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Myeloid-related protein-14 deficiency promotes inflammation in staphylococcal pneumonia

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**ABSTRACT**

**Introduction:** *Staphylococcus (S.) aureus* has evolved as an important cause of pneumonia in both hospital and community settings. Staphylococcal lung infection can lead to overwhelming pulmonary inflammation. During infection, neutrophils release complexes of myeloid-related protein (MRP)8 and MRP14 (MRP8/14). MRP8/14 has been shown to exert proinflammatory and chemotactic activity, and to assist in the killing of *S. aureus*. In the current study we sought to determine the role of MRP8/14 in the host response during *S. aureus* pneumonia.

**Methods:** Pneumonia was induced in wildtype (Wt) and MRP14 deficient mice (unable to form MRP8/14) by intranasal inoculation of $1 \times 10^7$ colony-forming units of *S. aureus* USA300. Mice were sacrificed at 6, 24, 48 or 72 hours after infection for analyses.

**Results:** *S. aureus* pneumonia was associated with a strong rise in MRP8/14 in bronchoalveolar lavage (BAL) fluid and lungs tissue. Surprisingly, MRP14 deficiency had a limited effect on bacterial clearance, and was associated with increased cytokine levels in BAL fluid and aggravated lung histopathology. MRP14 deficiency in addition was associated with a diminished transmigration of neutrophils into BAL fluid at late time points after infection together with reduced release of nucleosomes.

**Conclusion:** MRP8/14 serves an unexpected lung protective role in staphylococcal pneumonia.
INTRODUCTION

In the past few decades, methicillin resistant *Staphylococcus (S.) aureus* (MRSA) has evolved as the most important cause of nosocomial infections, including hospital-acquired and ventilator-associated pneumonia [1,2]. In recent years, virulent strains of community-associated MRSA have emerged as well, causing infections in individuals without recognized risk factors [1,3]. While these strains are mainly related to skin and soft tissue infections, they may cause more serious clinical presentations such as fulminant necrotizing pneumonia [3-5]. Indeed, infection of the lower respiratory tract by MRSA has an acute onset and can lead to overwhelming pulmonary inflammation contributing to organ damage and high fatality rates [4-7]. Progressive antibiotic resistance could make staphylococcal pneumonia an even larger health threat in the future [1], urging the need to gain more insight into host defence mechanisms that could influence outcome of this disease.

Acute *S. aureus* infection is associated with a variety of proinflammatory mechanisms to massively recruit neutrophils into the lung parenchyma [8,9]. In addition to phagocytosis of invaded microorganisms [10], these cells are stimulated to release constitutively available endogenous molecules (also known as alarmins) that are recognized by pattern recognition receptors and able to perpetuate inflammatory responses [11]. Among these alarmins are complexes of myeloid-related protein 8 (MRP)8 and MRP14 (MRP8/14 or calprotectin) [12]. Extracellular MRP8/14 induces a variety of innate immune responses including the enhancement of cytokine release via Toll-like receptor (TLR)4 in response to lipopolysaccharide [13] and leukocyte recruitment [14,15].

Next to its proinflammatory effects, MRP8/14 reduces microbial growth in infectious environments by virtue of its metal chelating properties [16]. In recent studies it was shown that MRP8/14 inhibited *staphylococcal* growth and virulence through binding of zinc and manganese [16-18]. In accordance, mice deficient for MRP8/14 showed an impaired defence after intravenous *S. aureus* injection [17,18]. The antimicrobial properties of MRP8/14 may be facilitated by neutrophil extracellular traps (NETs) [19]. We recently demonstrated that blocking MRP8/14 in NETs, reduced NET-mediated growth inhibition of staphylococci *in vitro* [20].

In the current study we investigated the role of MRP8/14 in inflammation and bacterial clearance in experimentally induced *staphylococcal* pneumonia. To this end, we used Wt and MRP14 deficient (*mrp14<sup>-/-</sup>*) mice that were infected with a strain of community associated MRSA (USA300) via the airways. *Mrp14<sup>-/-</sup>* mice lack MRP8 at a protein level, despite normal MRP8 mRNA levels and are therefore deficient for MRP8/14 [13]. We hypothesized that *mrp14<sup>-/-</sup>* mice would demonstrate an impaired clearance of staphylococci from the lungs but potentially with attenuated associated inflammation. Surprisingly, in the study reported here, *mrp14<sup>-/-</sup>* mice displayed only minimally enhanced bacterial
loads in the lungs compared to Wt mice, with enhanced pulmonary cytokine levels and aggravated lung histopathology.

METHODS

Mice
C57Bl/6 Wildtype (Wt) mice were purchased from Charles River Laboratories Inc. (Maas- tricht, the Netherlands). 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 Centre (Amsterdam, the Netherlands). Mrp14⁻/⁻ mice do not show any abnormalities in the healthy state [21,22]. Animal 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 (DIX100121AB).

Design
Mice were lightly anesthetized by inhalation of isoflurane (Abbot Laboratories, Queens- borough, Kent, UK) and intranasally inoculated with a sublethal dose of 1x10⁷ S. aureus USA 300 (BK 11540) in a 50 μl saline solution (n=7-8 per strain). This sublethal dose was determined in a previously described pilot study [23]. Mice were sacrificed 6, 24, 48 or 72 hours thereafter [23].

Preparation of plasma samples and homogenates
Collection and handling of samples were done as previously described [24,25]. In brief, blood was drawn into heparinized tubes, bronchoalveolar lavage (BAL) fluid (see below) was obtained and organs were removed aseptically and homogenized 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 (BA) plates and incubated at 37°C for 16 h. Lung homogenates were prepared for immune-assays as described before [24,25].

Bronchoalveolar lavage
BAL fluid was obtained as previously described [23]. 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 phosphate buffered saline (PBS). 0.7-0.9 ml of BAL fluid was retrieved per mouse. Total cell numbers in BAL fluid were counted using a Z2 Coulter particle count and size analyzer (Beckman-Coulter, Inc., Miami, FL). BAL fluid differential cell counts were carried
out on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, IL).

**Assays**
Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, keratinocyte-derived chemokine (KC), macrophage inflammatory protein 2 (MIP-2) and E-selectin (all R&D systems, Minneapolis, MN) and myeloperoxidase (MPO; Hycult Biotechnology BV, Uden, the Netherlands) were measured using specific ELISAs according to manufacturers’ recommendations. MRP8/14 [13] and nucleosomes [26] were measured as described before.

**Histology**
Lung pathology scores were determined as described before [24,25]. In brief, lungs were harvested at the indicated time points, fixed in 4% buffered formalin, and embedded in paraffin. 4 µm sections were stained with haematoxylin and eosin (HE) and analyzed by a pathologist blinded for groups as described earlier. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: bronchitis, oedema, 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. Granulocyte staining was done using FITC-labeled rat anti-mouse Ly-6 mAb (Pharmingen, San Diego, CA) as described earlier [27]. Ly-6G expression in the lung tissue sections was quantified by digital image analysis [28]; the amount of Ly-6G positivity was expressed as a percentage of the total surface area. MRP8 and MRP14 staining in lung tissue were performed as described [21]. Staining of anti-citrullinated histone H3 (H3Cit, Abcam, Cambridge, UK) of lung tissue was done as described [29,30]

**Statistical analysis**
Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. 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

MRP8/14 is released during staphylococcal pneumonia

To obtain insight into MRP8/14 expression during staphylococcal pneumonia, we measured MRP8/14 concentrations in BAL fluid, lungs and plasma from uninfected Wt mice and Wt mice intranasally challenged with $10^7$ *S. aureus*. MRP8/14 levels became detectable in BAL fluid already at 6 hours after infection and increased during the course of the disease; the highest levels were found after 72 hours (median 9 μg/ml; Fig. 1A). In uninfected whole lung homogenates, MRP8/14 was detectable at low levels and were highly elevated at 6 hours after infection (median; 150 μg/ml at 24 hours; Fig. 1B). Plasma MRP8/14 levels were only minimally elevated at 6 hours and reached basal levels shortly thereafter (data not shown).

To obtain insight into the cellular source of MRP8/14, we stained lung tissue slides obtained from naïve and infected Wt mice for MRP8 and MRP14. Naïve lungs did not exhibit any staining of MRP8 or MRP14. After 6 hours, expression of both MRP8 and MRP14 increased strongly, which was still present 48 hours after infection (Fig. 1C-D).

![Figure 1. Murine staphylococcal pneumonia results in an increase in pulmonary MRP8/14.](image)

MRP8/14 levels in BALF (A) and whole lung homogenates (B) in naive mice and 6, 24, 48 and 72 hours after intranasal *S. aureus* infection (1x10^7 cfu). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. MRP8 and MRP14 stainings in lungs of naive mice and mice 6 or 48 hours after infection with *S. aureus* (C). The boxed areas are also shown at a higher magnification (D) Scale bar indicates 200 μm.
MRP14 deficiency minimally influences bacterial clearance during *S. aureus* pneumonia

Earlier studies have shown that MRP8/14 inhibits staphylococcal growth *in vitro* [16] and *in vivo* [17,18]. To investigate a potential role for MRP8/14 in host defence during staphylococcal pneumonia, we harvested BAL fluid, lungs, livers and blood at 6, 24, 48 or 72 hours after infection and determined bacterial loads (Fig. 2). Remarkably, MRP8/14 deficiency did not influence the clearance of *S. aureus* from the airways to a significant extent, although at a single time point (24 hours) bacterial burdens were modestly, but statistically significantly, higher in lung homogenates of *mrp14*−/− mice. Staphylococcal loads were similar in all other body compartments at all time points in both mouse strains. These data suggest that MRP8/14 minimally contributes to bacterial clearance in a staphylococcal lung infection model.

**Figure 2. Mrp14−/− mice display minimally enhanced bacterial outgrowth in *S. aureus* pneumonia.** Bacterial loads in BALF (A), lung (B), liver (C) and blood (D) after intranasal inoculation of 1x10⁶ cfu *S. aureus* in Wt (grey) and *mrp14*−/− mice (white). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. (6-8 mice per group at each time point). *p*<0.05, versus Wt mice at the same time point.
MRP14 deficiency enhances early cytokine and chemokine release in *S. aureus* pneumonia

Previous investigations have shown that MRP8/14 enhances the proinflammatory response of bone marrow derived macrophages, which correlates to lower plasma TNF-α levels in *mrp14*−/− mice challenged with endotoxin [13]. To investigate the impact of loss of MRP14 on the inflammatory response in staphylococcal pneumonia, we measured cytokines (TNF-α, IL-6 and IL-1β) and chemokines (MIP-2 and KC) in BAL fluid from Wt and *mrp14*−/− mice at different time points after infection with *S. aureus* via the airways (Fig. 3A-E). The highest cytokine and chemokine levels were detected at 6 hours after infection and diminished during the course of the disease. Surprisingly, *mrp14*−/− mice displayed increased levels of all measured cytokines and chemokines 6 and 24 hours after infection (not significant for TNF-α and KC at 6 hours).

![Figure 3](image)

**Figure 3.** *Mrp14*−/− mice display elevated cytokine levels in BALF during *S. aureus* pneumonia. Cytokine (TNF-α, IL-6, IL-1β; A–C) and chemokine (MIP-2 and KC; D–E) levels in BALF, 6, 24, 48 and 72 hours after intranasal *S. aureus* infection in Wt (grey) and *mrp14*−/− mice (white). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (6-8 mice per group at each time point). **p<0.01, ***p<0.001 versus Wt mice at the same time point.

MRP14 deficiency enhances lung pathology during *S. aureus* pneumonia

We next analyzed HE stained lung tissue slides to investigate the role of MRP8/14 in lung pathology in *S. aureus* pneumonia (Fig.4A-C). The total histological scores were semi-quantitatively scored according to the scoring system described in the Methods section. Already at 6 hours, all mice displayed signs of severe pulmonary inflammation. Loss of MRP8/14 resulted in aggravated lung pathology at this early time point, with more advanced signs of interstitial inflammation and bronchitis. Lung pathology scores were
still higher in mrp14−/− mice 48 hours after infection, with more endothelialitis, bronchitis en edema. Histopathology analysis in this model is in correspondence to the cytokine data and suggests that MRP14 deficiency enhances pulmonary inflammation in murine *S. aureus* pneumonia.

To obtain additional insight in the inflammatory response at tissue level, we measured E-selectin levels in whole lung homogenates; the assay used does not discriminate between cell-bound and soluble E-selectin, and when performed on whole lung lysates can be used as a readout for endothelial cell activation [31]. Infection with *S. aureus* caused a brisk rise in whole lung E-selectin concentrations, peaking after 6 hours and strongly decreasing thereafter (Fig. 4D). Of notice, lung E-selectin levels were higher in mrp14−/− mice than in Wt mice at all time points, suggesting that MRP14 deficiency is associated with enhanced endothelial cell activation in the lungs.

![Figure 4](image)

**Figure 4. Mrp14−/− mice show enhanced lung pathology during *S. aureus* pneumonia.** Representative slides of lung HE staining of Wt and mrp14−/− mice 6 and 48 hours after intranasal *S. aureus* infection (A). The boxed areas are also shown at a higher magnification (B). Scalebar indicates 200 µm. Total pathology score at indicated time points post infection in Wt (grey) and mrp14−/− mice (white) was determined according to the scoring system described in the Methods section (C). E-Selectin levels were measured in lung homogenates as a marker for endothelial cell activation in Wt (Grey) and mrp14−/− mice (white) (D). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (6-8 mice per group at each time point). * p<0.05, ** p<0.01 versus Wt mice at the same time point.
Impact of MRP14 deficiency on neutrophil influx and degranulation

Staphylococcal pneumonia is associated with a massive influx of neutrophils, which is considered to play an important role in innate defence [8,9,23]. MRP8/14 has been implicated as an important mediator of neutrophil recruitment in various inflammatory conditions, including pneumonia [14,15]. We studied the recruitment of neutrophils into the lungs using various methods. Neutrophil numbers were similar in whole lungs as indicated by the numbers of Ly6-G positive cells in lung tissue slides prepared from mrp14−/− and Wt mice between 6 and 72 hours after infection (Fig. 5A,B). Remarkably, pulmonary MPO concentrations, reflecting the number and degranulation of neutrophils in tissue, were higher in mrp14−/− mice until 48 hours post infection (Fig. 5C). In BAL fluid, neutrophil numbers were similar as well in both mouse strains at 6 and 24 hours after infection. At 48 and 72 hours, however, when the largest portion of bacteria had already been cleared, mrp14−/− mice showed significantly lower neutrophil numbers in BAL fluid (Fig. 5D). These data suggest differential effects of MRP14 deficiency on neutrophil recruitment in different lung compartments and on degranulation during S. aureus pneumonia.

Figure 5. Mrp14−/− mice display diminished neutrophil transmigration to the bronchoalveolar space.
Representative neutrophil stainings (brown) of Wt and mrp14−/− mice at 6 and 48 hours after induction of S. aureus pneumonia (A). Scalebar indicates 200 µm. Quantitation of pulmonary Ly-6G positivity (B) and MPO levels in whole lungs (C) 6, 24, 48 and 72 hours after infection in Wt (grey) and mrp14−/− mice (white). Total neutrophil numbers in BALF at 6, 24, 48 and 72 hours after intranasal S. aureus infection in Wt (grey) and mrp14−/− mice (white) (D). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (6-8 mice per group at each time point). * p<0.05, ** p<0.01 versus Wt mice at the same time point.
Role of MRP14 deficiency on NET formation during S. aureus pneumonia.

Earlier investigations have shown that S. aureus is a potent inducer of NET release *in vitro* and *in vivo* [32,33]. To determine the release of NETs in lungs during S. aureus pneumonia, we stained lung tissue slides from Wt and *mrp14*−/− mice after staphylococcal infection for citrullinated histone H3, a marker for NETs [34]. Staining of citrullinated H3 showed that both mouse strains released NETs in staphylococcal pneumonia. We next quantified nucleosome levels (a NET degradation product and a biomarker for NETs [35] in BALF (Fig. 6B). Despite the abundant presence of bacteria and neutrophils at 6 hours, nucleosomes were hardly detected in either mouse strain. At 24 hours, *mrp14*−/− mice showed slightly higher levels of nucleosomes compared to Wt mice, while at later time points *mrp14*−/− mice showed reduced nucleosome levels compared to Wt mice.

![Figure 6](image_url)

**Figure 6.** Wt and *Mrp14*−/− mice release NETs in lungs after *S. aureus* infection in the lungs. Stainings of citrullinated H3 in lung tissue slides in Wt and *mrp14*−/− mice 48 hours after intranasal *S. aureus* infection (A). Scalebar indicates 100 µm. Nucleosomes, a NET-biomarker was measured in Wt (grey) and *mrp14*−/− mice (white) (B). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (6-8 mice per group at each time point). * p<0.05 versus Wt mice at the same time point.

**DISCUSSION**

MRSA pneumonia is associated with high morbidity and mortality rates in both immunocompromised patients [6], and in previously healthy individuals without recognized risk factors [4,5]. Staphylococci incite excessive inflammation upon lower airway infection, which may be perpetuated by alarmins [11], thereby contributing to lung injury and disease severity [7]. In the current study we aimed to determine the role of MRP8/14 in *S. aureus* induced pneumonia. Although MRP8/14 has been shown to facilitate the elimination of *S. aureus in vitro* [16] and after systemic infection *in vivo* [17,18], *mrp14*−/− mice surprisingly displayed a virtually unaltered capacity to clear *S. aureus* from their airways. Remarkably and in contrast to our hypothesis, MRP14 deficiency enhanced inflammation in lungs after intranasal *S. aureus* infection.
Human pneumonia is associated with local and systemic MRP8/14 release [36,37], which correlates with findings in murine pneumonia caused by *Klebsiella pneumoniae* [20] or pneumococci [14,37]. In accordance, in the current model of *S. aureus* pneumonia MRP8/14 levels in plasma and lungs were increased as well. The brisk rise of MRP8/14 at 6 hours in lung homogenates may be a reflection of the strong proinflammatory stimulus induced by a high intranasal dose of *S. aureus*. The gradual increase of bronchoalveolar MRP8/14, which was at least 20 times higher than in *Klebsiella* pneumonia [20], might have been due to accumulation of released MRP8/14 after a massive early influx of activated neutrophils, which represent a major source of this protein [38,39]. We previously showed that MRP8 and the neutrophil marker Ly6-G have a similar expression in pneumococci infected lungs [37].

Despite high concentrations at the primary site of infection, MRP8/14 appeared to have an insignificant role in local bacterial control as staphylococcal loads were largely similar in *mrp14*−/− and Wt mice. This finding is remarkable considering that MRP8/14 has been established as an antimicrobial protein in in vitro experiments, demonstrating reduced staphylococcal growth and virulence via the chelation of zinc and manganese [16,17]; additionally, the in vivo relevance of these data were shown in *mrp14*−/− mice which displayed enhanced bacterial growth when challenged with *S. aureus* intravenously [17,18]. It has previously been demonstrated that MRP8/14 is abundantly present within abscesses [40] and has its greatest antimicrobial potential within these infectious environments [17,18]. Distant abscess formation did not occur in our model of pneumonia. MRP8/14 may also inhibit bacterial growth via the formation of NETs [19,20]. We previously demonstrated that MRP8/14 is an important antimicrobial component within NETs to reduce *S. aureus* growth in vitro [20]. We here show that NETs are formed in *S. aureus* infected lungs of both mouse strains. Although Wt and *mrp14*−/− neutrophils have a similar capacity to release NETs [19], we observed enhanced levels of nucleosomes, a NET biomarker [35], in BALF of *mrp14*−/− mice, 24 hours after infection, which may be a reflection of the enhanced proinflammatory state in these mice at this time point. The enhanced nucleosome levels in Wt mice at 48 and 72 hours most likely reflect the increased number of neutrophils in the bronchoalveolar space. Interestingly, nucleosome levels were only present when the majority of bacteria were cleared. Previous investigations have already shown that NETosis may occur in vivo in the presence of *S. aureus* [33]. Not all neutrophils form NETs however, and only when bacteria are too large to be phagocytized, e.g. upon formation of large staphylococcal aggregates [41]. We speculate that a large portion of the inoculated bacteria was easily phagocytized by neutrophils, which therefore did not form NETs at this early time point [41]. The fact that nucleosomes were highly present in late stage disease may be due to NETosis in response to remaining bacteria that are not easily phagocytized and due to accumula-
tion of NET-degradation products. Together these data suggest that MRP8/14 (within or outside of NETs) has a limited antibacterial role in staphylococcal pneumonia.

While previous studies found proinflammatory effects of MRP8/14 under various pathological conditions [13], we were unable to demonstrate such a role for MRP8/14 in S. aureus pneumonia. In contrast, we unexpectedly observed higher cytokine and chemokine levels in lungs and more advanced signs of histopathology in mrp14−/− mice. This proinflammatory phenotype of mrp14−/− mice is unlikely explained by differences in bacterial loads, considering that many dissimilarities were already present at 6 hours after infection (when all bacterial counts were similar) and considering the only very modest variance in lung bacterial loads at 24 hours. It is also not explained by different baseline cytokine and chemokine levels, as these are similar in BAL fluid from naïve Wt and mrp14−/− mice [42]. Recent studies demonstrated that mrp14−/− mice have an enhanced maturation state of dendritic cells [43,44], and that these matured dendritic cells release more cytokines [44]. Notably, dendritic cells contribute to cytokine production during S. aureus pneumonia [45]. Further studies are needed to establish a possible role of these immune cells in the hyperinflammatory phenotype of mrp14−/− mice in this model.

Several animal studies have implicated MRP8/14 as a mediator of neutrophil recruitment [14,15]. In the current study, we found similar neutrophil numbers at 6 and 24 hours after infection in BALF and lung tissue of Wt and mrp14−/− mice. At 48 and 72 hours after infection, however, when the bulk of bacteria had been cleared from the lungs, mrp14−/− mice displayed markedly diminished neutrophil numbers in BALF, suggesting that MRP8/14 may play a role to attract neutrophils within the bronchoalveolar compartment but that this lack of a chemotactic effect in mrp14−/− mice may be compensated for by other mechanisms induced by viable bacteria at early time points. Remarkably, MRP8/14 did not influence neutrophil numbers in lung tissue, as shown by Ly-6G staining, suggesting that the high MRP8/14 levels in BALF at later time points impacts on neutrophil transmigration into the bronchoalveolar space. Indeed, taking the dilution factor introduced by the BAL procedure into account, BALF MRP8/14 levels were higher than those in lung tissue at 48 and 72 hours post infection. Despite similar neutrophil numbers in whole lungs, pulmonary MPO levels were increased in mrp14−/− mice, suggesting enhanced degranulation of neutrophils, which may be secondary to the enhanced inflammatory state. Notably, MRP14 deficiency did not impact on viability of neutrophils, or on neutrophil expression of adhesion receptors or respiratory burst in previous studies [21,22].

Previous studies have shown that endothelial cell activation as shown by E-selectin levels is enhanced by MRP8/14 [15,46]. Hence, we reason that the increased E-selectin levels in total lung homogenates of mrp14−/− mice may be due to the enhanced inflammatory state as well.
A limitation of our study is that mice are relatively resistant to *S. aureus* infection compared to humans, at least in part due to the inability of *this bacterium* to release iron from murine hemoglobin [47]. To model clinical pneumonia, mice have to be intranasally inoculated with high staphylococcal doses [7]. Extrapolation of our results to the human situation should therefore be done with caution. In addition, our study does not provide insight into which cell-type drives the phenotype of *mrp14*−/− mice. Although neutrophils are likely most important, adoptive transfer and/or bone marrow transplantation studies are required to obtain proof for this. While the former approach is technically challenging in mice, the latter approach might be less feasible considering incomplete replacement of alveolar macrophages [48].

In conclusion, we here document that *S. aureus* pneumonia is associated with accumulating MRP8/14 in the lungs, which promotes neutrophil transmigration to the bronchoalveolar space but has a limited role in clearance of bacteria from the airways. MRP14 deficiency is associated with enhanced pulmonary inflammation, neutrophil degranulation and endothelial cell activation in *S. aureus* pneumonia, suggesting that MRP8/14 may serve an unexpected lung protective role in severe *S. aureus* pneumonia.
REFERENCES


