RAGE and the innate immune response in Infection and Inflammation

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Receptor for advanced glycation end products facilitates host defense during *Escherichia coli* induced abdominal sepsis in mice

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ABSTRACT

Background
The receptor for advanced glycation end products (RAGE) mediates a variety of inflammatory responses.

Methods
To determine the role of RAGE in the innate immune response to abdominal sepsis caused by *Escherichia coli* (*E. coli*), RAGE deficient (*RAGE*^−/−^) and normal wild-type (*Wt*) mice were intraperitoneally injected with *E. coli*. In a separate experiment, Wt mice received either anti-RAGE IgG or control IgG.

Results
*E. coli* sepsis resulted in an upregulation of RAGE in the liver, but not in the lungs. RAGE deficient mice demonstrated an enhanced bacterial outgrowth in their peritoneal cavity and increased dissemination of the infection, accompanied by increased hepatocellular injury and exaggerated systemic cytokine release and coagulation activation, 20 h after intraperitoneal administration of *E. coli*. Wt mice treated with anti-RAGE IgG also displayed a diminished defense against the growth and/or dissemination of *E. coli*. RAGE was important for the initiation of the host response as reflected by a reduced immune and procoagulant response early after intraperitoneal injection of *E. coli* lipopolysaccharide.

Conclusion
These data are the first to suggest that intact RAGE signaling contributes to an effective antibacterial defense during *E. coli* sepsis, thereby limiting the accompanying inflammatory and procoagulant response.
**INTRODUCTION**

Sepsis is the most common cause of death in noncoronary critical care units in the United States with > 750,000 cases per year [1]. Peritonitis is the second most common cause of sepsis [2] with *Escherichia coli* (*E. coli*) as one of the major pathogens involved [3]. Since *E. coli* peritonitis is a life-threatening disease, an immediate and adequate host defense is necessary to contain and kill the pathogen.

The receptor for advanced glycation end products (RAGE) is a multiligand receptor of the immunoglobulin superfamily that is expressed in all tissues on a wide range of cell types, including cells involved in the innate and adaptive immune system [4-6]. RAGE is one of the major signal transduction receptors for advanced glycation end products (AGEs). Ongoing studies revealed, however, that RAGE is able to engage classes of unrelated (including non-AGE) molecules, recognizing their tertiary structures rather than amino-acid sequences [4]. Its known ligands include the damage-associated molecular patterns (DAMPs, i.e. endogenous molecules that signal tissue and cell damage [7]) high mobility group box 1 (HMGB1) [8, 9] S100A12 [10], S100B [11], and amyloid [12].

RAGE has been suggested to be involved in the inflammatory response in several ways. First of all, the cellular effects resulting from the activation of RAGE by above mentioned endogenous pro-inflammatory ligands are mediated by multiple intracellular signaling pathways, including NF-κB, leading to the transcription of pro-inflammatory factors [13, 14]. In addition to binding ligands that participate in inflammatory and immune responses, *in vitro* studies showed that RAGE on endothelial and epithelial cells [15, 16] can function as an adhesive receptor that interacts with leukocyte β2-integrins, thereby being directly involved in inflammatory cell recruitment [15, 16].

RAGE deficiency improved survival in a model of abdominal polymicrobial sepsis induced by cecal ligation and puncture (CLP) [17, 18]. These studies suggested that inhibition of RAGE during sepsis attenuates the systemic inflammatory response and ensuing organ damage. However, the CLP model is less suitable to study the influence of an intervention on bacterial growth and dissemination, considering that the infection is polymicrobial involving a large number of aerobic and anaerobic pathogens and considering that the antibacterial response is dependent on the extent of necrosis of the cecum and the formation of a local abscess [19]. Therefore, we here investigated the role of RAGE during abdominal sepsis induced by one of its major involved pathogens, *E. coli*, focusing on the outgrowth of bacteria at the primary site of infection and the subsequent dissemination, and the accompanying systemic inflammatory response syndrome.
Chapter 2

METHODS

Mice
8-10-week-old female RAGE+/− mice on a C57Bl/6 background (backcrossed ten times) were generated as described [17]. Age and sex matched wild-type (Wt) C57Bl/6 mice were obtained from Harlan Sprague Dawley Inc. (Horst, The Netherlands). The Institutional Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam, approved all experiments.

Experimental groups
E. coli peritonitis and LPS-induced inflammation were induced as described [20-22]. In some studies Wt mice were injected intraperitoneally (ip) with either rabbit anti-RAGE IgG polyclonal antibodies, produced as described before [23] (200 µg given 20 h before and 4 h after infection) or normal rabbit IgG (R & D Systems, Abingdon, United Kingdom). Mice were sacrificed 20 h after E. coli injection. Assays were performed as described [20, 21]. For measurements by ELISA liver and lung homogenates were lysed in lysis buffer and treated as described [20, 21].

Histological examination
Lungs and livers were harvested after 20 h, fixed in 4% formaldehyde, embedded in paraffin and cut in 4-µm thick sections for staining procedures. Immunostaining for RAGE was performed on paraffin slides after deparaffinization and rehydration using standard procedures. Primary antibodies used were goat anti-mouse RAGE polyclonal antibodies (Neuromics, Edina, MN) and secondary antibodies were biotinylated rabbit anti-goat antibodies (DakoCytomation, Glostrup, Denmark). Hematoxylin-eosin stainings were performed as described [24]. Liver and lung injury were semi-quantitatively scored as described [21]. Fibrin(ogen) stainings were performed as earlier described [20, 25].

Statistical analysis
All data are expressed as means ± SEs. Differences between groups were analyzed by Mann-Whitney U test. Values of \( P < .05 \) were considered to represent a statistically significant difference.
**Results**

RAGE is upregulated in the liver but not in lungs during *E. coli* peritonitis

To determine whether RAGE expression changes during *E. coli* peritonitis, we performed immunohistochemical stainings of RAGE of liver and lung tissue from Wt mice after ip administration of *E. coli*. In accordance with the literature [26-28], we found that normal, healthy mice showed modest if any RAGE staining in their livers (figure 1A). Livers from mice injected with *E. coli* displayed clearly more diffuse hepatic RAGE expression (figure 1B). Moreover, the surface of sinusoidal cells (capillaries between the hepatocytes, figure 1B), leukocytes in the vessels and some smooth muscle cells were stained positively for RAGE (figure 1B). Remarkably, areas of necrosis showed decreased RAGE staining (figure 1B, arrows). In the lungs, RAGE was extensively present in both healthy mice and mice infected with *E. coli* (figure 1D-E). Pulmonary RAGE expression was not increased in *E. coli* injected mice compared to healthy mice. Liver and lung tissue from RAGE−/− mice were used as negative controls for the RAGE staining and displayed some background staining only (figure 1C and F, respectively).

RAGE−/− mice have an enhanced bacterial outgrowth and dissemination to distant organs during *E. coli* peritonitis

To examine whether RAGE deficiency influences bacterial outgrowth during peritonitis, we established the number of *E. coli* cfu at 20 h after infection in PLF, blood, liver and lungs of Wt and RAGE−/− mice. RAGE−/− mice had significantly higher bacterial loads in their PLF than Wt mice (P< .01; figure 1G). In addition, blood, liver and lungs from RAGE−/− mice contained more bacteria compared with Wt mice (all P< .01 with the exception of lungs, P = .03; figure 1H-J). Hence, RAGE−/− mice showed a clearly increased outgrowth of *E. coli* at the primary site of infection, which was associated with an enhanced dissemination of bacteria to distant organs.

Inflammatory cell influx, chemokine and cytokine levels

At 20 h after infection, Wt and RAGE−/− mice had similar numbers of total leukocytes, neutrophils and macrophages in their peritoneal fluid (table 1). The mouse CXC chemokines KC and MIP-2 are important mediators in the attraction of neutrophils during inflammation [29, 30]. Therefore, we measured the concentrations of these chemokines in peritoneal fluid. RAGE−/− mice displayed higher MIP-2 levels than Wt mice (P< .05), whereas KC levels in PLF were similar in both mouse strains (table 1). To determine whether RAGE influences the production of cytokines during septic peritonitis, local and systemic concentrations of pro- and anti-inflammatory cytokines were measured in Wt and RAGE−/− mice (table 2). TNF-α, IL-6 and IL-10 were significantly higher in PLF
Figure 1. Expression of RAGE during *Escherichia coli* (*E. coli*) peritonitis and increased local bacterial outgrowth and dissemination in RAGE deficient (RAGE⁻/⁻) mice. Representative view of a liver and lung from a normal, uninfected wild-type (Wt) mouse (A and D, respectively) displaying modest if any staining in the liver and strong RAGE expression in the lung. Liver from a Wt mouse 20 h after intraperitoneal (ip) injection of 5 x 10⁴ cfu of *E. coli* showed more diffuse hepatic RAGE staining (B). The surface of sinusoidal cells (capillaries between the hepatocytes), leukocytes in the vessels and some smooth muscle cells were stained RAGE positive (B). Arrows indicate areas of necrosis (B). Absence of RAGE positivity in the liver of a RAGE⁻/⁻ mouse (C). Lungs from a Wt mouse 20 h after the inoculation of *E. coli* (E) and from a healthy Wt mouse (D). Absence of RAGE positivity in the lung of a RAGE⁻/⁻ mouse (F). RAGE staining; original magnification x10. Bacterial loads in peritoneal lavage fluid (PLF, G), blood (H), liver homogenate (I) and lung homogenate (J) were determined in Wt (white bars) and RAGE⁻/⁻ (black bars) mice 20 h after ip injection of 5 x 10⁴ cfu *E. coli* (n = 6-16 mice/genotype). Data are means ± SEs. ** *P* < .01 vs. Wt mice, *** *P* < .005 vs. Wt mice.
Table 1. Leukocyte counts and chemokine levels in peritoneal lavage fluid (PLF)

<table>
<thead>
<tr>
<th></th>
<th>Wt</th>
<th>RAGE&lt;/-</th>
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</thead>
<tbody>
<tr>
<td>Cell count (x 10^4/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>227.1 ± 32.9</td>
<td>179.5 ± 17.3</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>185.8 ± 28.5</td>
<td>150.7 ± 15.4</td>
</tr>
<tr>
<td>Macrophages</td>
<td>36.5 ± 6.6</td>
<td>28.3 ± 3.0</td>
</tr>
<tr>
<td>Chemokines (pg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KC</td>
<td>3758 ± 1982</td>
<td>17859 ± 10400</td>
</tr>
<tr>
<td>MIP-2</td>
<td>422 ± 126</td>
<td>763 ± 101*</td>
</tr>
</tbody>
</table>

NOTE Data are means ± SEs of 14-16 mice/group at 20 h after intraperitoneal injection of 5 x 10^4 cfu Escherichia coli. Wt, wild type; RAGE</-, receptor for advanced glycation end products gene deficient; KC, cytokine-induced neutrophil chemoattractant; MIP-2, macrophage inflammatory protein-2.

*P < .05 vs. Wt mice.

from RAGE</- mice (all P < .05 vs. Wt mice). Moreover, these three cytokines were higher in liver homogenates from RAGE</- mice, whereas in plasma IL-6 and IL-10 levels were elevated in these mice (table 2). In addition, RAGE</- mice displayed higher concentrations of MCP-1 in PLF and liver, whereas plasma MCP-1 levels tended to be higher in this mouse strain. The concentrations of these mediators were similar in lung homogenates of Wt and RAGE</- mice (data not shown). Hence, RAGE deficiency was associated with an increased release of cytokines, in particular in the peritoneal cavity and liver.

RAGE</- mice display more severe liver damage whereas lung inflammation is unaltered

Our model of E. coli peritonitis is associated with liver injury and focal hepatic necrosis [21]. Both Wt and RAGE</- mice showed signs of inflammation in liver tissue as characterized by the influx of leukocytes into the hepatic parenchyma (figure 2A-B). RAGE</- mice clearly showed more signs of liver necrosis (figure 2B, asterisks; figure 2C; P < .05 vs. Wt mice). In addition, RAGE</- mice demonstrated more thrombus formation (figure 2F, arrow; figure 2C, P < .05 vs. Wt mice). Clinical chemistry confirmed the existence of more profound hepatocellular injury in RAGE</- mice, i.e. RAGE</- mice had significantly higher plasma AST and ALT levels compared with Wt mice (figure 2D, both P < .05). MPO levels in liver homogenates were similar in Wt and RAGE</- mice (data
not shown). Pulmonary inflammation did not differ between the two mouse strains, as reflected by similar lung histology scores, relative lung weights and MPO levels in lung homogenates (data not shown). In conclusion, RAGE deficiency was associated with more extensive hepatocellular injury and necrosis and thrombus formation in the liver, while lung inflammation was unchanged.

### Table 2. Cytokine and chemokine concentrations in peritoneal lavage fluid, plasma and liver 20 h after intraperitoneal injection of *Escherichia coli*

<table>
<thead>
<tr>
<th>Cytokines (pg/mL)</th>
<th>Wt</th>
<th>RAGE⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNF-α</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLF</td>
<td>166 ± 51</td>
<td>266 ± 35ᵃ</td>
</tr>
<tr>
<td>plasma</td>
<td>366 ± 206</td>
<td>759 ± 250</td>
</tr>
<tr>
<td>liver</td>
<td>26 ± 11</td>
<td>34 ± 6ᵃ</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLF</td>
<td>3248 ± 1049</td>
<td>8269 ± 925ᵇ</td>
</tr>
<tr>
<td>plasma</td>
<td>3218 ± 1093</td>
<td>7146 ± 1455ᵃ</td>
</tr>
<tr>
<td>liver</td>
<td>221 ± 66</td>
<td>1251 ± 327ᵇ</td>
</tr>
<tr>
<td><strong>MCP-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLF</td>
<td>4674 ± 1128</td>
<td>10217 ± 793ᵇ</td>
</tr>
<tr>
<td>plasma</td>
<td>4355 ± 1260</td>
<td>5797 ± 1638</td>
</tr>
<tr>
<td>liver</td>
<td>4429 ± 865</td>
<td>8838 ± 1019ᵇ</td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLF</td>
<td>215 ± 144</td>
<td>454 ± 88ᵇ</td>
</tr>
<tr>
<td>plasma</td>
<td>78 ± 67</td>
<td>730 ± 233ᵃ</td>
</tr>
<tr>
<td>liver</td>
<td>501 ± 59</td>
<td>717 ± 88ᵃ</td>
</tr>
</tbody>
</table>

**NOTE** Data are means ± SEs of 14-16 mice/group at 20 h after intraperitoneal injection of 5 x 10⁴ cfu *Escherichia coli*. Wt, wild type; RAGE⁻/⁻, receptor for advanced glycation end products gene deficient; TNF, tumor necrosis factor; PLF, peritoneal lavage fluid; IL, interleukin; MCP-1, monocyte chemoattractant protein-1. ⁿᵃ P < .05 vs. Wt mice; ⁿᵇ P < .01 vs. Wt mice.

**RAGE⁻/⁻ mice demonstrate enhanced coagulation activation during *E. coli* peritonitis**

This model of abdominal sepsis is associated with thrombin generation and activation of the coagulation system [20, 21]. To determine the role of RAGE herein, we performed fibrin(ogen) stainings on liver tissue slides. RAGE⁻/⁻ mice demonstrated increased fibrin(ogen) depositions compared with Wt mice (figure 2F vs. figure 2E). Both Wt and RAGE⁻/⁻ mice showed strongly elevated TATc (figure 2G-H) and D-dimer (figure 2I-J) concentrations in plasma (figure 2G and I) and PLF (figure 2H and J). Importantly,
coagulation activation was more profound in RAGE−/− mice, as reflected by higher plasma and PLF TATc and D-dimer levels (figure 2G-J, all $P < .05$ vs. Wt mice). Together, these data indicate that RAGE deficiency enhances the activation of coagulation both locally and systemically during E. coli peritonitis.

**Figure 2.** RAGE−/− mice display more extensive liver necrosis and liver thrombi formation and enhanced systemic and local activation of coagulation. Wild-type (Wt) and RAGE−/− mice were intraperitoneally injected with 5 x 10^4 cfu Escherichia coli. Representative hematoxylin-eosin stainings of liver tissue at 20 h after injection in Wt (A) and RAGE−/− (B) mice. Original magnification x200. Arrow points out thrombi and asterisks point out necrotic areas. Graphical representation of the degree of liver thrombi and necrosis (C) determined according to the scoring system described in the Methods section. Plasma concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (D). Representative fibrin(ogen) staining of liver tissue of Wt (E) and RAGE−/− (F) mice. Arrow points out thrombi. Original magnification x20. Thrombin-anti-thrombin complex (TATc, G-H) and D-dimer (I-J) concentrations were measured systemically (plasma, G and I) and locally (PLF, H and J) in Wt (white bars) and RAGE−/− (black bars) mice (n = 6-16 mice/genotype). Dotted lines represent the mean values from normal (uninfected) mice. Data are means ± SEs. * $P < .05$ vs. Wt mice; ** $P < .01$ vs. Wt mice.
Table 3. Impact of RAGE deficiency on *Escherichia coli* lipopolysaccharide (LPS)-induced responses

<table>
<thead>
<tr>
<th></th>
<th>t = 0 h</th>
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<th>t = 2 h</th>
<th></th>
<th>t = 6 h</th>
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<tbody>
<tr>
<td></td>
<td>Wt</td>
<td>RAGE-/-</td>
<td>Wt</td>
<td>RAGE-/-</td>
<td>Wt</td>
<td>RAGE-/-</td>
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<tr>
<td><strong>PLF</strong> Cells (x 10^4/mL)</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Total cells</td>
<td>54.4 ± 3.5</td>
<td>37.5 ± 9.5</td>
<td>14.6 ± 2.5</td>
<td>11.5 ± 3.8</td>
<td>27.6 ± 7.6</td>
<td>17.3 ± 3.1</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.0 ± 0.0</td>
<td>1.0 ± 0.9</td>
<td>3.3 ± 0.7</td>
<td>2.4 ± 0.5</td>
<td>27.5 ± 5.8</td>
<td>11.2 ± 2.7^a</td>
</tr>
<tr>
<td>Macrophages</td>
<td>50.9 ± 4.1</td>
<td>34.1 ± 3.5</td>
<td>10.0 ± 2.2</td>
<td>5.5 ± 2.7</td>
<td>7.0 ± 4.2</td>
<td>4.7 ± 1.4</td>
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<tr>
<td><strong>Chemokines (pg/mL)</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KC</td>
<td>Nd</td>
<td>Nd</td>
<td>3881 ± 255</td>
<td>3175 ± 388</td>
<td>954 ± 320</td>
<td>1349 ± 307</td>
</tr>
<tr>
<td>MIP-2</td>
<td>Nd</td>
<td>Nd</td>
<td>954 ± 129</td>
<td>763 ± 106</td>
<td>167 ± 68</td>
<td>217 ± 49</td>
</tr>
<tr>
<td><strong>Plasma</strong> TNF-α (pg/mL)</td>
<td></td>
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<tr>
<td></td>
<td>7.4 ± 2.1</td>
<td>7.2 ± 0.8</td>
<td>5184 ± 836.5</td>
<td>2543 ± 370.9^b</td>
<td>39.2 ± 18.4</td>
<td>33.6 ± 7.4</td>
</tr>
<tr>
<td>TATc (µg/L)</td>
<td>5.8 ± 0.7</td>
<td>8.8 ± 1.6</td>
<td>29.5 ± 4.8</td>
<td>11.6 ± 1.4^a</td>
<td>13.6 ± 3.4</td>
<td>14.6 ± 1.9</td>
</tr>
</tbody>
</table>

**Note**: Data are means ± SEs of 4 (t = 0 h) to 12 mice (t = 2 and 6 h) mice/group at 0, 2 or 6 h after intraperitoneal injection of 250 µg lipopolysaccharide (LPS) from *Escherichia coli*. Wt, wild type; RAGE-/-, receptor for advanced glycation end products gene deficient; PLF, peritoneal lavage fluid; KC, cytokine-induced neutrophil chemoattractant; nd, not determined; MIP-2, macrophage inflammatory protein-2; TNF, tumor necrosis factor; TATc, thrombin-anti-thrombin complexes.

^a P < .05 vs. Wt mice; ^b P < .01 vs. Wt mice.
RAGE<sup>-/-</sup> mice demonstrate a diminished inflammatory response to *E. coli* LPS

We next investigated whether the exaggerated host response in RAGE<sup>-/-</sup> mice during *E. coli* peritonitis was the consequence of the higher bacterial loads in these animals or an inhibitory effect of RAGE. For this we injected Wt and RAGE<sup>-/-</sup> mice with *E. coli* LPS ip and harvested plasma and PLF 2 and 6 h later. RAGE<sup>-/-</sup> mice had two times lower neutrophil counts in their peritoneal fluid than Wt mice at 6 h after injection (table 3, *P* < .05); KC and MIP-2 levels did not differ in PLF of RAGE<sup>-/-</sup> and Wt mice (table 3). Plasma TNF-α levels were clearly diminished in RAGE<sup>-/-</sup> mice 2 h post LPS (table 3, *P* < .01), whereas MCP-1, IL-6 and IL-10 levels were similar in both genotypes (data not shown). Finally, at 2 h after LPS injection, the plasma concentrations of TATc were diminished in RAGE<sup>-/-</sup> mice (table 3, *P* < .05).

Anti-RAGE IgG enhances bacterial outgrowth and dissemination during *E. coli* peritonitis

To exclude the possibility that the results with live *E. coli* bacteria obtained in RAGE<sup>-/-</sup> mice were due to compensatory changes in these genetically modified animals unrelated to RAGE deficiency, we treated Wt mice with anti-RAGE IgG or control IgG antibodies and determined bacterial loads in various body compartments 20 h after induction of peritonitis. In strict accordance with the data generated in RAGE<sup>-/-</sup> mice, Wt mice with anti-RAGE IgG antibodies had more bacteria in their PLF and in distant body sites (data not shown). Furthermore, the numbers of total leukocytes, neutrophils, and macrophages in their PLF were similar in anti-RAGE and control antibody treated mice, whereas the local concentrations (in PLF) of the CXC chemokines KC and MIP-2 were higher in mice that had received anti-RAGE antibodies (data not shown), results that are in line with the data obtained in RAGE<sup>-/-</sup> mice.

Anti-RAGE IgG increased fibrin deposition during *E. coli* peritonitis

In line with the data obtained in RAGE<sup>-/-</sup> mice, livers of anti-RAGE IgG-treated mice showed more thrombi formation 20 h after infection (figure 3B, arrow, vs. figure 3A; figure 3C; *P* < .05 vs. control mice). These data were supported by fibrin(ogen) stainings (figure 3F vs. figure 3E). However, in contrast to the RAGE<sup>-/-</sup> mice, anti-RAGE IgG treatment was not associated with enhanced liver necrosis, hepatocellular injury (figure 3C-D) or TATc levels (figure 3G-H).
Figure 3. RAGE inhibition enhances hepatic fibrin(ogen) deposition during *Escherichia coli* (*E. coli*) induced sepsis. Mice were intraperitoneally injected with $5 \times 10^4$ cfu *E. coli* and treated with either anti-RAGE IgG antibodies (α-RAGE) or control IgG antibodies (control). Representative hematoxylin-eosin stainings of liver tissue at 20 h after injection in IgG control (A) and anti-RAGE IgG (B) treated mice. Original magnification x200. Arrow points out thrombi. Graphical representation of the degree of the liver thrombi and necrosis (C) determined according to the scoring system described in the Methods section. Plasma concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (D) ($n = 9-10$ mice/group). Representative fibrin(ogen) staining of liver tissue of control IgG (E) and anti-RAGE IgG (F) treated mice. Original magnification x20. Thrombin-anti-thrombin complex (TATc, G-H) concentrations were measured systemically (plasma, G) and locally (PLF, H). Data are means ± SEs. * $P < .05$ vs. control antibodies.
**Discussion**

Gram-negative peritonitis is a life-threatening condition frequently associated with systemic dissemination of bacteria and septic shock. Host defense in peritonitis is an established domain of the innate immune system as the rapid response to invading pathogens is essential for the host to survive. RAGE has the ability to activate signaling pathways leading to pro-inflammatory gene expression upon interaction with a range of distinct endogenous pro-inflammatory ligands. We here examined the *in vivo* role of RAGE during murine *E. coli* peritonitis, using RAGE−/− mice and anti-RAGE IgG. Our key finding was that RAGE contributes to an effective antibacterial host response during *E. coli* infection. Indeed, RAGE deficiency caused an enhanced outgrowth of *E. coli* at the primary site of infection together with increased spreading of bacteria to other body compartments and more severe liver injury.

Knowledge of the role of RAGE in host defense against bacterial infection is highly limited. RAGE−/− mice displayed a reduced mortality after induction of polymicrobial sepsis produced by CLP [17, 18]. Moreover, anti-RAGE therapy conferred a survival advantage even when administered 24 h after CLP in mice receiving antibiotic treatment [18]. In the latter investigation, RAGE deficiency or anti-RAGE therapy was reported not to influence bacterial loads in PLF, liver or spleen. It should be noted, however, that in this study all mice were treated with broad spectrum antibiotics and bacterial loads were only determined in mice that survived (i.e. not at predefined time points after CLP). Together with the fact that host defense against CLP at least in part relies on the extent of intestinal necrosis and the formation of a local abscess [19], the possible role of RAGE in antibacterial defense can not be easily determined from this earlier investigation [18]. Although our model does not resemble clinical abdominal sepsis as closely as CLP, it is a relevant tool to study the role of endogenous receptors and/or mediators in limiting the growth and dissemination of bacteria after a primary intraabdominal infection and to determine the contribution of these host proteins to specific immune responses [20, 21].

As such, we here provide evidence, using RAGE−/− mice and anti-RAGE IgG that RAGE signaling contributes to an effective antibacterial response. Most likely, RAGE exerts this effect indirectly and not via direct interaction with *E. coli*, considering that RAGE−/− leukocytes demonstrated an unaltered capacity to phagocytose and kill *E. coli* *in vitro* (data not shown). In addition, the observation that RAGE deficiency in general was associated with an exaggerated host response during *E. coli* sepsis and a reduced response to bolus *E. coli* LPS injection suggests that although RAGE is involved in the initiation of an immune reaction to *E. coli*, this function can be compensated for by other receptors in the presence of a growing bacterial load. Previously, *in vitro* data have shown that interaction of RAGE with its ligand HMGB1 can induce activation of intracellular signaling pathways [8, 9,
31] and thereby inflammation. Since HMGB1 has been reported to transduce cellular signals in vitro and in vivo by interacting with at least two other receptors, i.e. TLR2 and TLR4 [32-35], one possible explanation for the enhanced inflammation in the RAGE<sup>−/−</sup> mice could be that the absence of RAGE facilitates the interaction between HMGB1 and TLR2 and TLR4.

The current data do not necessarily contradict the previously described protective effect of RAGE deficiency with regard to CLP-induced mortality [17, 18]. The immune response to bacterial infection can act as a double-edged sword, on the one hand protecting the host against invading pathogens, on the other hand potentially damaging cells and tissues. It is conceivable that bacterial growth and dissemination do not impact significantly on the outcome of CLP-induced sepsis, in particular in the context of antibiotic therapy. Future research is warranted to investigate RAGE-mediated antibacterial activity against other pathogens associated with abdominal sepsis without the use of antibiotics. RAGE deficiency did not influence mortality in our <i>E. coli</i> model 14 out of 15 Wt mice and all 13 RAGE<sup>−/−</sup> mice died with the first lethality occurring 25 hours after infection in both groups; data not shown). In this respect it should be noted that the mortality curves after infection of previously healthy mice with this <i>E. coli</i> strain are very steep: whereas low doses do not cause lethality, doses that do cause lethality almost invariably do so. Hence, we consider this model less suitable to determine the impact on mortality and rather make use of it to study early host defense mechanisms.

Plasma IL-10 levels were 10-fold higher in the RAGE<sup>−/−</sup> mice, while other plasma cytokine levels doubled at most. Earlier, it has been established that the recovery of <i>E. coli</i> is diminished in IL-10<sup>−/−</sup> mice [36]. Similarly, treatment of mice with anti-IL-10 antibodies resulted in a lower bacterial outgrowth in a model of peritonitis induced by the intraperitoneal injection of the gram-negative bacterium <i>Klebsiella pneumoniae</i> [37]. In the latter study all mice received gentamicin. Together, these and our data suggest a (direct or indirect) association of high IL-10 levels with enhanced bacterial outgrowth during Gram-negative peritonitis. Of note, IL-10<sup>−/−</sup> mice demonstrated more organ damage during <i>E. coli</i> peritonitis despite an accelerated bacterial clearance [36]. In that report, anti-TNF-α partially attenuated neutrophil recruitment and multiple organ damage in the IL-10<sup>−/−</sup> mice. These results imply that although endogenous IL-10 facilitates the bacterial outgrowth during <i>E. coli</i> peritonitis, it protects mice from organ damage by a mechanism that involves inhibition of TNF-α release. In contrast to these IL-10<sup>−/−</sup> mice, our RAGE<sup>−/−</sup> mice show more severe organ (liver) damage and elevated TNF-α concentrations. Therefore, the role of elevated IL-10 in RAGE<sup>−/−</sup> mice during <i>E. coli</i> peritonitis remains to be elucidated.
RAGE deficiency resulted in enhanced organ injury with more necrosis of the liver in our model of *E. coli* induced sepsis. Sepsis is also associated with organ failure of the heart which contributes to hypotension, impaired perfusion and mortality [38]. The precise mechanism of this sepsis related myocardial dysfunction is unknown. Similar to some innate immune cells, cardiomyocytes are able to respond to “danger” signals with an innate immune inflammatory response [39, 40]. In addition, cardiomyocytes express multiple Toll-like receptors (TLRs) that signal predominantly through NF-κB when stimulated by pathogen-associated molecular patterns, leading to decreased cardiocyte contractility [41]. Boyd et al. demonstrated that RAGE coimmunoprecipitated with both S100A8 and S100A9 in hearts of mice injected with LPS [42]. Furthermore, it has been demonstrated that myocardial RAGE expression is upregulated in a model of ischemia/reperfusion in rats and that RAGE deficient mice are protected from ischemia/reperfusion injury of the heart [43, 44]. It remains to be established whether myocardial RAGE plays a role during *E. coli* sepsis. RAGE immunochemistry in mice has not been published before and unfortunately in our hands yielded unreliable results using multiple commercially antibodies.

In line with previous findings [20], *E. coli* peritonitis was associated with activation of the coagulation system. RAGE−/− mice displayed more activation of coagulation, as reflected by increased TATc and D-dimer concentrations in PLF and plasma and more fibrin deposition in the liver. Knowledge of the involvement of RAGE in activation of coagulation is limited. In a model of chronic vascular inflammation in diabetic apolipoprotein E deficient mice, administration of soluble RAGE for 6 weeks suppressed aortic levels of tissue factor [45], the main initiator of coagulation in sepsis in general [46] and in our model of abdominal sepsis in particular [20]. In addition, anti-RAGE IgG has been reported to inhibit tissue factor expression by monocytes stimulated with serum amyloid A in vitro [47]. Clearly, these previous data cannot be readily extrapolated to our model of severe acute bacterial infection. Our finding that RAGE−/− mice demonstrated lower plasma TATc levels upon bolus injection of LPS suggest that RAGE may play a role in acute activation of coagulation. The enhanced coagulation activation in mice with attenuated RAGE function during *E. coli* sepsis at least in part may have been caused by the higher bacterial loads in these animals. As such, our results provide evidence that although RAGE signaling may contribute to coagulation activation during acute infection, it is not essential for the procoagulant response during sepsis.

Although overall the effects of RAGE deficiency and anti-RAGE IgG on the host response to *E. coli* peritonitis were largely similar, some differences were observed. In this respect it should be noted that RAGE deficiency results in a complete absence of signaling via RAGE, whereas anti-RAGE IgG treatment most likely only partially prevents RAGE
signaling. Furthermore, these differences in effects could also be attributed to an additional deficiency of soluble RAGE in the RAGE deficient mice (in contrast to the anti-RAGE IgG treated mice that still can express soluble RAGE). In this respect it is important to note that most RAGE ligands (which can be bound by soluble RAGE) are promiscuous and are able to activate other cell-associated receptors besides RAGE [8-12, 48].

The present study is the first to document that intact RAGE signaling contributes to an effective antibacterial defense during abdominal sepsis caused by *E. coli*, thereby limiting the ensuing host systemic inflammatory and procoagulant response to infection. These data further illustrate the existence of a delicate balance between inflammation and anti-inflammation during severe bacterial infection where a certain degree of inflammation is required to combat invading pathogens and exaggerated inflammation can result in tissue injury.

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**REFERENCES**


