-/-^ mice against 1 out 8 Wt mice, developed a skin ulcer (Figure 1B). Skin ulcers quickly resolved in all mice, which led to accelerated skin recovery and a trend towards smaller lesional areas in *mrp14*^-/-^ mice when compared to Wt mice in the days thereafter.

Previous studies have shown that MRP8/14 reduces staphylococcal growth and virulence in infectious environments due to chelation of zinc and manganese [11,14,15]. Assessment of bacterial loads in standardized punch biopsies taken from the infection site at predetermined time points after infection showed enhanced bacterial outgrowth in *mrp14*^-/-^ mice at 1 and 4 days post infection (Figure 2). No differences in bacterial loads were detected thereafter. At day 12, the majority of bacteria were cleared in both Wt and *mrp14*^-/-^ mice, indicating that MRP8/14 is not indispensable for clearing *S. aureus* from the skin. The antimicrobial properties of MRP8/14 may be facilitated by neutrophil extracellular traps (NETs) [25]. We recently demonstrated that blocking MRP8/14 in NETs, reduced NET-mediated growth inhibition of staphylococci *in vitro* [26]. Staphylococci are potent inducers for neutrophils to form NETs *in vivo* [27]. It remains to be established however whether NETs play a role in *S. aureus* skin infection. Altogether, our data showed that while MRP8/14 does not influence the formation of abscesses, it can facilitate the elimination of *S. aureus*, establishing the role of MRP8/14 as a growth-inhibiting factor.
Chapter 11

MRP8/14 enhances the cutaneous inflammatory response after S. aureus infection

Neutrophil recruitment is the hallmark for abscess formation during S. aureus skin infection [7]. Previous data have implicated MRP8/14 as a mediator of neutrophil recruitment in various inflammatory conditions [17-20]. In murine air pouch models, leukocyte influx under the skin was attenuated after pretreatment with blocking antibodies directed against MRP8 and MRP14 in response to LPS [18] or monosodium urate crystals [20]. To determine local influx of neutrophils in S. aureus skin infected sites, we measured total MPO concentrations in skin homogenates, which correlates with the degree of infiltrated neutrophils [28]. Levels of this surrogate marker for neutrophils in the skin were reduced in mrp14−/− mice at 2 and 12 days after infection compared to Wt mice (Table 1), suggesting that MRP8/14 promotes leukocyte recruitment during infectious skin conditions as well.

Next to a role in chemotaxis, MRP8/14 has been shown to enhance the proinflammatory response in various pathological conditions [16]. To determine the role of MRP8/14 in the cutaneous cytokine and chemokine response we performed ELISAs on homogenized skin biopsies of infected sites (Table 1). We here showed reduced cytokine levels of TNF-α (1 to 8 days after infection), IL-1β (2 and 12 days after infection) and IL-6 (2 and 8 days after infection). Interestingly, IL-6 levels were enhanced in mrp14−/− mice, 24 hours after infection, which may have been a reflection of enhanced bacterial outgrowth at this time point. Of note, IL-1β levels showed a remarkable correspondence with MPO levels. Previous studies have shown that IL-1β is essential in S. aureus skin

Figure 2. MRP8/14 reduces bacterial loads after subcutaneous S. aureus infection.

Wt (grey) and mrp14−/− (white) mice were subcutaneously infected with 1x10⁵ cfu S. aureus. Bacterial loads were then determined in standardized punch biopsies of the infected site in euthanized mice after 6 hours or 1, 2, 4, 8 or 12 days. Data are expressed as cfu/ml per abscess in box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. (8 mice per group at each time point). ** p<0.01, versus Wt mice at the same time point.
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Table 1. MRP8/14 promotes the cutaneous cytokine response in *S. aureus* immune response. MPO, cytokine (TNF-α, IL-1β, IL-6) and chemokine (MIP-2) levels (pg/mg tissue) in skin homogenates at different time points after subcutaneous *S. aureus* infection in Wt and *mrp14<sup>-/-</sup>* mice. Data are expressed as medians and interquartile ranges (7-8 mice per group at each time point). *p<0.05, ** p<0.01, *** p<0.001 versus Wt mice at the same time point.
infections as it promotes neutrophil recruitment by inducing chemokines via activation of IL-1R/MyD88-signaling [29,30]. Conversely, these neutrophils (initially recruited after S. aureus-induced inflammation in the skin) are producers of IL-1β (which thereby sustain neutrophil recruitment and abscess formation) [28]. The chemokine MIP-2 was reduced in *mrp14*−/− mice as well (at 2 and 8 days post infection). These data indicate that MRP8/14 promotes neutrophil transmigration and the cutaneous inflammatory response in *S. aureus* skin disease. Together with enhanced bacterial elimination, our data suggest that MRP8/14 is a protective mediator in *S. aureus* skin disease.
REFERENCES


