Enterococcus faecium infections: where bacterial virulence meets innate immunity
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TLR2-DEPENDENT MYD88 SIGNALING CONTRIBUTES TO EARLY HOST DEFENSE IN MURINE ENTEROCOCCUS FAECIUM PERITONITIS

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Abstract

The incidence of infections with Enterococcus faecium is increasing worldwide. TLRs have been implicated in the recognition of pathogens and the initiation of an adequate innate immune response. We here sought to determine the roles of MyD88, the common adaptor protein involved in TLR signaling, TLR2, TLR4 and CD14 in host defense against E. faecium peritonitis. MyD88 knockout (KO) mice demonstrated an impaired early response to E. faecium peritonitis, as reflected by higher bacterial loads in peritoneal fluid and liver accompanied by a markedly attenuated neutrophil influx into the abdominal cavity. In vitro, not only MyD88 KO macrophages but also TLR2 KO and CD14 KO macrophages displayed a reduced responsiveness to E. faecium. In accordance, transfection of TLR2 rendered human embryonic kidney 293 cells responsive to E. faecium, which was enhanced by cotransfection of CD14. TLR2 KO mice showed higher bacterial loads in peritoneal fluid after in vivo infection with E. faecium and a diminished influx of neutrophils, whereas CD14 KO mice had an unaltered host response. E. faecium phagocytosis and killing were not affected by MyD88, TLR2 or CD14 deficiency. TLR4 did not play a role in the immune response to E. faecium in vitro or in vivo. These data suggest that MyD88 contributes to the effective clearance of E. faecium during peritonitis at least in part via TLR2 and by facilitating neutrophil recruitment to the site of the infection.
Introduction

Enterococci are normal inhabitants of the human and animal gastrointestinal tract. Although considered as relatively harmless nosocomial pathogens, enterococci have emerged as the third leading cause of serious bloodstream infections (1). Additionally, they are increasingly found in urinary tract infections, endocarditis, wound infections and abdominal infections. The emergence of infections with enterococci can largely be attributed to their ability to acquire high-level drug resistance through horizontal gene transfer and their multiresistant nature to various classes of antibiotics. In particular Enterococcus faecium has adapted to the abundant antibiotic use in hospitals by acquiring resistance to high dose aminoglycosides, β-lactam antibiotics, and vancomycin (2-4). Historically, 80 to 90% of clinical isolates of enterococci were Enterococcus faecalis, whereas E. faecium accounted for 5-10% (5). Currently, though, proportions of E. faecium isolates among all enterococcal infections is steadily increasing and exceeds 30% (6). These E. faecium isolates predominantly belong to a distinct genetic subpopulation, called clonal complex-17 (CC17), that has adapted extremely well to the hospital environment and that has spread world wide (7). CC17 is characterized by the acquisition of multiple adaptive mechanisms including ampicillin and quinolone resistance and a putative pathogenicity island harboring the esp virulence gene (8-10).

The innate immune system provides the first line of defense against invading pathogens by recognizing so called pathogen-associated molecular patterns (PAMPs). PAMPs are essential, conserved microbial patterns that are shared by large groups of pathogens and are not expressed by higher eukaryotes (11, 12). PAMPs are recognized by pattern recognition receptors, among which the family of Toll-like receptors (TLRs) prominently features. TLRs play an eminent role in the detection of microorganisms and the induction of an adequate immune response (13, 14). So far, 10 human TLRs have been described. TLR2 has been identified as a receptor that is central to the innate immune response to several Gram-positive bacteria and for cellular signaling by components of Gram-positive bacteria such as peptidoglycan, lipoteichoic acid (LTA) and lipoproteins (15-20). TLR4 recognizes lipopolysaccharide (LPS) and is therefore important in the immune response to Gram-negative bacteria (21, 22). Another pattern recognition receptor, CD14, is a glycosylphophatidylinositol surface-anchored molecule expressed by many cells. This receptor lacks an intracellular segment and functions as a coreceptor for numerous bacterial products, including peptidoglycan, LPS, and bacterial lipoproteins, thereby facilitating signaling through other receptors (23). During Gram-negative infection CD14
Chapter 2

play an important role in the induction of a protective immune response by its capacity to present LPS to the TLR4/MD2 signaling complex (13, 14, 24-27, 28). CD14 has been shown to be coexpressed and to form a complex with transmembrane TLR2 and to facilitate LTA signaling through TLR2 (23, 29, 30), yet, knowledge of the role of this receptor in host defense against Gram-positive bacteria is limited. Importantly, signaling of all known TLRs except TLR3 relies on the intracellular adaptor MyD88. Additionally, MyD88 serves as the essential adaptor for other IL-1/TLR family members, including the type I IL-1R and IL-18R. Activation of the intracellular pathway through MyD88 results in the activation of NF-κB and expression of proinflammatory mediators such as cytokines and chemokines (31, 32).

Very little is known about host defense mechanisms during infection with E. faecium. Such knowledge is important considering the increase in antibiotic resistance and clinical relevance of multiresistant E. faecium. Therefore, we here sought to investigate the roles of TLR2, TLR4, CD14 and MyD88 in the immune response to E. faecium using a newly developed model of peritonitis with this opportunistic pathogen.

Materials and Methods

Mice
Specific pathogen-free 10-wk-old female wild-type (WT) C57BL/6 mice were purchased from Harlan Sprague-Dawley. Tlr2 gene-deficient (TLR2 KO), TLR4 KO and MyD88 KO mice, backcrossed to a C57BL/6 background at least six times, were generated as described previously (22, 32, 33). CD14 KO mice, backcrossed to C57BL/6 background, were obtained from the Jackson Laboratories. All mice were bred in the animal facility of the Academic Medical Center (Amsterdam, the Netherlands). Age- and sex-matched C57BL/6 mice were used as controls in all experiments. The animals were housed in rooms with a controlled temperature and a 12-h light-dark cycle. They were acclimatized for 1 wk before usage, and received standard rodent chow and water ad libitum. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Bacterial strain
A vancomycin resistant E. faecium strain, E155, was used in all experiments. This clinical isolate from the Cook County Hospital (Chicago, IL), belongs to CC17. For all experiments, the bacteria were grown overnight on agar sheep blood plates and then grown for ~3.5 hours in Todd-Hewitt broth (Difco) to midlogarithmic phase at 37°C, while shaking.
Induction of peritonitis

*E. faecium* was cultured in Todd Hewitt broth at 37°C, harvested at mid-log phase, and washed twice in sterile saline to clear the bacteria of medium. Bacteria were then resuspended in sterile isotonic saline and mice were injected i.p. with ~10⁷, 10⁸, 10⁹ and 10¹⁰ CFU of *E. faecium* in 200 μl sterile isotonic saline. The inoculum was plated immediately after inoculation on sheep blood agar plates to determine viable counts.

Collection of samples

For comparison of bacterial outgrowth and to examine host responses in the experimental groups, mice were killed at different time points. To set up the *E. faecium* peritonitis model, mice were sacrificed 2, 6, 24 and 48 h after infection. In the other experiments, mice were killed at indicated time points. Mice were anesthetized by inhalation of isoflurane (Abbot, Laboratories)/O₂ (2%/ 2 liter) and a peritoneal lavage was performed with 5 ml of sterile PBS using a 18-gauge needle; the peritoneal lavage fluid was collected in sterile polypropylene tubes (Plastipack; BD Biosciences). After collection of peritoneal fluid, blood was drawn by cardiac puncture, with a sterile syringe, transferred to heparin-gel vacutainer tubes and immediately placed on ice. Next, the abdomen was opened and the liver, spleen and lungs were harvested.

Determination of bacterial outgrowth

The number of *E. faecium* CFU was determined in peritoneal lavage fluid, blood, liver and lung homogenates. To correct for the difference in organ weight, four times the weight (in milligrams) in microliters of sterile saline was added. The organs were homogenized at 4°C with a tissue homogenizer (Biospect Products), which was carefully cleaned and disinfected with 70% ethanol after each homogenization. Next, serial 10-fold dilutions were made of each sample in sterile saline and 50 μl of each dilution was plated onto blood agar plates. The plates were incubated at 37°C under 5% CO₂, and CFU were counted after 20 h and corrected for the dilution factor.

Cell counts and differentials

Total cell numbers were counted from each peritoneal lavage sample using a hemocytometer (Beckman Coulter). Differential cell counts were performed on cytospin preparations, stained with a modified Giemsa stain (Diff-Quick; Dade Behring). Peritoneal fluid supernatant was stored at -20°C until determination of cytokines.
Chapter 2

Histology
Directly after sacrifice, lung, liver and spleen were fixed in 4% formalin, and embedded in paraffin for routine histology. Sections of 4-μm thickness were stained with H&E. All slides were coded and scored by a pathologist without knowledge of the type of mice or treatment.

Assays
Macrophage inflammatory protein (MIP)-2, cytokine-induced neutrophil chemoattractant (KC) and LPS-induced C-X-C chemokine (LIX) were measured in peritoneal lavage fluid by ELISA’s (R&D Systems) according to the manufacturers’ instructions. TNF-α, IL-6, IL-10, IL-12p70 and MCP-1 were measured in peritoneal lavage fluid and plasma by using a commercially available cytometric bead array multiplex assay (BD Biosciences) in accordance with the manufacturer’s recommendations. Aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and creatinin were determined with commercially available kits (Sigma-Aldrich), using a Hittachi analyzer (Boehringer Mannheim).

Preparation of peritoneal macrophages
Peritoneal macrophages from untreated TLR2 KO, TLR4 KO, CD14 KO, MyD88 KO, and WT mice (n = 5–8/strain) were harvested by peritoneal lavage with 5 ml sterile PBS as described (34, 35). Total cell numbers were counted from each sample using a hemocytometer. Then, the cells were washed and resuspended in medium (RPMI 1640 containing 10% FCS, 1 mM pyruvate, 2 mM L-glutamine, penicillin, and streptomycin). Subsequently, cells were incubated in 96-well flat-bottom microtiter plates (Greiner Bioscience) (1 x 10^5 cells in 100 μl/well) for 2 h at 37°C, 5% CO₂, and then washed with medium to remove nonadherent cells. Next, the adherent monolayer cells were stimulated with LPS (from Escherichia coli O55:B5; 500 ng/ml; Sigma-Aldrich), LTA (from Staphylococcus aureus, 40 μg/ml, kind gift of Dr. T. Hartung, Univ. Konstanz, Germany (36)), living E. faecium (5 x 10^6 CFU/ml), or RPMI 1640 for 6h. MyD88 KO and CD14 KO cells were stimulated with mitomycin C (0.2 mg/ml; Sigma-Aldrich) treated (growth arrested) E. faecium (2.5 x 10^6 CFU/ml) for 20 hours. Supernatants were collected and stored at −20°C until assayed. Blood was drawn from the same mice and collected in heparin-gel vacutainer tubes, 50 μl/sample was divided in 96-well plates (Greiner). Stimulations were conducted as described for the macrophages.
Phagocytosis assays

Phagocytosis was evaluated in essence as described previously (34, 37). Peritoneal lavage was performed in WT, TLR2 KO, MyD88 KO and CD14 KO mice (n=5 per strain) using 5 ml of sterile PBS and blood was collected; macrophages were washed, counted, and resuspended in RPMI 1640 at a final concentration of 5x10^5 cells/ml. Cells were allowed to adhere in 48-well microtiter plates (Greiner) overnight. Mitomycin C (0.2 mg/ml) was used to inhibit bacterial growth before labeling the bacteria with CFSE dye (Invitrogen, Life Technologies). Macrophages were incubated with E. faecium (2.5x10^7 CFU/ml) for 10 and 60 minutes. Phagocytosis was stopped by placing cells on ice, then cells were washed in PBS and suspended in Quenching solution (Orpegen). To determine the neutrophil phagocytosis capacity, 50 μl of whole blood was incubated with CFSE-labeled, growth-arrested bacteria (1x10^7 CFU/ml) and incubated for 10 or 60 minutes at 37°C. Cells were suspended in Quenching solution, incubated in FACS lysis/fix solution (BD Biosciences) and neutrophils were labeled using anti-Gr-1-PE (BD Pharmingen), using concentrations as recommended by the manufacturer. Cells were washed with ice-cold FACS buffer after which the degree of phagocytosis of neutrophils and macrophages was determined using the FACS Calibur (BD Biosciences).

Bacterial killing assay

Bacterial killing was determined as described previously (38). In brief, peritoneal macrophages were harvested from WT, TLR2 KO, MyD88 KO and CD14 KO mice and plated as described above (n=5 per strain). E. faecium was added at a multiplicity of infection of 50 and spun onto cells at 2000 rpm for 5 min, after which plates were placed at 37°C for 10 min. Each well was then washed 5 times with ice-cold PBS to remove extracellular bacteria. To determine bacterial uptake after 10 min, cells were lysed with sterile dH2O and designated as t=0. RPMI 1640 was added to remaining wells and plates were placed at 37°C for 10 and 60 min after which cells were again washed 5 times with ice-cold PBS and lysed with dH2O. Cell lysates were plated on blood agar plates and bacterial counts were enumerated after 16h. Bacterial killing was expressed as the percentage of killed bacteria in relation to t=0.

Human embryonic kidney (HEK) cells

To establish the responsiveness of TLR2 and TLR4, in the presence or absence of CD14, to E. faecium, stable cell lines of HEK 293 cells expressing TLR2, TLR4/MD-2, and/or CD14 were used. These cells were provided by Dr. D. Golenbock (University of Massachusetts Medical School, Worcester, MA) (39). The cells were maintained in DMEM supplemented...
with 10% FCS, 2 mM L-glutamine, penicillin, and streptomycin. Cells were trypsinized and seeded into 96-well plates at a density of \(1 \times 10^5\) cells/well. The cells were stimulated the following day with \(10^6\) CFU/ml viable \(E.\) \(faecium\), LTA (10 \(\mu\)g/ml) or LPS (100 ng/ml). TLR4 requires MD-2, a small glycosylated protein, to optimally sense LPS. We therefore used a HEK cell line expressing soluble MD2; supernatant of this cell line was used for HEK-TLR4 stimulation. IL-8 release was used as read-out and measured by using a commercially available ELISA (Bio-source International). Cells were stimulated for 5 h and each stimulation was performed in quadruplicate.

**Statistical analysis**

All data are expressed as mean ± SEM. Difference between groups were analyzed with Mann-Whitney \(U\) test or Kruskal-Wallis analysis with Dunn post test where appropriate. These analysis were performed using Graph-Pad Prism version 4 (GraphPad Software). Values of \(p < 0.05\) were considered statistically significant.

**Results**

**E. faecium peritonitis mouse model**

To investigate host defense mechanisms in \(E.\) \(faecium\) peritonitis in vivo, we developed a suitable mouse model, in which healthy mice are able to generate an effective immune response and clear the enterococci. Such a model would allow the evaluation of natural host defense pathways. A clinical vancomycin resistant \(E.\) \(faecium\) isolate, E155, was selected for this purpose. Mice were inoculated i.p. with \(1 \times 10^7, 10^8, 10^9,\) or \(10^{10}\) CFU \(E.\) \(faecium\) \((n = 5/group)\) and observed for 48 h. One day after infection 4 of 5 mice infected with \(10^{10}\) CFU had died, and 2 mice infected with \(10^9\) CFU were severely ill; these mice died within the next hours. The other mice from this group were ill, but survived the 48 h. Mice that were injected with either \(10^7\) or \(10^8\) CFU did not show any signs of illness during the entire experiment. After 48 h the surviving mice were sacrificed. From the 3 surviving mice that were infected with \(10^9\) CFU, enterococci were isolated from all cultured organs: blood, peritoneal wash fluid, liver and lungs. From the other mice, enterococci could no longer be cultured.

Insights into the kinetics of the clearance of enterococci and in the accompanying immune response were obtained by infecting two groups of mice with either \(10^7\) or \(10^9\) CFU \(E.\) \(faecium\) and sacrificing 5 mice per group 2, 6, 24 (on two occasions) and 48 h after infection. In accordance with our first set of experiments, of mice infected with
10⁹ CFU i mouse died after 24 h and 2 mice died between 24 en 48 h after enterococcal injection. The remaining mice were severely ill; these mice did not clear the infection and remained bacteremic throughout (Fig. 1). In contrast, mice infected with 10⁸ CFU were able to gradually eliminate the enterococci during this period (Fig. 1). In these animals, a rapid increase in the number of peritoneal cells was seen; the early increase was mainly caused by a strong influx of neutrophils, which peaked 6 h after E. faecium infection (Table I). After an initial decrease, the number of macrophages increased in peritoneal fluid from 24 h postinfection onwards (Table I). Both macrophages and neutrophils were observed with phagocytosed enterococci (data not shown). Peritoneal cells harvested from mice infected with 10⁹ CFU were counted and differentiated as well, yet these cells appeared lysed and no reliable conclusions could be drawn from these results. E. faecium peritonitis, induced by either 10⁸ or 10⁹ CFU, was not associated with a strong induction of cytokines or chemokines. Modestly elevated levels of TNF-α, IL-6, IL-12p70, IL-10, MCP-1, KC, and MIP-2 were detected in blood and peritoneal fluid between 2 and 6 h after infection (data not shown; for the values of 10⁹ CFU i.p. see experiments described below). In accordance with the mild proinflammatory response and the clearance of the bacteria, the bacterial challenge of 10⁸ CFU did not result in histopathological changes in liver, lung and spleen, and clinical chemistry analyses did not show evidence for liver injury (ALAT, ASAT) or renal failure (creatinin) (data not shown). In accordance with the increased signs of illness in mice injected with 10⁹ CFU i.p., histopathological changes were seen in lung and liver that increased in time. In lungs mild pleuritis, interstitial inflammation and edema were seen. Twenty-four hours after the infection, inflammation was seen with necrosis and some thrombi in liver (data not shown).

Table I. Leukocyte counts and differentials in peritoneal fluid after intraperitoneal injection of 10⁹ CFU E. faecium.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Total cells (x10⁴/ml)</th>
<th>Neutrophils</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>69.3 ± 7.1</td>
<td>0.9 ± 0.4</td>
<td>60.4 ± 7.0</td>
<td>7.3 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>81.6 ± 11.7</td>
<td>59.9 ± 8.1 *</td>
<td>21.2 ± 5.1</td>
<td>0.5 ± 0.2 *</td>
</tr>
<tr>
<td>6</td>
<td>201.6 ± 17.8 *</td>
<td>179.2 ± 14.6***</td>
<td>21.5 ± 4.6</td>
<td>1.9 ± 1.0</td>
</tr>
<tr>
<td>24</td>
<td>226.4 ± 67.4 *</td>
<td>79.3 ± 17.0 *</td>
<td>1271 ± 42.5</td>
<td>6.5 ± 2.2</td>
</tr>
<tr>
<td>48</td>
<td>181.8 ± 22.4</td>
<td>55.0 ± 13.5</td>
<td>108.7 ± 16.8</td>
<td>8.8 ± 1.6</td>
</tr>
</tbody>
</table>

Mice received an i.p. injection with E. faecium (10⁹ CFU) at t = 0 h. Data are mean ± SE of 5 mice per time point. Data at 0 h were obtained in uninfected mice. *, p < 0.05 vs t = 0; †, p < 0.05 vs t = 2.
Figure 1. *E. faecium* outgrowth in peritoneal fluid (A), blood (B), liver (C) and lung (D). Mice were injected with either $10^8$ or $10^9$ CFU i.p. (n = 5/time point). Intraperitoneal injection resulted in systemic infection; enterococci were isolated from all cultured organs. Mice were able to clear a high inoculum of $10^8$ CFU, whereas $\geq 30\%$ mortality was seen in 48 h after injecting $10^9$ CFU. Data are means ± SEM.

**MyD88 deficiency results in an impaired early immune response to *E. faecium***

Having established a murine model of *E. faecium* peritonitis, we next sought to determine the role of TLR signaling in the observed immune response. Considering that MyD88 is the common adaptor protein for all TLRs (except TLR3), IL-1R and IL-18R, we first infected MyD88 KO and WT mice with $5 \times 10^7$ CFU *E. faecium* and compared bacterial loads in different organs and the associated immune response at 2 and 24 h after infection. These time points, based on our experiments described above, were considered most appropriate to investigate the early immune response (2 h postinfection) and the impact on the clearance of *E. faecium* (2 and 24 h postinfection). An inoculum of $\sim 10^8$ CFU was chosen because WT mice are able to clear this high inoculum in $\sim 48$ h and because this infectious dose caused a measurable inflammatory response (see above). MyD88 KO mice
displayed a diminished early host response when compared with WT mice. In particular, at 2 h after infection, MyD88 KO mice had almost 1 log more *E. faecium* at the primary site of infection (peritoneal lavage fluid) and the liver (Fig. 2A and C, *p* < 0.05 vs WT mice); at 24 h MyD88 KO mice still had modestly higher bacterial loads in peritoneal fluid but the difference with WT mice was not statistically significant anymore. Bacterial loads did not differ between MyD88 KO and WT mice at other body sites at 2 h postinfection; at 24 h more enterococci were recovered from blood (*p* = 0.05) and lungs (*p* < 0.05) of MyD88 KO mice. These data suggest that the early defense of MyD88 KO mice is impaired at the primary site of infection, which leads to a modestly increased dissemination later on.

To obtain insight into the mechanisms underlying the elevated bacterial loads in the peritoneal cavity of MyD88 KO mice early after infection, we determined the number of leukocytes recruited to the site of infection. At both 2 and 24 h after infection MyD88 KO mice demonstrated a diminished number of neutrophils in their peritoneal lavage fluid (Fig. 3A, *p* < 0.01 vs WT mice). This impaired response was accompanied by lower concentrations of the CXC chemokine LIX in the peritoneal cavity, whereas KC and MIP-2 levels did not differ between groups; at 24 h the concentrations of these three chemokines were below the detection limit of the assays in both mouse strains (Fig. 3B-3D). Similarly, the early (2 h) cytokine response was indistinguishable between MyD88 and WT mice: TNF-α, IL-6, IL-10 and MCP-1 concentrations did not differ in either peritoneal lavage fluid or plasma (Table II). At later time points, the levels of these mediators were either very low or undetectable (data not shown).

Together these data suggest that MyD88 deficiency results in an impairment of the early immune response to *E. faecium* peritonitis primarily due to a reduced recruitment of neutrophils to the site of the infection.

### Table II. Concentration (pg/ml) of cytokines in WT and MyD88 KO mice in plasma and peritoneal fluid.

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Peritoneal concentrations</th>
<th>Plasma concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>MyD88 KO</td>
</tr>
<tr>
<td>TNF-α</td>
<td>60.4 ± 25.3</td>
<td>61.2 ± 21.3</td>
</tr>
<tr>
<td>IL-6</td>
<td>301.2 ± 71.9</td>
<td>290.7 ± 78.9</td>
</tr>
<tr>
<td>IL-10</td>
<td>22.7 ± 6.5</td>
<td>23 ± 6.3</td>
</tr>
<tr>
<td>MCP-1</td>
<td>146.0 ± 31.9</td>
<td>176.9 ± 67.9</td>
</tr>
</tbody>
</table>

Mice were i.p. infected with 5 x 10⁷ CFU *E. faecium* and sacrificed 2 h thereafter. Data are means ± SEM (n = 7-8 mice/group).
Figure 2. MyD88 KO mice show an impaired early host defense during *E. faecium* peritonitis. MyD88 KO (open bars) and WT mice (closed bars) were i.p. infected with $5 \times 10^7$ CFU *E. faecium* and killed 2 and 24 h thereafter (6-7 mice/group). Two hours after infection MyD88 KO mice showed higher bacterial loads in peritoneal fluid and liver. Twenty-four hours after the infection, bacterial loads were similar in MyD88 KO and WT mice, except for in lungs. Peritoneal fluid (A), blood (B), liver (C) and lung (D). Results are expressed as mean ± SEM. *, $p < 0.05$. 
Figure 3. MyD88 KO mice show reduced peritoneal neutrophil recruitment. Neutrophil numbers in PLF (A) at 2 and 24 h after infection of MyD88 KO (open bars) and WT mice (closed bars) with 5 x 10^7 CFU *E. faecium*. The reduced influx of neutrophils in MyD88 KO mice was accompanied by reduced levels of CXC chemokine LIX in MyD88 KO mice (B), but not with reduced KC or MIP-2 levels (C and D). Data are means ± SEM (n = 6-8 mice per group at each time point). **, p < 0.01; ***, p < 0.0001.

*E. faecium* stimulates peritoneal macrophages via TLR2

To determine the contribution of TLR2, TLR4 and CD14 we assessed the responsiveness of peritoneal macrophages recovered from TLR2, TLR4, CD14 KO and WT mice toward *E. faecium*, and compared these with the responsiveness of peritoneal macrophages recovered from MyD88 KO mice (Fig. 4). In contrast to the modest *in vivo* cytokine response to *E. faecium*, WT macrophages demonstrated strong cytokine release upon stimulation with this bacterium *in vitro* as shown for TNF-α. MyD88 KO, TLR2 KO and CD14 KO macrophages all showed a strongly reduced TNF-α release upon incubation with *E. faecium* (Fig. 4). TLR4 KO macrophages tended to release less TNF-α, but the difference with WT cells was not significant. Additionally, we performed stimulations on whole blood drawn from the aforementioned mice. Comparable results were found for these blood cell stimulations (data not shown). In control experiments and as expected, TLR2 KO macrophages responded normally to LPS but not to LTA, whereas TLR4 KO macrophages responded normally to LTA but not to LPS (data not shown).
To acquire further insight into the role of the TLR family in the host response to *E. faecium* we tested HEK 293 cells expressing TLR2, TLR4, and/or CD14. Cells were incubated with living *E. faecium*, LTA and LPS were used as positive controls for, respectively, TLR2 and TLR4. Cell activation was determined by IL-8 levels in the supernatant. HEK-TLR2, but not HEK-TLR4 cells released IL-8 upon stimulation with *E. faecium*, a response that was significantly enhanced in cells that also expressed CD14 (Fig. 5). As expected, LTA activated HEK-TLR2 but not HEK-TLR4 cells, whereas LPS activated HEK-TLR4, but not HEK-TLR2 cells (data not shown). Together these data indicate that TLR2 recognizes *E. faecium* in vitro, most likely at least in part by an interaction with CD14.

Figure 4. MyD88 KO, TLR2 KO and CD14 KO macrophages are less responsive to *E. faecium*. (A) Peritoneal macrophages were harvested from WT, TLR2 KO and TLR4 KO mice and incubated with living *E. faecium* (5 x 10^6 CFU/ml) for 6 h. (B) In another experiment, peritoneal macrophages were harvested from WT, CD14 KO and MyD88 KO mice, and stimulated with growth arrested *E. faecium* (2.5 x 10^6 CFU/ml) for 20 h. Data are means ± SEM (n = 6-8/group). *, p < 0.05; **, p < 0.01; ***, p < 0.0001.

Figure 5. TLR2 expressed by HEK 293 cells recognizes *E. faecium*, while TLR4 does not. HEK 293 cells expressing no TLR, TLR2 and/or CD14, or TLR4 and/or CD14 were stimulated for 5 h with living *E. faecium* (10^6 CFU/ml). TLR2-expressing cells produced significantly more IL-8 compared to control cells and this response was enhanced by the concurrent expression of CD14. No IL-8 could be measured after incubation of TLR4-expressing cells with *E. faecium*. Data are means ± SEM (n = 4). *, p < 0.05.
TLR2 deficiency results in an impaired early immune response to *E. faecium*

To further investigate the *in vivo* role of TLR2 and CD14, TLR2KO and CD14KO mice were infected i.p. with 8x10⁷ CFU *E. faecium*. In accordance to the MyD88 KO mice, TLR2 KO mice displayed a diminished early host response when compared with WT mice. At 2 h after infection, TLR2 KO mice had more *E. faecium* at the primary site of infection (peritoneal lavage fluid) (Fig. 6A, *p < 0.05* vs WT mice); no differences in bacterial loads were seen in other organs. Furthermore, at later time points no differences in bacterial loads were detected in any body site. As was seen in MyD88 KO mice, TLR2 KO mice showed a diminished peritoneal neutrophil influx during the entire experiment (Fig. 7A, *p < 0.01* vs WT mice). This reduced neutrophil recruitment was accompanied by lower LIX and KC levels in peritoneal fluid 2 h after the infection, whereas MIP-2 levels were not different between the two mouse strains. Slightly diminished IL-6, IL-10 and MCP-1 levels were seen in TLR2 KO mice 2 h after the infection (significantly so for plasma IL-6 and MCP-1, and peritoneal fluid IL-10, Table III). In contrast to the *in vitro* studies described above, TNF-α levels were similar in both mouse strains. Twenty-four hours after the infection, the levels of these mediators were either very low or undetectable (data not shown). Despite of the clear role for CD14 in cellular responsiveness to *E. faecium* *in vitro*, CD14 KO mice displayed a host defense response to *E. faecium* infection *in vivo* that was indistinguishable from that in WT mice; i.e., bacterial loads, neutrophil recruitment into the peritoneal cavity and local and systemic cytokine concentrations were similar in CD14 KO and WT mice (data not shown). Finally, TLR4 KO mice were also indistinguishable from WT mice with regard to clearance of enterococci and host immune responses in this model of *E. faecium* peritonitis (data not shown).

Table III. Concentration (pg/ml) of cytokines in WT and TLR2 KO mice in plasma and peritoneal fluid.

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Peritoneal concentrations</th>
<th>Plasma concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>TLR2 KO</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1673± 61.9</td>
<td>234.6± 84.0</td>
</tr>
<tr>
<td>IL-6</td>
<td>1329 ± 426.9</td>
<td>677.4 ± 148.0</td>
</tr>
<tr>
<td>IL-10</td>
<td>83.4 ± 17.8</td>
<td>34.48 ± 2.1 *</td>
</tr>
<tr>
<td>MCP-1</td>
<td>710.1 ± 103.2</td>
<td>600.9 ± 42.6</td>
</tr>
</tbody>
</table>

Mice were i.p. infected with 8 x 10⁷ CFU *E. faecium* and sacrificed 2 h thereafter. Data are means ± SEM (*n = 8 mice/group*). *, *p < 0.05* vs. WT.
Figure 6. TLR2 KO mice demonstrate an impaired early host defense during *E. faecium* peritonitis. TLR2 KO (open bars) and WT mice (closed bars) were i.p. injected with $8 \times 10^7$ CFU *E. faecium* (8 mice/group) and sacrificed at given time points. TLR2 KO mice showed more enterococci in peritoneal fluid compared to WT mice, 2 h after infection.

At later time points WT and TLR2 KO mice demonstrated comparable *E. faecium* loads. The early delay in peritoneal enterococcal clearance was not seen in other cultured organs. Peritoneal fluid (A), blood (B), liver (C) and lung (D).

Data are means ± SEM. *, $p < 0.05$. 

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Figure 7. TLR2 KO mice show reduced peritoneal neutrophil recruitment. Neutrophil numbers in PLF of TLR2 KO (open bars) and WT mice (closed bars) at 2 and 24 h after infection with $8 \times 10^7$ CFU *E. faecium* (A). In parallel with the reduced neutrophil influx, peritoneal fluid LIX and KC levels were reduced in TLR2 KO mice at 2 h postinfection (B and C), whereas MIP-2 levels (C) were similar in both mouse strains. Data are means ± SEM (n = 8 mice/group at each time point). **, $p < 0.01$; ***, $p < 0.0001$.

**No role for MyD88 or TLR2 in phagocytosis or killing of *E. faecium***

In contrast to the reduced antibacterial defense in MyD88 and TLR2 KO mice, as described above, MyD88 and/or TLR2, as well as CD14, did not seem to contribute to phagocytosis and/or killing of *E. faecium*. As shown in Fig. 8, MyD88 KO, TLR2 KO and CD14 KO cells demonstrated an unaltered capacity to phagocytose and kill *E. faecium*.
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Figure B. No difference in *E. faecium* phagocytosis or killing capacity of MyD88 KO, TLR2 KO or CD14 KO cells. (A) Phagocytosis capacity of peripheral blood neutrophils are expressed as mean fluorescence intensity (MFI) per neutrophil (representing the number of phagocytosed CFSE-*E. faecium*), mean MFI ± SEM. (B) Killing capacity of peritoneal macrophages are shown as percentage of killed *E. faecium* compared with t = 0 (n = 5/mouse strain)

Discussion

Enterococci are increasingly causing nosocomial infections worldwide, which is mainly attributed to a rise in multiresistant *E. faecium* strains, specifically adapted to the hospital environment en belonging to CC17 (4, 5). Especially severely ill, immunocompromised patients are vulnerable to infections with enterococci. In contrast, in healthy human individuals, *E. faecium* only very rarely causes infections. Knowledge of how the intact innate immune system deals with this bacterium is highly limited. We here investigated the interaction between the host immune system and *E. faecium* focusing on the role of members of the TLR family. As such, the primary objective of the current studies was to answer the question whether intact TLR signaling is the reason why a healthy host is not susceptible to *E. faecium* infection. Using a newly developed mouse model of *E. faecium* peritonitis, we show that MyD88, the common adaptor protein for TLR signaling, contributes to an effective clearance of *E. faecium* during the early phase after infection, most likely by stimulating the recruitment of neutrophils to the primary site of infection. The protective effect of MyD88 appeared to be mediated at least in part by TLR2, because deficiency of this receptor resulted in a similar, albeit less strong phenotype early after i.p. infection with *E. faecium*, characterized by an initially higher bacterial load in peritoneal fluid and a diminished neutrophil influx. We could not detect a role for either TLR4 or CD14 in host defense against *E. faecium* peritonitis. Our data are the first to investigate the role of TLRs in the immune response to this emerging pathogen.
To enable studies on the innate immune response to *E. faecium* peritonitis we first set up a model in which a peritoneal infection was induced in healthy WT mice from which they were able to recover. To induce peritonitis we used a clinically isolated strain of *E. faecium* belonging to CC17. Injecting $10^9$ CFU *E. faecium* caused lethality in some mice, whereas $10^8$ CFU caused a systemic infection that was eventually cleared. In this model an early and strong peritoneal influx of neutrophils was seen, which, however, was not accompanied by a profound cytokine or chemokine response; even in the model using $10^9$ CFU *E. faecium* the release of cytokines and chemokines remained relatively modest. These data are in accordance with the low cytokine responses during *E. faecalis* peritonitis described by Papassin et al. (40). Indeed, after i.p. injection of *E. faecalis* only low level localized TNF-$\alpha$ responses were measured in the absence of detectable plasma levels, even in mice that died following infection. Similar to the findings of Papasin et al. (40) for *E. faecalis*, we found that *E. faecium* is able to induce a rapid and robust TNF-$\alpha$ and IL-6 release by peripheral blood cells and peritoneal macrophages upon stimulation *in vitro*, indicating that the weak TNF-$\alpha$ response during either *E. faecium* or *E. faecalis* infection is not attributable to an inability of the organisms to activate immune cells. *E. faecium* peritonitis was not accompanied by significant organ damage as indicated by histopathology of lung, liver and spleen and the plasma levels of ASAT, ALAT and creatinin. Of note, we specifically chose to validate the model in healthy WT mice in order to allow subsequent studies in mice lacking components of the innate immune response. In this regard, the absence of mortality and distant organ injury is in line with the fact that the intact immune system can mount an effective response against this intrinsically modestly virulent microorganism.

TLRs are regarded the major molecular mechanism by which the host recognizes invading microorganisms (21, 41, 42). Signaling by all TLRs, except TLR3, relies on MyD88, the common intracellular TLR adaptor protein. To obtain a first insight into a possible role of TLR family members in defense against *E. faecium* peritonitis, we compared bacterial loads and host responses in MyD88 KO and WT mice. MyD88 deficiency resulted in a diminished clearance of the bacteria early in the infection which was accompanied and probably caused by a reduced migration of neutrophils to the peritoneal cavity, the primary site of infection. No difference in macrophage composition was seen between the two groups. The diminished neutrophil recruitment was accompanied by a reduced level of CXC chemokine LIX only. No other demonstrable changes in the cytokine or chemokine responses were seen, probably due to the already modest response in normal mice. A recent report by Jeyaseelan et al. (43) on the role of Toll/IL-1R domain-containing adaptor
inducing IFN-β-mediated signaling during *E. coli* pneumonia, showed comparable results for the CXC chemokines LIX, KC, and MIP-2 and neutrophil influx. They found reduced neutrophil numbers in the pulmonary compartment that was accompanied by reduced levels of LIX, but no differences in KC and MIP-2 levels. Additionally, *Pseudomonas aeruginosa* pneumonia in TRIF-deficient mice resulted in lower amounts of pulmonary neutrophils and lower levels of KC compared with WT mice, but comparable levels of MIP-2 (44). Although LIX has been identified as the most potent murine neutrophil attracting chemokine (45), it is questionable whether this difference in LIX response is the sole explanation for the reduced peritoneal neutrophil influx in the MyD88 KO mice in our model. Remarkably, in our study, the loss of TLR signaling was compensated for as 24 h after the initial infection MyD88 KO mice had started to clear the infection and displayed largely similar bacterial loads as WT mice at different body sites. This indicates that although the TLR family contributes to the innate immune response to *E. faecium* peritonitis, it is not indispensable. This is of interest since MyD88 KO blood leukocytes and peritoneal macrophages failed to respond to *E. faecium* in vitro. These results should be viewed upon in the context of several previous studies that investigated the role of MyD88 in host defense against infection, which have yielded rather different outcomes. For example, MyD88 KO mice failed to mount an effective defense against intrapulmonary challenge with *P. aeruginosa* but controlled lung infection with *S. aureus*, although in both models MyD88 appeared essential for induction of a local cytokine response (46). Our laboratory recently reported that MyD88 KO mice showed higher bacterial loads and higher cytokine levels in their lungs after infection with nontypeable *Haemophilus influenzae* (27). Moreover, MyD88 KO demonstrated a reduced host defense against systemic infection with either *S. aureus* (18) or *Listeria monocytogenes* (47), but were protected from severe polymicrobial peritonitis (48). Together these data indicate that although MyD88 is essential for the recognition of a variety of pathogens, its role in the host response *in vivo* may vary, presumably at least in part depending on the microorganism, the localization, and the severity of the infection.

There is abundant evidence from *in vitro* observations that TLR2 is the predominant receptor signaling the presence of cell wall components of Gram-positive bacteria, such as peptidoglycan and LTA (15, 16, 49, 50). In accordance, we found that TLR2 is important for cellular responsiveness toward *E. faecium* in vitro. Indeed, transfection of TLR2 into HEK cells rendered these cells responsive to *E. faecium*, whereas TLR2 deficiency was associated with a loss of responsiveness of macrophages to this bacterium. In addition, we demonstrated that TLR2 is involved in the early immune response to *E. faecium* in
TLRs in E. faecium peritonitis

vivo. TLR2 KO mice displayed higher peritoneal bacterial loads 2 h after infection and a reduced number of peritoneal neutrophils during the entire experiment. Notably, TLR2 KO mice differed in some aspects from MyD88 KO in this model. First, bacterial loads clearly differed more between MyD88 KO and WT mice than between TLR2 KO and WT mice. Furthermore, in contrast to MyD88 KO mice, TLR2 KO mice showed reduced levels of KC, MCP-1, IL-6 and IL-10 in plasma or peritoneal fluid. It is difficult to envision why a complete absence of MyD88 signaling (disturbing signaling of all TLRs except TLR3, as well as of IL-1 and IL-18) does not influence the production of these mediators whereas isolated TLR2-dependent MyD88 signaling does, even when considering the small difference in the bacterial doses (5 x 10^7 CFU and 8 x 10^7 CFU in the experiments with MyD88 KO and TLR2 KO mice, respectively). Clearly, more research is warranted to address this intriguing finding. Our current data exclude a role for TLR4 dependent MyD88 signaling in host defense against E. faecium; the roles of other TLRs and IL-1/IL-18 remain to be determined. With regard to the results obtained in TLR2 KO mice, Kau et al. (51) studied the role of TLR2 during an urinary tract infection with E. faecalis in mice. Twenty-four hours after the induction of the infection, these authors did not see any difference in enterococcal load in kidneys from TLR2 KO compared with WT mice, neither did they find any differences on histological examination. The inflammatory response was not discussed in this study. Differences in the bacterial strain and/or the route and localization of infection may account for the fact that we did detect a role for TLR2 in the response to infection with enterococci in vivo.

The pattern recognition receptor CD14 is well known to facilitate LPS signaling through TLR4 and LTA signaling through TLR2 (23, 30). CD14 has been shown to be coexpressed and to form a complex with transmembrane TLR2 (29). We identified an important role for CD14 in the activation of TLR2 by E. faecium in vitro: HEK cells transfected with both CD14 and TLR2 were much more responsive to E. faecium than HEK cells transfected with TLR2 alone, whereas both CD14 KO and TLR2 KO macrophages were markedly less responsive to E. faecium than WT macrophages. Nonetheless, in our in vivo studies, CD14 did not play a detectable role in E. faecium infection: we did not find any difference in enterococcal clearance, neutrophil recruitment, or cytokine/chemokine responses between CD14 KO and WT mice. The apparent discrepancy between our in vitro and in vivo results on the role of CD14 suggest that other components of the immune system not present in our in vitro systems may compensate for the absence of CD14. CD14 is considered to contribute to an antibacterial response during Gram-negative infection by virtue of its capacity to present LPS to the TLR4/MD2 signaling complex (13, 14). In Gram-positive infections
the role of CD14 may be more variable. Haziot et al (52) saw no difference in survival and/or bacterial clearance after i.v. or i.p. injection of S. aureus, whereas our laboratory recently revealed that CD14 facilitates invasive infection during pneumonia caused by Streptococcus pneumoniae (53).

Our studies focused on the role of TLR signaling in host defense against E. faecium in the previously healthy host. Thereby we sought to determine whether TLR signaling is a prerequisite for the resistance of the healthy host to E. faecium infections. Clearly, follow-up studies should address host defense mechanisms against E. faecium in the host that is more vulnerable due to predisposing conditions such as neutropaenia and/or glucocorticoid treatment. The present data show that MyD88 and TLR2 are involved in the early immune response to E. faecium peritonitis. The mechanism by which MyD88 and TLR2 deficiency result in higher bacterial loads most likely - at least in part - involves a diminished recruitment of neutrophils to the primary site of the infection. MyD88 KO and TLR2 KO immune cells did not show a reduced capacity to phagocytose or kill E. faecium in vitro, suggesting that a reduced cell number rather than a diminished function contributes to a less efficient clearance of E. faecium. Considering that MyD88 deficiency does not result in overwhelming infection and that differences in bacterial loads between MyD88 KO and WT mice were relatively modest, our data further indicate that in addition to the TLR family (and IL-1/IL-18) other components of the innate immune system may be involved in controlling peritonitis caused by E. faecium in our model.

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References


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