RAGE and the innate immune response in Infection and Inflammation

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Expression and role of myeloid-related protein-14 in clinical and experimental sepsis

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**Abstract**

**Rationale**
Myeloid related protein (MRP) 8 and 14 can form heterodimers that elicit a variety of inflammatory responses. We recently showed that MRP8/14 is a ligand for Toll-like receptor 4, and that mice deficient in MRP8/14 are protected against endotoxic shock-induced lethality.

**Objectives**
To determine 1) the extent of MRP8/14 release in patients with sepsis and/or peritonitis and in healthy humans exposed to lipopolysaccharide (LPS) and 2) the contribution of MRP(8/14) to the host response in murine abdominal sepsis.

**Methods**
MRP8/14 was measured in 51 severe sepsis patients, 8 subjects after intravenous injection with LPS and 17 patients with peritonitis. Host responses to sepsis were compared in *mrp14* gene deficient (and thereby MRP8/14 deficient) and wild-type mice intraperitoneally injected with *Escherichia (E.) coli*.

**Measurements and Main Results**
Sepsis patients displayed elevated circulating MRP8/14 concentrations at both days 0 and 3, and LPS injection resulted in systemic MRP8/14 release in healthy humans. In patients with peritonitis, MRP8/14 levels in abdominal fluid were >15-fold higher than in plasma. MRP14 deficient mice displayed an improved defense against *E. coli* abdominal sepsis in an early phase, as indicated by a diminished dissemination of the bacteria at 6 h. In addition, MRP14 deficient mice demonstrated decreased systemic inflammation, as reflected by lower cytokine plasma concentrations, and less severe liver damage.

**Conclusion**
Human sepsis and endotoxemia are associated with enhanced release of MRP8/14. In abdominal sepsis, MRP8/14 likely primarily occurs at the site of the infection, facilitating bacterial dissemination in an early phase and liver injury.
**INTRODUCTION**

Sepsis is the second leading cause of death in non-coronary intensive care units and the 10th leading cause of death overall in developed countries (1, 2). During the last two decades the incidence of sepsis has shown an annual increase of 9% to 240 per 100,000 population in the United States up to 2000 (3). Whereas the overall mortality rate of sepsis is 25-30%, mortality in patients with abdominal sepsis can be as high as 60% (4). Clearly, sepsis, and in particular sepsis with an abdominal source, represents a major clinical and therapeutical challenge. Of note, among the different bacteria identified as causative organisms in peritonitis, *Escherichia (E.) coli* remains one of the most common pathogens (4, 5).

The host response to sepsis is orchestrated by a variety of inflammatory mediators and pathways (6, 7). Recently it has become clear that invasive infection commonly is associated with the release of endogenous proteins that serve to warn the host for eminent danger. These proteins have collectively been called “damage associated molecular patterns” (DAMPs) or “alarmins” (8). S100 proteins, which mediate inflammatory responses and are involved in the recruitment of inflammatory cells to sites of injury (9, 10), have been suggested to be alarmins. S100 proteins comprise a family with more than 20 members, three of which have been linked to innate immune functions by their expression by myeloid cells: S100A8 (also called calgranulin A or myeloid-related protein (MRP) 8), S100A9 (MRP14 or calgranulin B) and S100A12 (MRP6 or calgranulin C). Of these, MRP8 and MRP14 form heterodimers, which are the biologically relevant forms of these proteins (11-13). MRP8/14 complexes induce a variety of inflammatory reactions and the extent of MRP8/14 expression correlates with disease activity in several inflammatory disorders (10, 14). Recently, we showed that the Toll-like receptor (TLR) 4 complex interacts with MRP8/14 (13). MRP8/14 was found to amplify lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α release in vitro and in vivo, and mice with a targeted deletion of the *mrp14* gene were protected against LPS-induced lethal shock. MRP8 is also almost not detectable at protein level in mature phagocytes of MRP14−/− mice despite normal MRP8 mRNA levels probably due to a elevated metabolism of MRP8 in absence of its binding partner. Thus, targeted deletion of MRP14 leads to a complete lack of a functional MRP8/14 complex in the mouse (13, 15). Moreover, MRP14 deficient (MRP14−/−) mice showed an improved survival after intraperitoneal injection of *E. coli* (13).

In the present study, we first aimed to determine the extent of systemic MRP8/14 release in patients with severe sepsis and to examine local MRP8/14 levels at the site of infection in patients with peritonitis. In addition, we determined the role of MRP14 in specific host responses to murine *E. coli* abdominal sepsis. For this, we compared inflammatory
reactions in MRP14^{-/-} and normal wild-type (Wt) mice using our established model of abdominal sepsis induced by intraperitoneal injection of live *E. coli*, resulting in severe peritonitis and sepsis with rapid dissemination of bacteria to distant organs and multiple organ damage (16-18).

**METHODS**

**Human studies**

All studies were approved by the scientific and ethics committees of the Academic Medical Center (Amsterdam, the Netherlands), the St. Luke University Hospital (Brussels, Belgium) and/or the St. Pierre’s Hospital (Ottignies, Belgium). Written informed consent was obtained from all subjects or their relatives.

**Patients**

The study included two patient populations described in detail previously (19): (i) 51 patients with severe sepsis (68 ± 2 years, 31 males) of whom serum was obtained at the day severe sepsis was diagnosed (day 0) and 3 days thereafter (day 3); 31 healthy subjects served as controls; (ii) 17 peritonitis patients (61 ± 4 years, 9 males) requiring emergency laparotomy because of perforation (n = 8), anastomotic leakage (n = 7) or other causes (n = 2). EDTA anticoagulated blood and abdominal fluid samples (from an abdominal drain in *cavum Douglasi*) were taken at index laparotomy for peritonitis (t = 0) and after 1, 2 and 3 days.

**Healthy subjects**

Eight healthy males (22.6 ± 0.6 years) were studied after intravenous injection of *E. coli* LPS (4 ng/kg; lot G, United States Pharmacopeial Convention, Rockville, MD). EDTA anticoagulated blood was obtained before and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 24 h after challenge.

**Assays**

MRP8/14 was measured as described before (20, 21).

**Mouse studies**

**Mice**

Sex and age matched MRP14^{-/-} mice (15) and Wt littermates, backcrossed six times to a C57BL/6 background, were used in all experiments. The Animal Care and Use Committee of the University of Münster approved all experiments.
Design
Abdominal sepsis was induced by intraperitoneal injection of *E. coli* O18:K1 (10⁴ colony forming units (CFUs)) as described (16-18). Sample harvesting and processing, and determinations of bacterial loads and cell counts were done as described (16-18).

Assays
MRP8/14 was measured by ELISA (13). Keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP-2) were measured by ELISA (R&D systems, Abingdon, United Kingdom). Tumor necrosis factor (TNF)-α, interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1 and IL-10 were measured by cytometric bead array multiplex assay (BD Biosciences, San Jose, CA). Aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were determined with commercially available kits (Sigma-Aldrich, St. Louis, MO), using a Hittachi analyzer (Boehringer Mannheim, Mannheim, Germany).

Histology
MRP8/MRP14 and BM8 stainings were performed as described previously (15, 22, 23). Semi-quantitative pathology scores of lung and liver tissue were generated as described (16).

Statistical analysis
Data are presented as means ± SEM. Differences between sepsis groups were performed with non-parametric repeated measures analysis of variances or Kruskal-Wallis test. Differences in time after intravenous LPS in healthy volunteers were compared using non-parametric repeated measures of variances. Differences between time points within groups were compared using the Wilcoxon signed-rank test. MRP8/14 levels between abdominal fluid and plasma and between survivors and non-survivors were compared with a Mann-Whitney U test. Correlations were calculated using Spearman’s rho test. Differences between MRP14⁻/⁻ and Wt mice were analyzed by Mann-Whitney U test. Values of p < 0.05 were considered to represent a statistically significant difference.

Results
Sepsis results in elevated serum MRP8/14 levels irrespective of the source of infection
The overall in-hospital mortality of patients with severe sepsis enrolled in the study was 45%. The primary source of infection was the lungs in 29 patients (52%), the abdomen in 12 patients (24%), and the urinary tract in ten patients (20%). Sepsis patients displayed elevated serum MRP8/14 concentrations both at day 0 and at day 3 (both p < 0.005; Figure 1A). All severe sepsis subgroups, with peritonitis, pneumonia or urinary tract infection as
primary infection, showed increased MRP8/14 levels at day 0 and 3 (all p < 0.05; Figure 1B-D). Of note, patients with severe sepsis caused by pneumonia displayed the highest MRP8/14 serum concentrations (p < 0.05 vs severe sepsis caused by peritonitis). There was no apparent correlation between serum MRP8/14 and the severity of disease: serum MRP8/14 did not correlate with either APACHE II (r = -0.200, p = 0.159) or SOFA scores (r = -0.153, p = 0.284). Furthermore, survivors and non-survivors had similar MRP8/14 serum levels (3676 ± 541.7 and 2425 ± 374.9 ng/ml, respectively, p = 0.06).

**Figure 1. Sepsis results in elevated serum MRP8/14 levels.**

Serum MRP8/14 levels in patients with severe sepsis (all patients, n = 51, A) and subgroups of sepsis patients with different infectious sources; abdomen (peritonitis, n = 12, B), lungs (pneumonia, n = 29, C) and urinary tract (UTI, n = 10, D) and from healthy controls (n = 31). UTI = urinary tract infection. Data are means ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.005 vs healthy controls.

**Human endotoxemia is associated with systemic MRP8/14 release**

To determine whether intravenous LPS induces MRP8/14 release in humans in vivo, healthy subjects were studied after induction of endotoxemia (Figure 2). MRP8/14 concentrations started to rise as early as 1.5 h after LPS administration, peaking after 5 h. Remarkably, MRP8/14 was still elevated in plasma at 24 h after LPS injection.

**Human peritonitis is associated with local MRP8/14 release**

We next wished to investigate whether MRP8/14 is released at the site of infection. Therefore, we measured MRP8/14 in abdominal fluid of patients with peritonitis. None of the 17 patients with peritonitis died within 28 days. During peritonitis, MRP8/14 levels in abdominal fluid were more than 15-fold higher than in concurrently obtained plasma and these local levels remained elevated throughout the 3-day sampling period (p < 0.0005 at all time points, Figure 3).
MRP8/14 expression is enhanced during *E. coli* induced abdominal sepsis in mice

Having established that MRP8/14 is released systemically in patients with severe sepsis and predominantly at the site of infection in patients with peritonitis, we next studied the role of MRP8/14 during *E. coli* abdominal sepsis in mice. Therefore, we first aimed to obtain insight into local, systemic and organ MRP8/14 complex concentrations in mice injected with *E. coli* by measuring MRP8/14 levels in PLF, plasma, lungs and liver homogenates of uninfected Wt mice and mice after administration of *E. coli*. Intraperitoneal injection of *E. coli* caused significantly increased MRP8/14 levels in all compartments after 6 and 20 h (all p < 0.01 vs t = 0 h; Figure 4A-D). In order to study the localisation of MRP8/14 in lungs and liver in our model, we studied the expression of MRP8/14 complexes in lung and liver tissue obtained from mice 20 h after injection with *E. coli*. Immunohistochemical staining of MRP8/14 revealed a strong increase in the number of MRP14+ cells, (predominantly neutrophils) in lungs and liver tissue of mice with *E. coli* (arrows, Figure 5B vs A and 5D vs C, respectively).
Figure 4. Endogenous MRP8/14 levels increase during *E. coli* peritonitis.

MRP8/14 levels in peritoneal lavage fluid (PLF, A), plasma (B), lung (C) and liver homogenates (D) were determined before and 6 and 20 h after intraperitoneal administration of *E. coli*. Data are means ± SEM of 8-10 mice per group. ** p < 0.01, *** p < 0.001 vs \( t = 0 \) h.

Figure 5. Increased MRP8/14 tissue expression in lungs and liver after *E. coli* injection.

Representative view of the lungs (A and B) and liver (C and D) of a non-infected mouse (A and C). Whereas tissues from uninfected mice show absence of positive MRP8/14 staining, the lungs and liver of a mouse 20 h after *E. coli* inoculation show an influx of MRP8/14 positive neutrophils (arrows, B and D). MRP8/14 staining; original magnification x40.

Impact of MRP14 deficiency on leukocyte recruitment and bacterial loads

To obtain a first insight into the role of MRP8/14 in specific host responses to severe bacterial infection, we determined the influx of leukocytes to the primary site of infection and bacterial loads in several body compartments at 6 and 20 h after inoculation. We considered this of particular interest considering that MRP8 has been implicated in neutrophil migration (21, 24-27) and considering that the early recruitment of neutrophils to the peritoneal cavity contributes to an effective antibacterial defense in this model (28, 29). Moreover, we recently showed that
MRP8/14 signals via TLR4 (13); this receptor is of eminent importance for host defense against gram-negative infection in general (30) and in this model of gram-negative abdominal sepsis in particular (31). Leukocyte counts and differentials were similar in PLF obtained from MRP14−/− and Wt mice at both time points (table 1). Moreover, the local (PLF) levels of the neutrophil attracting CXC chemokines KC and MIP-2 did not differ between both mouse strains (table 1). MRP14−/− mice displayed lower bacterial loads at 6 h post inoculation at the primary site of infection (PLF; p = 0.06 vs Wt mice; Figure 6A) and at the more distant sites in blood and lungs (both p < 0.05; Figure 6B and C). At 20 h after inoculation bacterial loads were similar in all body compartments in both mouse strains.

Table 1. Cell counts and chemokine levels in peritoneal lavage fluid

<table>
<thead>
<tr>
<th></th>
<th>Wt</th>
<th>MRP14−/−</th>
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<tr>
<td></td>
<td>6 h</td>
<td>20 h</td>
<td>6 h</td>
<td>20 h</td>
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<tr>
<td>Cells (x 10^5/ml)</td>
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<td></td>
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</tr>
<tr>
<td>Total cells</td>
<td>12.2 ± 1.9</td>
<td>13.0 ± 1.1</td>
<td>583.1 ± 81.7</td>
<td>671.1 ± 42.1</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>7.5 ± 1.2</td>
<td>6.6 ± 0.8</td>
<td>503.0 ± 69.2</td>
<td>553.8 ± 40.9</td>
</tr>
<tr>
<td>Macrophages</td>
<td>4.5 ± 1.2</td>
<td>6.0 ± 1.3</td>
<td>62.3 ± 19.8</td>
<td>93.3 ± 6.6</td>
</tr>
<tr>
<td>Chemokines (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KC</td>
<td>5842 ± 2664</td>
<td>2147 ± 486</td>
<td>17320 ± 1542</td>
<td>16478 ± 2477</td>
</tr>
<tr>
<td>MIP-2</td>
<td>409 ± 212</td>
<td>155 ± 36</td>
<td>2717 ± 412</td>
<td>2793 ± 575</td>
</tr>
</tbody>
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Definition of abbreviations: Wt = wild-type; KC = cytokine-induced neutrophil chemoattractant; MIP = macrophage inflammatory protein.

Mice were intraperitoneally injected with 10^4 CFUs E. coli. At indicated time points, mice were killed and cell counts and chemokine levels were determined in peritoneal lavage fluid. Data are means ± SEM of 8-10 mice per genotype at each time point.

**MRP14−/− mice have a reduced systemic cytokine response**

We recently demonstrated that MRP14−/− bone marrow cells are less responsive to LPS, which correlated with lower TNF-α levels in the circulation of MRP14−/− mice challenged with LPS in vivo (13). To study the impact of MRP14 deficiency on cytokine release during gram-negative sepsis, we measured the levels of TNF-α, IL-6, IL-10 and MCP-1 in PLF (Figure 7, left panels) and plasma (Figure 7, right panels) at 6 and 20 h after inoculation. Overall, cytokine responses were lower in MRP14−/− mice at 6 h post infection: in PLF TNF-α, IL-6 and MCP-1 levels were lower in MRP14−/− mice at this time point, although these differences did not reach significance, whereas in plasma TNF-α (p < 0.05), IL-6 (p
< 0.05) and MCP-1 (p = 0.07) concentrations were lower in MRP14<sup>−/−</sup> mice. At 20 h post infection, the levels of these mediators were similar in PLF and plasma of both mouse strains.

**Figure 6.** MRP14<sup>−/−</sup> mice demonstrate a diminished bacterial outgrowth and dissemination in the early phase of sepsis.

Bacterial loads in peritoneal lavage fluid (PLF, A), blood (B), lungs (C) and liver (D) at 6 and 20 h after intraperitoneal injection of *E. coli* in Wt (open bars) and MRP14<sup>−/−</sup> mice (closed bars). Data are means ± SEM of 8-10 mice per genotype at each time point. * p < 0.05 vs Wt mice at the same time point.

**Figure 7.** Lower cytokine levels in MRP14<sup>−/−</sup> mice in the early phase of sepsis.

TNF-α, IL-6, MCP-1 and IL-10 levels in peritoneal lavage fluid (PLF, left panels) and plasma (right panels) of Wt (open bars) and MRP14<sup>−/−</sup> mice (closed bars) at 6 and 20 h after intraperitoneal administration of *E. coli*. Data are means ± SEM of 8-10 mice per genotype at each time point. * p < 0.05 vs Wt mice at the same time point.

MRP14<sup>−/−</sup> mice demonstrate unaltered lung inflammation

This model of abdominal sepsis is associated with an inflammatory response in the lungs (16-18). Considering the enhanced expression of MRP8/14 in the lungs, we were interested to determine the impact of MRP14 deficiency on sepsis-induced lung inflammation. Therefore, we analyzed lung tissue slides obtained from Wt and MRP14<sup>−/−</sup> mice 20 h after inoculation with *E. coli*. The lungs of both mouse strains displayed signs of...
inflammation as reflected by the accumulation of neutrophils in the interstitium and along vessel walls (Figure 8A and B). The total histological scores (semi-quantified according to the scoring system described in the Methods section) of the two mouse strains were similar (Figure 8C). In line, granulocyte stainings showed an equal granulocyte influx in the lungs of Wt and MRP14−/− mice (Figure 8D and E).

**Figure 8. MRP14−/− mice show unaltered lung inflammation.**

Representative HE stainings of lung tissue 20 h post injection of *E. coli* in Wt (A) and MRP14−/− (B) mice. Original magnification x20. Graphical representation of the degree of lung inflammation (C), determined according to the scoring system described in the Methods section. Data are means ± SEM of 8-10 mice per genotype. Representative neutrophil stainings of lung tissue of Wt (D) and MRP14−/− (E) mice. Ly-6G staining; original magnification x20.

**MRP14−/− mice are protected from liver damage**

Our model of *E. coli* sepsis is characterized by liver injury associated with parenchymal inflammation (16-18). To obtain insight into the role of MRP8/14 in liver injury during *E. coli* induced sepsis, we determined liver damage in Wt and MRP14−/− mice 20 h after inoculation. Upon histopathological examination, both Wt and MRP14−/− mice displayed signs of inflammation of the hepatic parenchyma (Figure 9A and B). Interestingly, liver inflammation in MRP14−/− mice was less profound compared with that in Wt mice as reflected by lower total histology scores (semi-quantified according to the scoring system described in the Methods section) (p < 0.05; Figure 9C). The histological findings of less severe liver inflammation in MRP14−/− mice were confirmed by granulocyte stainings of liver sections (Figure 9D and E). Clinical chemistry confirmed the existence of less profound hepatocellular injury in MRP14−/− mice, *i.e.* MRP14−/− mice had lower plasma ALAT (p < 0.05; Figure 6F) and ASAT levels, although this latter difference did not reach statistical significance (p = 0.06; Figure 9G). In conclusion, MRP14 deficiency was associated with less extensive liver inflammation and injury.
Figure 9. MRP14−/− mice display reduced liver injury.
Representative HE stainings of liver tissue 20 h post injection of *E. coli* in Wt (A) and MRP14−/− (B) mice. Original magnification x10. Graphical representation of the degree of liver inflammation (C), determined according to the scoring system described in the Methods section. Data are means ± SEM of 8-10 mice per genotype. Representative neutrophil stainings of liver tissue of Wt (D) and MRP14−/− (E) mice. Ly-6G staining; original magnification x20. Alanine aminotransferase (ALAT, F) and aspartate aminotransferase (ASAT, G) levels in plasma from Wt and MRP14−/− mice. Data are means ± SEM of 8-10 mice per genotype. * p < 0.05 vs Wt mice.

Kupffer cells and infiltrating neutrophils both express MRP8/14 in the liver during *E. coli* sepsis
In Figure 5C and D we showed that MRP8/14 expression is enhanced in liver tissue 20 h after injection with *E. coli*. MRP8 and 14 are specifically released during the activation of phagocytes (32, 33). In our sepsis model, candidates for MRP8/14 expressing phagocytes in the liver are Kupffer cells and infiltrating neutrophils (the latter cells are stained in Figure 9D and E.) To investigate what cells express MRP8/14 in the liver during *E. coli* sepsis (and thus might be responsible for the enhanced hepatic injury), we performed immunohistochemical double stainings with antibodies against MRP14 and BM8 (the latter being an indicator for Kupffer cells in liver tissue (22, 23)). The livers of healthy Wt mice displayed that all BM8 positive Kupffer cells are MRP14 negative with one exception (Figure 10A). Liver tissue of mice with *E. coli* revealed a mixture...
of MRP14 expressing cells composed of BM8 positive Kupffer cells (Figure 10B, black arrows) and BM8 negative neutrophils (Figure 10B, white arrow). These data suggest that both Kupffer cells and infiltrating neutrophils express MRP8/14 and may - at least in part - attribute to the enhanced liver injury during E. coli sepsis.

Figure 10. Kupffer cells and infiltrating neutrophils both express MRP8/14 during E. coli sepsis.
Representative MRP14 and BM8 double stainings of liver tissue of healthy Wt mice (A; MRP14 red, BM8 blue) and Wt mice 20 h post injection of E. coli (B; MRP14 blue, BM8 red). Original magnification x10. Black arrows indicate double stained (MRP14 and BM8 positive) Kupffer cells. White arrow indicates MRP14 positive, BM8 negative neutrophil. Dotted arrow indicates monostaining with BM8.

Discussion
Severe sepsis remains a major challenge in the care of critically ill patients. The outcome is poor and mortality rates remain up to 30-40%. Peritonitis is the second most common cause of sepsis, and especially abdominal sepsis bears a grim prognosis (1, 34). During local and systemic bacterial infections, inflammatory responses may act as double-edged swords, fighting pathogens on one hand, but potentially causing tissue damage on the other hand. Previously, we showed that MRP8/14 deficiency protects against mortality induced by both endotoxic shock and E. coli induced sepsis (13), suggesting that MRP8/14 has a net detrimental role in both systemic inflammatory response syndrome and sepsis. We here aimed to investigate MRP8/14 release in severe sepsis and, subsequently, the role of MRP8/14 in abdominal sepsis. We made the following key observations: i) patients with severe sepsis and healthy humans intravenously injected with LPS have increased circulating MRP8/14 levels, ii) MRP8/14 is released at the site of infection in patients with peritonitis and iii) MRP14 deficiency is associated with a diminished spreading of E. coli to blood and lungs during the early phase of the infection and with a reduced liver damage.
MRP8 and -14 proteins can form heterodimers and these heterodimers represent 40% of the cytosolic proteins in neutrophils (35). MRP8/14 is released during inflammation (14, 36-39) and there is a strong correlation between systemic MRP8/14 levels and the presence of inflammation (40). In addition, several inflammatory disorders, such as rheumatoid arthritis, cystic fibrosis and chronic bronchitis are associated with elevated plasma concentrations of MRP8/14 (41, 42). Knowledge about MRP8/14 expression during peritonitis is highly limited. Lagasse and colleagues found that recruited neutrophils to the peritoneal cavity after a thioglycollate injection were MRP8+ and 14+ (43). We here demonstrate for the first time that circulating MRP8/14 levels are elevated in patients with severe sepsis. In addition, MRP8/14 release occurs in sepsis irrespective of the primary source of infection, with pneumonia patients displaying the highest concentrations. MRP8/14 concentrations did not correlate with the severity of sepsis, as reflected by APACHE II and SOFA scores, or mortality. LPS administration in healthy human volunteers induced increased circulating MRP8/14 concentrations as early as 1.5 h. Remarkably, MRP8/14 was still elevated in plasma at 24 h after LPS injection. To the best of our knowledge the exact mechanism of the clearance of MRP8/14 is not known. However, a recent study has suggested that the half-life of circulating MRP8/14 is about one day (44). In that study, performed in patients with acute Kawasaki disease, MRP8/14 levels decreased from 3251 ng/ml at day 0 to 1265 ng/ml at day 1 after intravenous immunoglobulin therapy.

The fact that intravenous LPS rapidly elicits a rise in systemic MRP8/14 concentrations implicates that LPS induces the release of a danger signal that interacts with its own receptor, TLR4, amplifying its responses (13).

The observed elevated systemic and local MRP8/14 levels in patients and mice in our studies can be explained by active release of MRP8/14 from stimulated, viable neutrophils and other phagocytes (9, 10, 13). Possibly, MRP8/14 is released passively too, but this has not been reported before. Indeed, although MRP8/14 (and other S100 family members) have been suggested to be alarmins, passive release of MRP8/14 following non-programmed cell death (necrosis) has not been demonstrated (8). Therefore, further research is warranted to study mechanisms contributing to elevated MRP8/14 levels during sepsis.

To the best of our knowledge, local MRP8/14 levels at sites of infection have not been reported in patients before. We here show that patients with peritonitis displayed increased MRP8/14 levels in their abdominal fluid that were about 15-fold higher than in concurrently obtained plasma. In our mouse model of *E. coli* induced abdominal sepsis MRP8/14 complexes were not only elevated locally (PLF) and systemically (plasma), but also in distant organs (lungs and liver). These results expand data from Raquil et al. who showed that MRP8/14 levels are elevated in lung homogenates and bronchoalveolar lavage fluid from mice infected with *S. pneumoniae* (39).
Abdominal sepsis is characterized by recruitment of neutrophils to the site of infection (28, 29). MRP8/14 has been suggested to be involved in neutrophil recruitment. MRP8 and 14 have been reported to increase the binding capacity of the integrin receptor CD11b/CD18 on neutrophils to ICAM-1 on endothelial cells (24). In addition, MRP8 and 14 can induce the adhesion molecules ICAM-1 and VCAM-1 as well as the CXC chemokines IL-8, MCP-1 and GRO-α, (21), all important players during neutrophil migration. Furthermore, blocking (the interaction of) MRP8 and 14 inhibits transendothelial migration of leukocytes (25) and neutrophil migration in response to LPS (26). One other in vitro study reported on chemotactic activities of MRP8/14 (27); however, the concentrations of MRP8 that were used in that study were 100-10,000 fold lower compared to those found in the circulation of healthy controls or of patients with inflammatory disorders (10). In our study, the numbers of neutrophils migrated to the peritoneal cavity did not differ between Wt and MRP14−/− mice, which contrasts with recent findings in mice with pneumococcal pneumonia, in which pretreatment with anti-MRP8 and/or anti-MRP14 antibodies attenuated neutrophil influx into the lungs (39). Together these data suggest an organ and/or pathogen specific role for MRP8/14 in neutrophil recruitment and argue against a role of importance for MRP8/14 in neutrophil influx into the peritoneal cavity during E. coli peritonitis.

At 6 h post inoculation, MRP14−/− mice demonstrated lower bacterial loads at the primary site of infection (PLF, p = 0.06) and at the distant sites blood and lungs. However, at 20 h, bacterial burden did not differ any more at any site. The early difference in bacterial outgrowth between the two mouse strains probably is not due to an enhanced ability of MRP14−/− neutrophils to generate reactive oxygen species, since we found that Wt and MRP14−/− neutrophils and monocytes display a similar capacity to mount a respiratory burst response (data not shown), confirming earlier data (45). Additionally, MRP14 deficiency was reported not to impact on other key neutrophil functions, including chemotaxis, phagocytosis (of E. coli) and apoptosis (45).

Targeted deletion of MRP14 leads to a complete lack of a functional MRP8/14 complex in the mouse (13, 15). Recently, we showed that MRP14−/− mice have lower systemic TNF-α levels 2 h after intraperitoneal LPS administration (13). Moreover, we found that MRP8/14 complexes amplify LPS induced signal transduction via TLR4-MD2 in vitro (13). To determine the impact of MRP14 deficiency on TNF-α release and that of other proinflammatory cytokines during sepsis caused by viable E. coli, we measured cytokines locally (PLF) and systemically (plasma) at 6 and 20 h after inoculation. In line with the observed lower circulating TNF-α levels early after LPS treatment (13), we found that not only the levels of TNF-α were lower in plasma of MRP14−/− mice at 6 h after inoculation, but also the plasma concentrations of IL-6 and MCP-1. Of note, MRP14 deficiency
did not significantly influence the local concentrations of these mediators in PLF. The decreased systemic cytokine levels in the MRP14 mice in the early phase of *E. coli* sepsis could be explained by a diminished activation of TLR4 due to the absence of endogenous MRP8/14 complexes (13). However, neither plasma nor PLF cytokine concentrations differed between the two mouse strains at 20 h after inoculation, supporting the notion that the lower bacterial load at 6 h could also have contributed to the reduced cytokine levels in the MRP14−/− mice at that time point and/or that the reduced TLR4 signaling can be compensated for by other pathways during the later phase of the infection. Steinbakk et al. showed in an *in vitro* experiment that MRP8/14 inhibits and, at higher concentrations, kills blood culture isolated *E. coli* (46). Our finding of lower bacterial loads at 6 h in the MRP14−/− mice suggests that the lack of this potential antibacterial effect is overruled by other mechanisms induced by MRP14 deficiency in our model of severe infection with *E. coli*. It has to be established whether MRP14−/− mice challenged with a lower bacterial load of *E. coli* would have increased bacterial outgrowth.

It is possible that MRP8(/14) at least in part functions as a carrier protein for pro-inflammatory bacterial or host derived molecules similar to what has been reported for high mobility group box 1 (47). However, thus far, data indicate that the observed effects of MRP8(/14) are mediated by the protein itself rather than via a protein carrier function. We previously demonstrated that the TLR4 activation by MRP8 was not due to acquisition of LPS, a pathogen associated molecular pattern relevant for our *E. coli* sepsis model. Indeed, MRP8 effects could not be blocked by addition of the LPS inhibitor polymyxin B, whereas they could be prevented by heat inactivation (13). Moreover, we reported that MRP8 directly and specifically interacts with the TLR4-MD2 complex in the absence of LPS (13) and we found that MRP8/14 is not able to bind LPS using a fluorescence assay according to Yu and Wright (48) (data not shown). Although these findings make an indirect role for LPS in MRP8/14 effects unlikely, it remains to be established whether MRP8/14 can bind and present other pro-inflammatory molecules to immune cells.

Although pulmonary MRP8/14 expression clearly increased during *E. coli* sepsis, lung injury associated with abdominal sepsis did not differ between Wt and MRP14−/− mice. In line with our findings, earlier studies have shown that pulmonary (or circulating) levels of MRP8 and 14 and MRP8/14 are upregulated during pulmonary infection and/or inflammation. Indeed, MRP8/14 concentrations were increased during murine *S. pneumoniae* pneumonia (39) and patients with pneumonia displayed more MRP8/14+ alveolar macrophages in their bronchoalveolar lavage fluid compared to healthy volunteers (49). Furthermore, patients with pulmonary tuberculosis and patients with sarcoidosis have higher MRP8/14 plasma levels than healthy volunteers (50). In addition, MRP8/14
is produced in the lungs after intratracheally administered LPS in rats (51) and bronchial epithelium produces MRP8 and 14 proteins in response to incubation with LPS from *Pseudomonas aeruginosa* in vitro (52). Moreover, MRP8/14 stimulates IL-8 production in alveolar epithelial cells (53). Hence, although our study identifies the lung as a source for MRP8/14 during abdominal sepsis, this protein did not contribute to lung inflammation in our model.

In contrast to the observed similar pulmonary inflammation in the two mouse strains, MRP14\(^{-/-}\) mice displayed significantly less hepatocellular injury, as indicated by histopathology and plasma transaminases. These data suggest that MRP8/14 deficiency protects against sepsis-induced liver damage. In accordance with our findings, Arai and coworkers and Yang and colleagues found that normal, uninfected livers from mice and humans, respectively, do not contain MRP8 or MRP14\(^{+}\) cells (54, 55), whereas infection with *Schistoma mansoni* led to an increased expression of MRP8 and 14 in liver tissue (55). To the best of our knowledge, no other data have been published about MRP8/14 expression in the liver or its involvement during infection. We found that hepatic MRP8/14 levels are upregulated during *E. coli* sepsis at 6 and 20 h after inoculation and that MRP14\(^{-/-}\) mice display diminished liver damage at 20 h. Further experiments are needed to investigate which mechanism(s) underlie(s) the role of MRP8/14 in liver injury. One explanation might be that there is a diminished activation of TLR4 in the MRP14\(^{-/-}\) mice during the early response to infection (13), resulting in less inflammation and reduced tissue injury. In line with this possibility we found lower hepatic levels of TNF-\(\alpha\) and IL-6 in MRP14\(^{-/-}\) mice at 6 h after infection (data not shown). Of note, MRP14\(^{-/-}\) mice demonstrated a modest but significantly improved survival in this model of abdominal sepsis (13); it remains to be established to which extent the reduced hepatocellular injury plays a role herein.

Biologically active MRP8/14 heterodimers cannot be formed in MRP14\(^{-/-}\) mice and therefore, these mice are MRP8/14 deficient. Previously, we and others demonstrated that peripheral myeloid cells of healthy MRP14\(^{-/-}\) mice do not express MRP8 protein (15, 45), possibly due to the need of MRP8 for its binding partner MRP14 for stability. Since it is theoretically possible that septic MRP14\(^{-/-}\) mice do express MRP8 protein in their myeloid cells (in contrast to healthy MRP14\(^{-/+}\) mice), it would be of interest to measure MRP8 homodimers in MRP14\(^{-/-}\) mice subjected to *E. coli* sepsis. Unfortunately, currently there are no adequate assays that can distinguish between MRP8 homodimers and MRP8/14 heterodimers. Therefore, our data do not exclude the possibility that unbound MRP8 plays a role in the pathogenesis of *E. coli* sepsis.

We used MRP14\(^{-/-}\) mice and Wt littermates backcrossed six times to a C57BL/6 genetic
background. Thus, although the genetic background of these animals was not pure C57BL/6, we consider the use of Wt littermates as controls adequate.

The present study is the first to document that MRP8/14 release occurs in severe sepsis and that MRP8/14 is released locally during severe infection in patients with peritonitis. Investigations seeking to provide insight into the functional role of MRP8/14 revealed that MRP14 contributes to bacterial dissemination (transiently) and liver injury during abdominal sepsis. Inhibition of MRP8/14 may be a useful adjunctive therapy for severe sepsis.

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