Enterococcus faecium infections: where bacterial virulence meets innate immunity

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Citation for published version (APA):

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THE COMPLEMENT SYSTEM FACILITATES CLEARANCE OF ENTEROCOCCUS FAECIUM DURING MURINE PERITONITIS

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Submitted
Abstract

Background: Infections with multiresistant enterococci are a growing problem worldwide. Little is known about host defense against enterococcal diseases. In vitro studies demonstrated an important role for complement proteins in neutrophil-mediated phagocytosis. In this study we investigated the importance of complement in an in vivo model of E. faecium peritonitis. Methods: Peripheral neutrophils and peritoneal macrophages were incubated with E. faecium, preincubated with decomplemented or normal plasma and phagocytosis and killing was examined. E. faecium peritonitis was induced in C57BL6 mice rendered complement deficient by intraperitoneal Cobra Venom Factor (CVF) injections and Complement 3 knockout (C3 KO) mice. The course of the infection at several time points up to 48 hours was compared to saline control and WT mice, respectively. Results: Opsonisation by complement enhanced phagocytosis by neutrophils and macrophages. CVF treated and C3 KO mice were severely hampered in clearing E. faecium from all cultured organs (peritoneal fluid, blood, lung and liver). Higher peritoneal cytokines and chemokines were measured in decomplemented mice, whereas no differences in systemic or peritoneal cell kinetics were detected. Conclusion: Complement deficiency severely hampers the clearance of E. faecium peritonitis and subsequent systemic infection.
Complement in *E. faecium* peritonitis

**Introduction**

Systemic infections with multiresistant enterococci are a major problem in hospitals worldwide. From relative triviality, enterococci have become a leading cause of nosocomial infection, currently ranking the top 3 of microorganisms causing nosocomial bacteremia in the United States [1]. The rise in incidence has, in part, been attributed to changes in medical care, e.g. the growth in the numbers of immunocompromised and critically ill patients, the increased use of intravascular devices, more prolonged hospital stays, and the widespread use of antibiotics to which the enterococci are resistant [2, 3]. Particularly the ability to acquire resistance genes by *E. faecium* facilitated the rise in incidence of enterococcal infection and led to an increase in the relative importance of *E. faecium* as a pathogen. Currently, β-lactam and vancomycin-resistance has almost completely penetrated the clinical population of *E. faecium*, with 90 and 80% of all *E. faecium* from nosocomial infections being resistant against ampicillin and vancomycin, respectively [1].

Enterococci are frequently isolated from ascending urinary tract infections and from more severe infections like endocarditis, (neonatal) bloodstream infections, urosepsis and intra-abdominal sepsis [1, 4]. Surprisingly little is known about either bacterial virulence factors or host defense mechanisms operative in enterococcal infections. The immediate host defense after recognition of the microorganism consists of the opsonic bactericidal action of complement as well as phagocytosis by macrophages and/or neutrophils [5]. Previously, we investigated the involvement of several components of the innate immune response during *E. faecium* infection. We found Toll-like receptor 2, signaling via Myeloid Differentiation protein 88 [6], neutrophils [7] and macrophages [8] contributing importantly to the initial host defense against *E. faecium* infection. In this study we investigated the role of the complement system. Previous *in vitro* studies demonstrated that complement proteins are of primary importance in neutrophil-mediated killing of *E. faecalis* and *E. faecium* and therefore were postulated to be essential in mounting an efficient immune response during *E. faecium* infection. We found Toll-like receptor 2, signaling via Myeloid Differentiation protein 88 [6], neutrophils [7] and macrophages [8] contributing importantly to the initial host defense against *E. faecium* infection. In this study we investigated the role of the complement system. Previous *in vitro* studies demonstrated that complement proteins are of primary importance in neutrophil-mediated killing of *E. faecalis* and *E. faecium* and therefore were postulated to be essential in mounting an efficient immune response [9, 10]. No studies, however, have subsequently been performed to investigate the role of the complement system in host defense against *E. faecium* infections *in vivo*.

To investigate the role of the complement system *in vivo*, we used a non-lethal mouse model of systemic *E. faecium* infection, caused by induction of *E. faecium* peritonitis [6]. Cobra Venom Factor (CVF) was used to deplete mice of complement; CVF forms a C3 convertase that rapidly and uncontrollably cleaves C3, resulting in hyperactivation of the complement cascade, thereby degrading the complement components, resulting
in nearly complete depletion of complement activity [11, 12]. In additional experiments, C3-gene deficient mice were used, which are completely deficient in the late steps of complement activation regardless of the route used to initiate the complement pathway [13].

Materials and Methods

Mice
Specific pathogen-free 10-wk-old female wildtype (WT) C57BL/6 mice were purchased from Harlan Sprague-Dawley (Horst, The Netherlands). Age- and gender-matched C3-gene knockout (KO) mice, backcrossed on a C57BL/6 background for at least six generations, were kindly provided by Dr. S. Verbeek (Department of Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands). The animals were housed in rooms with a controlled temperature and a 12-h light-dark cycle. They were acclimatized for 1 week prior to usage, and received standard rodent chow and water ad libitum. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Depletion of complement by Cobra Venom Factor
To deplete mice of complement, 4 units of CVF (Quidel, Clindia Benelux, Leusden, The Netherlands) was injected intraperitoneally; control mice were intraperitoneally injected with saline. To evaluate the efficiency and additional effects of CVF injection, 5 sex- and age-matched mice were injected with CVF or saline and sacrificed 16 hours thereafter, e.g. at the moment experimental mice were subjected to E. faecium peritonitis (see below). In these control mice C3 levels were measured in plasma and peritoneal fluid, cell counts and –differentials were performed and baseline cytokine levels were measured according to methods described below. From our own (unpublished) experience and available literature [12] it was known that mice would have severely reduced levels of complement at least up till 3 days after CVF injection (<90% of normal values).

Bacterial strain
VRE strain, E155, was used in all experiments. This clinical isolate from the Cook County Hospital, Chicago, IL, belongs to a genetic subpopulation of hospital-associated E. faecium that is responsible for the worldwide emergence of nosocomial multiresistant E. faecium, characterized by high-level quinolone and ampicillin resistance, a pathogenicity
island, containing the variant *esp* gene, and the presence of five cell surface protein genes [14, 15]. For all experiments the bacteria were grown overnight on sheep blood agar (BA) plates and then grown for approximately 3.5 hours in Todd-Hewitt (TH) broth (Difco, Detroit, MI) to midlogarithmic phase at 37°C, while shaking.

**Induction of peritonitis**

Peritonitis was induced as described previously [6]. *E. faecium* was cultured as described above and washed twice in sterile saline to clear the bacteria of medium. Bacteria were then resuspended in sterile isotonic saline and mice were injected intraperitoneally with approximately 10⁴ colony-forming units (CFU) of *E. faecium* in 200 μl saline. The inoculum was plated immediately after inoculation on BA plates to determine viable counts. Peritonitis was induced 16 hours after CVF or saline injection. Additional peritonitis experiments were performed with C3 KO mice.

**Collection of samples**

For comparison of bacterial outgrowth and to examine host responses in the experimental groups, mice treated with either CVF or saline were killed at 6, 24 or 48 hours after infection. In the experiments comparing C3 KO and WT mice, animals were killed after 6 or 24 hours. Mice were anesthetized by inhalation of isoflurane (Abbot, Laboratories Ltd., Kent, UK)/ O₂ (2%/ 2 liter) and a peritoneal lavage was performed with 5 ml sterile phosphate-buffered-saline (PBS) using a 18-gauge needle; the peritoneal lavage fluid (PLF) was collected in sterile polypropylene tubes (Plastipack; Beckton-Dickinson, Mountain View, CA). After collection of PLF, blood was drawn by cardiac puncture, with a sterile syringe, transferred to heparin-gel vacutainer tubes and immediately placed on ice. Next, the abdomen and thorax were opened and the liver and lungs were harvested.

**Determination of bacterial outgrowth**

The number of *E. faecium* CFU was determined in PLF, blood, liver and lung homogenates. Organs were weighed and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospect Products, Bartlesville, UK). 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 hours and corrected for the dilution factor.
Chapter 5

Cell counts and differentials
Erythrocytes were lysed with ice-cold isotonic NH4Cl solution (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.4) and the remaining cells were washed with PBS. These cells and cells in the PLF samples were counted using a Coulter Counter (Beckman Coulter, Fullerton, CA). Differential cell counts for the determination of neutrophils, macrophages/monocytes and lymphocytes were performed on cytopsin preparations, stained with Giemsa (Diff-Quick; Dade Behring). PLF supernatant and plasma were stored at -20˚C until determination of cytokines.

Opsonophagocytosis and bacterial killing assay
The role of complement in phagocytosis by neutrophils and peritoneal macrophages and killing by macrophages of this strain of *E. faecium* was first tested *ex vivo*. Heat killed (70˚C, 20 min) and FITC-labeled bacteria were first incubated (15 minutes) in 20 % heparin-plasma from mice treated with saline or CVF (e.g. C3 depleted plasma) 16 hours earlier. To determine the neutrophil phagocytosis capacity, 50 μl of whole blood was incubated with the two groups of preincubated bacteria (1x10⁷ CFU/ml), and incubated for 10, 60 or 120 minutes at 37˚C. Cells were suspended in Quenching solution, incubated in FACS lysis/fix solution (Beckton-Dickinson, Mountain View, CA) and neutrophils were labeled using anti-Gr-1-PE (BD Pharmingen, San Diego, CA), using concentrations recommended by the manufacturer.

Phagocytosis and killing by peritoneal macrophages were evaluated in essence as described before [6]. Peritoneal macrophages were harvested and cell numbers were counted using a Coulter Counter. Then the cells were washed, and resuspended in RPMI 1640 at a final concentration of 5x10⁵ cells/ml and were allowed to adhere in 48-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) overnight. For the phagocytosis assay macrophages were incubated with the two groups of preincubated *E. faecium* (2.5x10⁷ CFU/ml), for 10, 60, or 120 minutes. Phagocytosis was stopped by placing cells on ice, after which cells were washed in PBS and suspended in Quenching solution (Orpegen, Heidelberg, Germany). Neutrophils and macrophages were washed with ice-cold FACS buffer after which the degree of phagocytosis by neutrophils and macrophages was determined using the FACSCalibur (Becton Dickinson). For the bacterial killing assay by peritoneal macrophages, cells were treated as described above (*n=5* per strain). *E. faecium* was grown to midlog-phase, and 1x10⁹ CFU/ml was incubated in 20% plasma from CVF or saline treated mice, for 15 minutes. Then bacteria were washed, added on top of the seeded macrophages 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 minutes. Each well was
then washed with ice-cold PBS to remove extracellular bacteria. To determine bacterial uptake after 10 minutes, cells were lysed with sterile dH₂O and designated as T = 0. RPMI was added to remaining cells and plates were placed at 37°C for 15, 30 and 60 minutes after which cells were washed and lysed with dH₂O. Cell-lysates were plated on BA plates and bacterial counts were enumerated after 20h. Bacterial killing was expressed as the percentage of killed bacteria in relation to T = 0.

Assays

C₃ was detected by sandwich ELISA as described [7]. Macrophage inflammatory protein (MIP)-2, cytokine-induced neutrophil chemoattractant (KC) and LPS-induced C-X-C chemokine (LIX) were measured by ELISA’s (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, and monocyte chemoattractant protein (MCP)-1 were measured by using a commercially available cytometric bead array multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturer’s recommendations.

Statistical analysis

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

Results

Complement improves phagocytosis of \( E. faecium \) by neutrophils and macrophages, but does not influence intracellular killing

We first sought to obtain insight into the consequence of opsonisation by complement of \( E. faecium \) on subsequent phagocytosis and killing by macrophages and neutrophils \textit{ex vivo}. For this, \( E. faecium \) was preincubated in plasma of saline or CVF treated mice. CVF treatment led to a systemic and peritoneal reduction of the C₃ protein of ≥ 90 % (Fig. 1). \( E. faecium \) preincubated in normal (saline) plasma was phagocytosed more rapidly by neutrophils and primary peritoneal macrophages compared to \( E. faecium \) incubated in plasma depleted of complement by CVF (\( p < 0.001 \), Fig. 2A and B). Once internalized, opsonisation by complement did not influence killing by macrophages (Fig. 2C).
Impaired clearance of *E. faecium* in complement depleted mice

To determine the importance of complement in host defense against *E. faecium* peritonitis, mice were first depleted of complement by injecting CVF (or as a control saline). CVF treated mice remained C3 depleted during the entire experiment (data not shown). Sixteen hours after CVF or saline treatment, mice were subjected to *E. faecium* peritonitis and sacrificed 6, 24 or 48 hours thereafter. Complement depletion caused a severe delay in *E. faecium* clearance (Fig. 3). Six hours after the start of the infection > 2 log higher numbers of *E. faecium* were cultured from PLF of CVF treated mice compared to saline controls (Fig. 3A). Higher peritoneal bacterial loads resulted in higher systemic spread and 6 hours after the start of the infection 1 log more *E. faecium* CFU was cultured from blood (Fig. 3B), liver (Fig. 3C) and lungs (Fig. 3D). Both CVF and saline treated mice were able to reduce peritoneal and systemic *E. faecium* burdens. At 24 and 48 hours after infection, peritoneal *E. faecium* numbers were comparable in both groups of mice, whereas the amount of *E. faecium* in CVF treated mice in blood, liver and lungs remained elevated compared to control mice during the entire experiment.
Complement in *E. faecium* peritonitis

Figure 2. Diminished phagocytosis of *E. faecium* preincubated with C3-depleted plasma. The effect of opsonisation by complement on phagocytosis and killing of *E. faecium* was examined. Peripheral blood neutrophils (A) and peritoneal macrophages (B) harvested from untreated mice were incubated with FITC-labeled *E. faecium*, preincubated in plasma of mice treated with saline or CVF 16 hours earlier. Phagocytosis was determined by FACS analysis and expressed as mean fluorescence x percentage positive cells. For killing, peritoneal macrophages (C) were incubated with *E. faecium*, preincubated in plasma of saline or CVF treated mice (multiplicity of infection, 50) and bacterial killing was assessed over time, shown as percentage of killed *E. faecium* relative to T = 0. Data are means ± SEM of n = 5 mice per group.
Figure 3. Mice depleted of complement are impaired in peritoneal and systemic *E. faecium* clearance. Mean (± SEM) *E. faecium* CFU in PLF (A), blood (B), liver (C), and lungs (D) 6, 24 and 48 hours after inoculation with 10^8 CFU *E. faecium* (at T = 0). Mice were infected 16 hours after intraperitoneal CVF or saline injection. N = 8 mice per group at each time point. *P* values in figures represent the overall difference between groups; asterisks indicate differences between groups at one time point, *p*<0.05; **p**<0.01 compared to saline-injected mice.

Figure 4. No influence of complement depletion on peritoneal neutrophil and macrophage counts during *E. faecium* infection. Sixteen hours after intraperitoneal CVF or saline injection, mice were inoculated with 10^8 CFU *E. faecium* (at T = 0) and sacrificed 6, 24 and 48 hours thereafter. Peritoneal neutrophils (A) and macrophages (B) are shown. Data are means ± SEM of n = 8 mice per group at each time point.
**Complement in *E. faecium* peritonitis**

**Effect of complement depletion on the inflammatory response**

At the time of infection, leukocyte counts and differentials in PLF and blood were similar in CVF and saline treated mice (Fig. 4 and data not shown). Induction of *E. faecium* peritonitis was associated with a brisk influx of neutrophils to the primary site of infection. Six hours after infection CVF and saline treated mice had attracted similar amounts of neutrophils into the peritoneal cavity (Fig. 4A). As the amount of peritoneal *E. faecium* decreased neutrophil numbers in PLF declined, returning to near baseline values after 48 hours in both groups (Fig. 4A). *E. faecium* peritonitis resulted in an initial decrease in the number of peritoneal macrophages, as measured 6 hours post infection (Fig. 4B), confirming our previous results [8]. Thereafter, macrophage numbers increased again, showing modestly higher counts from 24 hours onward. Although after 24 hours CVF treated mice tended to have lower macrophage numbers in their PLF, the difference with saline mice was not statistically significant. Complement depletion did not influence the alterations in peripheral blood neutrophil or monocyte counts either (data not shown).

During the infection no differences were found with regard to circulating neutrophils and monocytes (data not shown).

To further evaluate the inflammatory response in complement deficient and sufficient mice, we measured the levels of the proinflammatory cytokines TNF-α and IL-6 and the anti-inflammatory cytokine IL-10, as well as MCP-1, in PLF and blood and neutrophil attracting CXC chemokines LIX, KC and MIP-2 in PLF. At the time of infection, CVF and saline treated mice had low or undetectable cytokine and chemokine levels that were not different between groups (data not shown). CVF treated mice sacrificed 6 hours after the infection showed higher peritoneal TNF-α and IL-6 levels than saline controls, corresponding with the 2 log higher CFU in the peritoneal cavity (Table 1). Peritoneal IL-10 and MCP-1 levels were comparable in both groups as were plasma cytokine levels. The concentrations of KC, MIP-2 and LIX were all higher in CVF treated mice. Cytokine levels measured at later time points were undetectable or low in CVF and saline treated mice, and not different between groups (data not shown).
Chapter 5

Table 1. Effect of complement depletion by Cobra Venom Factor on cytokine and chemokine responses during *E. faecium* peritonitis.

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>Plasma</th>
<th>PLF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>CVF</td>
</tr>
<tr>
<td>TNFα</td>
<td>98 ± 21</td>
<td>126 ± 45</td>
</tr>
<tr>
<td>IL-6</td>
<td>398 ± 159</td>
<td>465 ± 191</td>
</tr>
<tr>
<td>IL-10</td>
<td>152 ± 27</td>
<td>192 ± 49</td>
</tr>
<tr>
<td>MCP-1</td>
<td>553 ± 76</td>
<td>620 ± 186</td>
</tr>
<tr>
<td>KC</td>
<td>26 ± 3</td>
<td>225 ± 105 *</td>
</tr>
<tr>
<td>LIX</td>
<td>43 ± 11</td>
<td>208 ± 86 *</td>
</tr>
<tr>
<td>MIP-2</td>
<td>61 ± 16</td>
<td>197 ± 76 *</td>
</tr>
</tbody>
</table>

Mice were inoculated with 10⁸ CFU *E. faecium*, 16 hours after CVF or saline injection and sacrificed 6 hours after infection. Data are means ± SEM of 8 mice per group, *p<0.05 compared to saline injected mice.

Impaired clearance of *E. faecium* in C3 KO mice

CVF treatment did not render mice absolutely depleted of complement (Fig. 1) and may have other (toxic) effects. Therefore, we wished to confirm the data reported above by using C3 KO mice in the same model of *E. faecium* peritonitis. C3 KO mice are absolute deficient of C3, as we confirmed by ELISA (not shown) and has previously been described [13].

Six hours after the start of the infection C3 KO mice had > 3 log more *E. faecium* CFU in their PLF as compared to WT mice (Fig. 5A). In blood, liver and lung about 2 log more *E. faecium* was cultured from C3 KO mice (Fig. 5B-D). Twenty-four hours after the infection C3 KO mice still had higher numbers of *E. faecium* in their PLF, blood and lungs compared to WT mice, yet they had been able to reduce the amount of bacteria, even to a similar extent as WT mice in liver. No differences were found in peritoneal and peripheral cell composition or cytokine profiles between uninfected WT and C3 KO mice (data not shown). During the infection no differences were found in the peritoneal and peripheral cell composition (Fig. 6 and data not shown).

Higher peritoneal and blood IL-6 levels and peritoneal MIP-2, KC, LIX and MCP-1 chemokines were measured in C3 KO mice 6 hours after the infection (Table 2). TNF-α and IL-10 were not found different in either plasma or PLF. No differences were measured after 24 hours.
Complement in *E. faecium* peritonitis

Figure 5. C3 KO mice are impaired in peritoneal and systemic *E. faecium* clearance. C3 KO and WT mice were intraperitoneally infected with $10^8$ CFU *E. faecium* and killed 6 or 24 hours thereafter. *E. faecium* outgrowth in PLF (A), blood (B), liver (C) and lung (D) are shown. Data are means ± SEM of n = 7-8 mice per group at each time point. * $p < 0.05$; ** $p < 0.01$ compared to WT mice.

Table 2. Plasma and peritoneal cytokines in WT and C3 KO mice intraperitoneally infected with $10^8$ CFU *E. faecium*

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Plasma</th>
<th>C3 KO</th>
<th>WT</th>
<th>C3 KO</th>
<th>PLF</th>
<th>C3 KO</th>
</tr>
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<tbody>
<tr>
<td>TNFα</td>
<td>65 ± 11</td>
<td>99 ± 17</td>
<td>37 ± 9</td>
<td>54 ± 17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>317 ± 53</td>
<td>2077 ± 1055$^*$</td>
<td>199 ± 36</td>
<td>870 ± 258$^*$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>118 ± 29</td>
<td>156 ± 37</td>
<td>145 ± 20</td>
<td>146 ± 38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>416 ± 90</td>
<td>605 ± 199</td>
<td>137 ± 24</td>
<td>680 ± 230$^*$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KC</td>
<td>44 ± 7</td>
<td>356 ± 138$^*$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIX</td>
<td>32 ± 6</td>
<td>291 ± 139$^*$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-2</td>
<td>52 ± 9</td>
<td>324 ± 121$^*$</td>
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</table>

Complement 3 knockout (C3 KO) and wildtype (WT) mice were inoculated with $10^8$ CFU *E. faecium* and sacrificed 6 hours thereafter. Data are means ± SEM of 8 mice per group, * $p<0.05$ compared to WT mice.
Chapter 5

Discussion

In this study, we demonstrate for the first time the involvement of the complement system in host defense against \textit{E. faecium} infection \textit{in vivo}. \textit{E. faecium} has emerged as an important pathogen worldwide causing severe nosocomial infections. Because of the growing resistance to almost all antibiotics used in the hospital setting, infections with this multidrug resistant pathogen are difficult to treat. To improve therapeutic options, a better understanding of the pathogenesis of these infections and the interaction with host defense systems is needed. Previous \textit{in vitro} studies indicated an important role for complement proteins in opsonizing enterococci and subsequent phagocytosis by neutrophils [9, 10]. After confirming that opsonisation by complement enhances neutrophil, and additionally, macrophage phagocytosis of our clinical \textit{E. faecium} isolate, we investigated the role of the complement system during \textit{E. faecium} infection \textit{in vivo}.

Both groups of complement deficient mice (decomplemented by use of CVF injection and C3 KO mice) were severely hampered in clearing \textit{E. faecium} peritonitis. Especially early in the infection, complement deficient mice had 2-3 log higher numbers of \textit{E. faecium} cultured from the peritoneal fluid, resulting in higher systemic dissemination and a severe delay in \textit{E. faecium} clearance. Higher \textit{E. faecium} burden was accompanied by higher peritoneal cytokine and chemokine levels and higher systemic IL-6 levels in C3 KO mice. Interestingly, complement deficiency did not hamper cell recruitment to the primary site of infection during \textit{E. faecium} peritonitis.
The complement system is a key player in the systemic defense system as well as in the peritoneal cavity [16]; it comprises one of the major groups of pattern recognition molecules, consisting of >30 serum and cell surface proteins. Activation of complement can occur through the classical, alternative, or mannose binding lectin pathways, all of which rely on the function of complement component C3. Cleavage of C3 by the C4b2a (classical and lectin pathways) or C3bBb complexes (alternative pathway) is a critical step that results in release of the soluble anaphylatoxin C3a and deposition of C3b and iC3b on the surface of the bacterium. C3b and iC3b serve as ligands or ‘opsonins’ for cellular receptors on leukocytes and receptor-mediated binding of these C3 opsonins leads to microbial clearance by phagocytes. Additionally, cleavage of C3 results in the formation of the membrane attack complex (C5-9). In a complete absence of C3, almost all of the biological properties mediated by complement are absent, including opsonization and phagocytosis of bacteria, directed migration of inflammatory cells and amplification of the immune response [17-19]. In general, the thick cell wall of gram-positive bacteria, like E. faecium, prevents complement-mediated lysis; therefore, complement primarily plays an opsonic role in host defense against gram-positive infections [20, 21]. Interestingly, Nordahl et al. [22] demonstrated a direct antimicrobial effect of C3a and C3a-desArg, which efficiently killed E. faecalis.

Previous in vitro studies demonstrated neutrophil-mediated bactericidal activity towards enterococci was ablated by inactivation of complement. C4-deficient serum promoted neutrophil killing consistently, suggesting that complement mediated neutrophil activity may proceed by the alternative pathway of complement [9, 10]. Additional studies demonstrated enhanced killing of enterococci when specific antibodies were involved. Huebner et al. [23] found that enterococcal glucose-glycerol-teichoic acid capsular polysaccharides (LTA) are targeted by antibodies in rabbits, and that anti-enterococcal rabbit serum had a protective effect in mice infected with enterococci. In an opsonophagocytic killing assay, antisera raised against purified polysaccharide from E. faecalis killed a variety of heterologous strains of E. faecalis, as well as E. faecium, including vancomycin-resistant strains. Interestingly, certain strains of E. faecium were found resistant to phagocytosis by neutrophils, probably caused by a carbohydrate-containing moiety expressed by these strains. Specific antibodies could overcome this resistance, most likely by promoting deposition of complement [24-26]. In addition, extracellular gelatinase (GelE) expressed by E. faecalis destroys complement in human serum, by hydrolyzing C3a and degeneration of C3b [30, 31]. Expression of GelE was also described in E. faecium [32], besides the described expression of capsular polysaccharides, this may contribute to the resistance to phagocytosis as well.
The clinical isolate of *E. faecium* (E155) we used in our experiments belongs to a genetic subpopulation of hospital-associated *E. faecium* that is responsible for the worldwide emergence of nosocomial multiresistant *E. faecium*, characterized by high-level quinolone and ampicillin resistance, a pathogenicity island, containing the variant esp gene, and the presence of specific cell surface protein genes [14, 15]. We first established by an *in vitro* assay, that opsonization by complement of this *E. faecium* strain enhanced phagocytosis by neutrophils in whole blood. Additionally, peritoneal macrophages were demonstrated to more easily internalize the bacteria when these were previously opsonized by complement. This is of importance as peritoneal macrophages are the cells that first encounter the bacteria when introduced in the normally sterile peritoneal cavity [16]. This strain was clearly not resistant to phagocytosis.

We then tested the influence of complement *in vivo*. By injection of CVF, mice remain decomplemented for up to 3 days [12]. Decomplementation by CVF resulted in 90% reduction of C3 levels. CVF treated mice were severely debilitated in clearing enterococci, which was especially apparent early after infection. Six hours after the start of the infection they had up to 3 log more *E. faecium* in their peritoneal cavity, compared to the saline injected control mice, with increased systemic dissemination. Despite this prolonged increased bacterial load, no mortality was seen and apparently bacteria were not able to multiply *in vivo*, as at later time points the bacterial numbers were reduced also in decomplemented mice. Although CVF treatment caused a severe reduction in systemic complement proteins, some C3 could still be measured, potentially still playing a role in the reduction of the bacteria. We then examined if an absolute deficiency in C3, using C3 KO mice, would render the host even more hampered in enterococcal clearance. Like in the CVF treated mice, these experiments resulted in higher loads at the 6 hours time point in C3 KO mice, compared to the WT control mice, and also a reduction in load later in the infection.

Both cleavage products C3a and C5a are known anaphylatoxins, playing a role in attracting leukocytes to the site of inflammation [27-29]. Interestingly, we did not find differences in the attraction of neutrophils or macrophages into the peritoneal cavity in either model of complement deficient mice. As such, the increased numbers of *E. faecium* in the peritoneal cavity were not due to reduced peritoneal cellular recruitment. Apparently, in the absence of an intact complement system, multiple compensatory mechanisms exist for the recruitment of neutrophils into the PLF after *E. faecium* infection. In this respect it should be noted that the peritoneal levels of the neutrophil attracting CXC chemokines KC, MIP-2 and LIX were elevated in complement deficient mice 6 hours post infection, likely caused by the markedly higher bacterial loads at this time point, providing a
more potent proinflammatory stimulus for chemokine release; these elevated local CXC chemokine concentrations possibly compensated for the absent complement attracting function.

Our data show the requirement of complement for the early containment of *E. faecium* infection, with increased dissemination and severely hampered clearance in the absence of complement, which strongly suggests that complement is pivotal in the early antibacterial response during *E. faecium* infection primarily by facilitating phagocytosis.

**Acknowledgements**

We thank J. Daalhiusen and M. ten Brink for providing expert technical assistance.

**References**

Chapter 5