Enterococcus faecium infections: where bacterial virulence meets innate immunity
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NEUTROPHILS ARE ESSENTIAL FOR RAPID CLEARANCE OF ENTEROCOCCUS FAECIUM IN MICE

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Abstract

A progressive increase in infections with multiresistant *E. faecium* has been reported, especially in cancer and neutropenic patients. Despite its increasing importance as nosocomial pathogen, knowledge on the pathogenesis of *E. faecium* infections is highly limited. In this study we investigated the role of neutrophils during peritonitis with subsequent bacteremia caused by *E. faecium*. We therefore depleted neutrophils by intraperitoneal injections of monoclonal antibody RB6-8C5. Mice were followed for 5 days, and the enterococcal outgrowth and the inflammatory response were compared between neutropenic mice and IgG-injected control mice. Neutropenic mice demonstrated a severe delay in enterococcal clearance from all cultured organs (peritoneal fluid, blood, lung and liver). In particular, neutropenic mice remained bacteremic for up to 3 days, whereas all nonneutropenic mice had cleared the bacteria from the circulation by 2 days. Furthermore, neutropenic mice displayed elevated peritoneal cytokine and chemokine levels 1 day after the infection and attracted fewer macrophages into the peritoneal cavity. In the circulation, a prolonged elevation of tumor necrosis factor-α, interleukin-6 and the acute phase proteins serum amyloid A and complement 3 were measured in neutropenic mice. In conclusion, attraction of neutrophils to the primary site of *E. faecium* infection is important for a rapid clearance of this bacterium, thereby attenuating a systemic inflammatory response.
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**Introduction**

Enterococci are part of the normal bacterial flora of the human and animal gastrointestinal tracts. Although enterococci were once not regarded as virulent, they are now recognized as a major cause of nosocomial infections worldwide (13). They are the third most common cause of nosocomial bacteremia in the United States and the fourth most common in Europe (http://www.earss.rivm.nl/). Although enterococci rarely cause disease in healthy individuals, they can become pathogenic in patients in intensive care units and in hospitalized patients with severe underlying diseases or an impaired immune system, and in elderly people (23). Severely ill patients with hematologic malignancies and deep neutropenia are at an increased risk of developing enterococcal bacteremia (6, 7, 18, 29, 43).

The emergence of infections with enterococci can largely be attributed to their multiresistant nature to various classes of antibiotics. Especially *Enterococcus faecium* has acquired resistance to high-dose aminoglycosides, β-lactam antibiotics and vancomycin (5, 20, 37). Hospital-acquired *E. faecium* isolates belong predominantly to a distinct genetic subpopulation currently known as clonal complex-17 (CC17), which has adapted extremely well to the hospital environment and that has spread worldwide (39). CC17 is characterized by the acquisition of multiple adaptive mechanisms, including ampicillin and quinolone resistance, a putative pathogenicity island harboring the esp virulence gene, and other cell surface protein genes (16, 19-21).

Despite the clinical importance of enterococci, little is known about defense mechanisms that protect the normal host against invasive enterococcal infections. The innate immune system represents the first line of defense against bacterial infections (27, 46). In previous studies, we described the normal immune response during primary *E. faecium* peritonitis (22). In a nonlethal model, we found a fast and brisk peritoneal neutrophil influx and a consecutive, rapid decline in peritoneal and systemic enterococcal load. In Toll-like receptor 2 (TLR2) and myeloid differentiation protein 88 knockout mice, a significantly reduced amount of neutrophils was attracted to the peritoneal cavity, which was accompanied by a delay in enterococcal clearance (22). These data, together with the fact that neutropenic patients are more vulnerable to acquiring *E. faecium* infections, prompted us to investigate the role of neutrophils during nonlethal *E. faecium* peritonitis with subsequent bacteremia.
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Materials and Methods

Mice
Specific pathogen-free 10-wk-old female C57BL/6 mice were purchased from Harlan Sprague-Dawley (Horst, 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.

In vivo neutrophil depletion
To characterize the role of neutrophils during E. faecium peritonitis, mice were depleted of neutrophils before E. faecium challenge. For depletion, mice were treated intraperitoneally (i.p.) with the rat anti-mouse monoclonal antibody (MAb) RB6-8C5 directed against Ly-6G, previously known as Gr-1, an antigen on the surface of murine granulocytes (36). The Ab (αLy-6G) was produced by TSD BioServices (Germantown, NY) by i.p. injection of RB6-8C5 hybridoma into nude mice and by subsequent ascites collection. A total of 100 μg of RB6-8C5 was administered i.p. 1 day before the challenge with E. faecium. When mice were followed up until 5 days after induction of E. faecium peritonitis, mice were injected with the Ab on days 1 and 3 as well. The specificity and efficacy of RB6-8C5 have been well established (9, 35, 42). Control mice were given the equivalent amount of purified rat immunoglobulin G (ri gG) (Sigma, St. Louis, Missouri).

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 a genetic subpopulation of hospital-associated E. faecium, currently labeled CC17. For all experiments, the bacteria were grown overnight on agar sheep blood plates and then grown for ~ 3.5 h in Todd-Hewitt broth (Difco, Detroit, MI) to mid-logarithmic phase at 37°C, with shaking.

Induction of peritonitis
Peritonitis was induced as described (22). Briefly, 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 10⁸ CFU of E. faecium in 200 μl sterile isotonic saline. This bacterial dose is gradually cleared by normal C57BL/6 mice and is not associated with lethality (22). The inoculum was plated immediately after inoculation on sheep blood agar plates to determine viable counts.
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**Collection of samples**
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 using a 18-gauge needle; peritoneal lavage fluid was collected in sterile polypropylene tubes (Plastipack; Beckton-Dickinson, Mountain View, CA). After collection of peritoneal fluid, blood was drawn by cardiac puncture, transferred to heparin-gel vacutainer tubes, and immediately placed on ice. Next, the abdomen was opened and the liver 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. Lungs and livers were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFU were determined from serial dilutions; 50 μl of each dilution was plated onto blood agar plates and incubated at 37°C at 5% CO₂ for 20 h before colonies were counted and corrected for the dilution factor.

**Cell counts and differentials of blood and peritoneal lavage fluid**
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 phosphate-buffered saline. These cells and cells in the peritoneal lavage 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 cytospin preparations, stained with a modified Giemsa stain (Diff-Quick; Dade Behring). Peritoneal fluid supernatant and plasma were stored at -20°C until determination of cytokines.

**FACS analysis**
As RB6-8C₅ MAb also effects Gr-1+ subpopulations of monocytes/macrophages [10], FACS analysis of blood monocytes and peritoneal macrophages from RB6-8C₅ and control Ab treated mice was performed. Immunostaining for cell surface molecules was performed for 30 min at 4°C, using directly labelled antibodies against F4/80 and Gr-1. Antibodies were used in concentrations recommended by the manufacturer (BD Pharmingen). Cells were analysed using FACSCalibur (BD Biosciences, Mountain View, CA).
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Histology
Directly after sacrifice, lungs and livers 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 and cytokine-induced neutrophil chemoattractant (KC) were measured in peritoneal lavage fluid by ELISAs (R&D Systems, Minneapolis, MN) according to the manufacturers’ instructions. TNF-α, IL-6, IL-10, IL-12p70, IFN-γ and MCP-1 were measured in peritoneal lavage fluid and plasma by using a commercially available cytometric bead array multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturer’s recommendations. Serum amyloid A (SAA) was measured by a commercially available ELISA for mouse SAA, in accordance with the manufacturer’s instructions (Biosource International). Complement 3 (C3) was detected by sandwich ELISA as described previously (32). Aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), lactate dehydrogenase (LDH) and creatinine were determined with commercially available kits (Sigma-Aldrich, St. Louis, MO), using a Hittachi analyzer (Boehringer Mannheim, Mannheim, Germany).

Statistical analysis
All data are expressed as means ± SEM. Serial data were analyzed by two-way analysis of variance followed by a post hoc Bonferroni test. Two group comparisons were done by using the Mann-Whitney U test. For all analyses, GraphPad Prism version 4 (GraphPad Software, San Diego, CA) was used. A p-value < 0.05 was considered statistically significant.

Results
Neutropenia is associated with a strongly reduced early clearance of E. faecium
To determine the importance of neutrophils in host defense against E. faecium peritonitis, mice were depleted of neutrophils by administration of αly-6G 1 day prior to infection and where applicable 1 and 3 days after infection. Treatment with αly-6G was successful for inducing neutropenia (< 50 neutrophils/μl) (Fig. 1A). E. faecium peritonitis was induced in neutropenic and rIgG-treated control mice, and groups of mice were sacrificed on days
1, 2, 3, and 5 after infection. An inoculum of $10^8$ CFU was chosen, as we previously found that healthy mice are able to clear this infection in 2 to 3 days, whereas an inoculum of $10^9$ CFU resulted in 30% mortality (22). Mice injected with αLy-6G remained neutropenic during the entire 5-day observation period (< 50 neutrophils/μl; p<0.01 vs control mice) (Fig. 1A). αIgG- treated control mice showed an early rise in peripheral blood neutrophil numbers, peaking at day 1; thereafter neutrophil counts returned to baseline values. In addition, control mice displayed a strong increase in the number of neutrophils in their peritoneal lavage fluid 1 day after infection; such an increase was not observed in neutropenic mice (p<0.001) (Fig. 1C). Remarkably, although before infection with *E. faecium* neutropenic mice had unaltered macrophage numbers in their peritoneal lavage fluid, they demonstrated a reduced influx of macrophages into their peritoneal cavities relative to that in control mice after i.p. infection (p<0.01) (Fig. 1D). Of note, monocyte counts in peripheral blood were similar in neutropenic and control mice (Fig. 1B). FACS analysis of peritoneal and blood cells obtained from mice 1 day after antibody treatment (e.g. the moment of *E. faecium* challenge) revealed that 0.69% (± 0.83%) of peritoneal macrophages of control mice were Gr-1+. After RB6-8C5 MAb treatment, this amount was reduced to 0.05% (± 0.03%). Of blood monocytes, 2.82% (± 0.62%) were Gr-1+ in control mice and 0.20% (± 0.08%) in RB6-8C5 MAb-treated mice.

One day after the infection, *E. faecium* was cultured from all organs investigated: peritoneal fluid (the primary site of the infection), blood, lungs, and livers (Fig. 2A-D). At this time point, all organs from neutropenic mice contained at least 10-fold more *E. faecium* CFU than αIgG-treated control mice (p<0.01 to p<0.0001). Neutropenic mice stayed bacteremic for up to 3 days after the infection, whereas blood cultures in control mice were sterile from day 2 after infection onward (p<0.01 for the difference between groups). Similarly, in the peritoneal fluid of neutropenic mice, 100-fold more *E. faecium* CFU were cultured than in peritoneal fluid of control animals 2 and 3 days postinfection (p<0.001, p<0.01, respectively). Moreover, the lungs of neutropenic mice displayed very high bacterial loads at day 2 and 3 postinfection; at these time points, *E. faecium* could not be recovered from lungs of control mice anymore (p<0.001). Of note, in spite of the strongly impaired clearance during the first 3 days after infection, neutropenic mice eventually cleared *E. faecium* from all body compartments; i.e., cultures from all tested body sites were sterile 5 days after infection. Figure 3 displays a cytospin preparation of peritoneal cells 24 h after infection in control mice, showing the importance of neutrophils in phagocytosing *E. faecium*. Of note, αLy-6G treatment per se (i.e. before infection with *E. faecium*) did not result in positive cultures of peritoneal fluid or blood; in addition, cultures obtained from mice infected with *E. faecium* only grew this pathogen.
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Figure 1. Mice injected with αLy-6G were neutropenic during the entire experiment. Mice were injected with αly-6G (open symbols) or rIgG (closed symbols) 24 h before and 1 and 3 days after i.p. injection of 10^8 CFU E. faecium. (A) At the moment of infection, αly-6G-treated mice had < 50 circulating neutrophils/μl; these mice stayed neutropenic during the entire experiment. (B) αly-6G did not influence blood monocyte counts. Neutropenic mice did not attract neutrophils (C) and attracted less macrophages (D) to the peritoneal cavity than control mice. Data are mean ± SEM (n = 8 per group at each time point). P values in the figures represent the overall difference between groups. **, p < 0.01; ***, p < 0.001 vs control mice at the time points indicated.
Figure 2. Neutropenic mice demonstrate a severely delayed clearance of *E. faecium*. Mice were injected with αLY-6G (open symbols) or rIgG (closed symbols) 24 h before and 1 and 3 days after i.p. injection of $10^8$ CFU *E. faecium*. Bacterial loads were cultured in peritoneal lavage fluid (PLF) (A), blood (B), lung (C) and liver (D) at 1, 2, 3 and 5 days after inoculation. Data are mean ± SEM (n = 8 per group at each time point). P values in the figures represent the overall difference between groups. **, $p<0.01$; ***, $p<0.001$ vs control mice at the time points indicated.
Prolonged high *E. faecium* load associated with higher cytokine response and prolonged, elevated acute-phase protein levels.

At the moment of i.p. *E. faecium* injection (i.e., 1 day after injection of RB6-8C5 or control Ab), mice had no signs of an inflammatory response, as reflected by unaltered cytokine levels (not shown). In accordance with our previous study (22), control mice displayed low or undetectable levels of TNF-α, IL-6, MCP-1, IL-10, IL-12p70, IFN-γ, KC and MIP-2 in plasma and peritoneal fluid 1 day after infection and beyond (Fig. 4) (Table I, and data not shown). In contrast, neutrophil-depleted mice had detectable plasma TNF-α and IL-6 levels up to 3 days after the infection (Fig. 4A and B). Moreover, neutropenic mice had detectable levels of TNF-α, IL-6, KC and MCP-1 in their peritoneal lavage fluid (Table I). Additionally, in these mice, plasma C3 and SAA levels were persistently elevated (Fig. 5A and B).

Neutrophil depletion does not cause organ damage

In accordance with our earlier study (22), *E. faecium* peritonitis and bacteremia were not associated with organ injury, as determined by histopathology of liver and lungs and by clinical chemistry (ASAT, ALAT, LDH, creatinin)(data not shown). In spite of the prolonged enterococcal load in multiple body sites, neutrophil-depleted mice did not show any sign of organ injury either (data not shown).
Figure 4. Neutropenic mice display elevated plasma TNF-α and IL-6 concentrations. Mice were injected with αLy-6G (open symbols) or rIgG (closed symbols) 24 h before and 1 and 3 days after i.p. injection of $10^8$ CFU *E. faecium*. Plasma TNF-α (A) and IL-6 (B) levels were measured at the time points indicated. Data are mean ± SEM (n = 8 per group at each time point). P values in the figures represent the overall difference between groups. *, $p < 0.05$; ***, $p < 0.001$ vs control mice at the time points indicated.

Table I. Elevated cytokine and chemokine levels in peritoneal fluid of neutropenic mice 24 hours after infection with *E. faecium*.

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<tr>
<th>Cytokine</th>
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<tr>
<td></td>
<td>Control mice</td>
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<td>TNF-α</td>
<td>BD</td>
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<td>IL-6</td>
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<td>MCP-1</td>
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Mice were inoculated i.p. with $10^8$ CFU *E. faecium* after depletion of neutrophils by i.p. injection of αLy-6G; control mice received an i.p. injection with rIgG. BD, below detection. Data are mean ± SEM, n = 8 mice/group. *, $p<0.05$; ***, $p<0.001$ compared to controls.
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Figure 5. Neutropenic mice display a prolonged acute-phase protein response. Mice were injected with αLy-6G (open symbols) or rIgG (closed symbols) 24 h before and 1 and 3 days after i.p. injection of 10^8 CFU E. faecium. Plasma (A) C3 and SAA (B) levels were measured at the time points indicated. Data are mean ± SEM (n = 8 per group at each time point). P values in the figures represent the overall difference between groups. ***, p < 0.001 vs control mice at the time points indicated.

Discussion

Since the 1970s, a progressive increase of enterococcal bacteremia has been described, and enterococci are currently ranked as the third most common cause of bloodstream infections (7, 34). Especially vancomycin-resistant enterococci (VRE) are of main concern, as only few antibiotics are effective against this multiresistant pathogen. The vast majority of VRE infections are caused by E. faecium, which is specifically adapted to the hospital environment and belongs to a genetic subpopulation, currently labeled CC17 (20, 40, 47). CC17 is characterized by the acquisition of multiple adaptive mechanisms, including ampicillin and quinolone resistance, a putative pathogenicity island harboring the esp virulence gene, and other cell surface protein genes (16, 19-21).

Patients with hematologic malignancies and severe neutropenia display the highest risk of acquiring enterococcal bacteremia (6, 7, 18, 29, 43). Knowledge of how the intact innate immune system deals with E. faecium is limited. In an earlier study, we showed that E. faecium is recognized by immune cells through TLR2 and that interruption of TLR-dependent MyD88 signaling after i.p. administration of this pathogen is associated with a reduced early neutrophil recruitment to the primary site of the infection and a modestly delayed bacterial clearance (22). In the current study, we focused on the role of neutrophils in this model of E. faecium peritonitis. The main finding was a strongly impaired early clearance of E. faecium from all body sites examined in mice with neutropenia. Indeed, neutropenic mice had prolonged high enterococcal loads in all
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(cultured) organs and remained bacteremic for at least 3 days after infection, whereas control mice were no longer bacteremic after 2 days. Of note, bacteria were eventually cleared even in neutropenic mice.

Interestingly, *E. faecium* peritonitis and bacteremia did not convert to a lethal infection in neutrophil-depleted mice, although in previously healthy mice i.p. infection with 10 times more bacteria of the same *E. faecium* strain resulted in 30% lethality (22). This discrepancy might be explained by the absence of neutrophils, as neutrophils play a dual role during infection and inflammation. On the one hand, these cells are of great importance in killing bacteria and clearing infections. On the other hand, attraction of neutrophils to the site of inflammation can cause serious tissue damage and propagate the inflammatory response by the release of neutrophil-generated oxygen free radicals and proteases (45, 46). Indeed, Walley et al. (44) and Ness et al. (28) demonstrated that in a lethal model of polymicrobial peritonitis, inhibition of neutrophil recruitment into the peritoneal cavity by elimination or inhibition of neutrophil-attracting chemokines was associated with a reduced mortality, supporting the notion that enhanced neutrophil influx can cause tissue damage during abdominal infection. One can hypothesize that the low virulence nature of *E. faecium* does not cause serious organ damage without the participation of neutrophils during infection. Nonetheless, neutropenic mice did show evidence for an enhanced systemic inflammatory response, as reflected by sustained elevations of the plasma concentrations of TNF-α and IL-6, as well as of the acute phase proteins SAA and C3.

Healthy mice clear an infection with a large inoculum of $10^8$ CFU *E. faecium* without showing important signs of illness, which resembles the scenario in healthy humans who are unlikely to develop infection and disease by *E. faecium*. Clearly, the healthy innate immune system is able to control *E. faecium* infections and prevent the development of severe disease. Previously, we found the recruitment of large numbers of neutrophils to the primary site of infection in the model of *E. faecium* peritonitis used here (22). We now firmly establish that this early reaction significantly contributes to an effective antibacterial response. Of note, although neutrophils are crucial in host defense against a wide array of pathogens, their contribution is not the same for all infections or even for different routes of infection with the same pathogen (3, 4, 8, 9, 11, 24, 25, 33, 36, 42). Neutrophils were found to impact on the efficacy of therapeutic interventions (i.e., granulocyte-colony stimulating factor or IFN-γ given together with appropriate antibiotics) after i.p. infection with *E. faecalis* (30, 31). However, these studies, in which neutropenia was induced by cyclophosphamide, did not directly investigate the role of neutrophils in host defense against *E. faecalis* peritonitis (30, 31).
To deplete mice of neutrophils, MAb RB6-C5 was used. This antibody recognizes an antigen, Ly-6G, present on the cell surface of mature neutrophils and eosinophils, and specifically depletes these granulocytes in vivo (12,38). Additionally, Gr-1 is intermediately expressed by other cells, such as a small proportion of monocytes, dendritic cells, CD8+ and CD4+ T cells (10,14,26,41). We found a depletion of Gr-1-expressing monocytes/macrophages after treatment with the RB6-C5 MAb. It is unlikely, however, that the depletion of this Gr-1+ subset of monocytes/macrophages explains the reduced antibacterial defense seen in the RB6-C5 MAb-treated mice, as the proportion of these cells (in uninfected mice) is < 3%. Furthermore, during infection, the amount of circulating monocytes did not differ between the two groups.

Previous studies that used MAb RB6-C5 to investigate the role of neutrophils during Legionella pneumophila and Acinetobacter baumannii infections showed that its primary effect is depletion of neutrophils (36,42). During E. faecium peritonitis, MAb RB6-C5-treated mice attracted fewer macrophages to the peritoneal cavity than their controls, despite a greater enterococcal burden and higher cytokine and chemokine concentrations in their peritoneal fluids. These results are similar to those previously reported by LaFleur et al. [17], which showed that neutrophil depletion by MAb RB6-C5 and subsequent i.p. thioglycollate treatment resulted in 33% reduced peritoneal macrophage recruitment in neutrophil-depleted mice despite unchanged numbers of circulating monocytes. The underlying mechanism for this phenomenon remains to be established but could be related to the release of macrophage-attracting mediators (not investigated in our study or by LaFleur et al. [17]) by neutrophils that have migrated into the peritoneal cavity in the normal situation.

Improving our knowledge on the pathogenesis of E. faecium infections is necessary in face of the increasing prevalence of multiresistant E. faecium isolates. In this study we demonstrate that the attraction of neutrophils to the site of E. faecium infection is an important facet in the early and rapid clearance of this bacterium. Nonetheless, in a neutrophil-deficient but otherwise healthy host, other components of the immune system, e.g., complement proteins, natural antibodies (1,2,15) and monocytes/macrophages, are eventually able to compensate for the deficit in neutrophils.

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References

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