Interaction between inflammation, coagulation and fibrinolysis during infection

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Chapter 2

Endogenous Interleukin 12 Improves the Early Antimicrobial Host Response to murine Escherichia coli Peritonitis

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Abstract

Interleukin (IL)-12 is a heterodimeric proinflammatory cytokine formed by a p35 and a p40 subunit. To determine the role of IL-12 in abdominal sepsis, p35 gene deficient (IL-12 knockout, KO) mice and normal wild type (WT) mice were injected intraperitoneally with Escherichia coli. Peritonitis was associated with a bacterial dose dependent increase in IL-12p40 and IL-12p75 concentrations in peritoneal fluid and plasma. While at 6 hours postinfection IL-12 KO and WT mice displayed similar bacterial counts, at 20 hours IL-12 KO mice had significantly more bacteria in liver homogenates and were more susceptible for progressing to systemic infection. In addition, IL-12 KO mice demonstrated higher levels of proinflammatory cytokines in peritoneal fluid and increased lung and liver injury. IL-12 deficiency did not influence the recruitment of cells to the site of the infection. These data suggest that endogenous IL-12 is involved in the early antibacterial host response during abdominal sepsis.

Introduction

Severe peritonitis is an important cause of death in adult intensive care units (1). Abdominal sepsis is associated with a mortality rate up to 60%, which is much higher than the 25-30% overall mortality rate of sepsis in general (2). Peritonitis is almost invariably caused by a perforation in the intestinal tract. As a result, enteric Gram-negative bacteria are the most commonly encountered pathogens, among which Escherichia coli (E. coli) can be found in up to 60% of cases (2, 3).

Interleukin (IL)-12 is a heterodimeric proinflammatory cytokine (IL-12p70), formed by a p35 and p40 subunit, which is mainly produced by antigen-presenting cells (4, 5). IL-12 is a potent stimulator of T cell functions, inducing the production of interferon (IFN)-γ and facilitating the differentiation of naïve CD4+ T cells into T helper 1 cells. Ample evidence exists that IL-12 also plays an important role in the pathogenesis of Gram-negative bacterial infection and the associated inflammatory response. Administration of live E. coli or purified endotoxin (lipopolysaccharide, LPS), the toxic moiety of the Gram-negative bacterial cell wall, elicits the release of biologically active IL-12p70 into the circulation of experimental animals (6-9). Neutralization of this endogenously produced IL-12 provides a clear survival benefit after a high dose LPS challenge to mice (7, 9, 10). Taken together with the observation that exogenously administered IL-12 can provoke systemic inflammatory responses (11, 12), these data indicate that IL-12 is involved in the toxic sequelae of severe endotoxemia.

Several studies have investigated the role of endogenous IL-12 in host defense against peritonitis. In a model of septic peritonitis induced by cecal ligation and puncture (CLP), treatment with a polyclonal anti-IL-12 antibody was reported to either increase (13, 14) or not to influence (15) mortality. In another peritonitis model, produced by intraperitoneal (i.p.) injection of live E. coli, anti-IL-12 treatment did not influence lethality (7). Both after CLP and i.p. administration of E. coli, the anti-IL-12
antiserum enhanced the outgrowth of bacteria, suggesting that endogenous IL-12 contributes to an effective antibacterial defense (7, 13). Of note, all of these studies made use of polyclonal antibodies raised against recombinant IL-12, and in none of these reports the extent of IL-12 neutralization was established. Moreover, since the p40 component of IL-12p70 is also part of IL-23, a heterodimeric cytokine consisting of a p40 and a p19 subunit, it is possible that these antisera influenced the activity of endogenous IL-23 (5, 16). Therefore, in the present study we sought to determine the role of endogenous IL-12 in the host response to septic peritonitis by making use of p35 gene deficient mice, animals in which IL-12 is the only cytokine that cannot be produced.

Material and methods

**Animals**

The Institutional Animal Care and Use Committee approved all experiments. p35 gene deficient (IL-12 KO) C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Normal wild type C57BL/6 (WT) mice were obtained from Harlan CPB (Zeist, the Netherlands). Sex- and age-matche d (8- to 12-wk old) mice were used in all experiments.

**Induction of peritonitis**

Peritonitis was induced as described previously (17, 18). In brief, *E. coli* O18:K1 was cultured in Luria Bertani medium (LB; Difco, Detroit, MI) at 37°C, harvested at mid-log phase, and washed twice with sterile saline before injection to clear the bacteria of medium. Mice were injected i.p. with $10^2$ to $10^4$ *E. coli* O18:K1 colony-forming units (CFU) in 200 μl sterile isotonic saline. The inoculum was plated immediately after inoculation on blood agar plates to determine viable counts. Control mice received 200 μl normal saline.

**Monitoring of mortality and sample harvesting**

Our laboratory previously established that in this model mortality occurs predominantly between 18h and 24h after *E. coli* challenge (17, 18); therefore, mortality was assessed every hour in this time period, and with 6h intervals thereafter. Mice surviving more than 3 days appeared to be long-term survivors. At the time of sacrifice, mice were first anesthetized by inhalation of isoflurane (Abbott Laboratories Ltd., Kent, UK) / O₂ (2% / 2l). A peritoneal lavage was then performed with 5 ml sterile isotonic saline using an 18-gauge needle, and peritoneal lavage fluid was collected in sterile tubes (Plastipack; Becton-Dickinson, Mountain View, CA). The recovery of peritoneal fluid was > 90% in each experiment and did not differ between groups. After collection of peritoneal fluid, deeper anesthesia was induced by i.p. injection of 0.07 ml/g FFM mixture (Fentanyl (0.315 mg/ml)-Fluanisone (10 mg/ml) (Janssen, Beersen, Belgium), Midazolam (5 mg/ml) (Roche, Mijdrecht, The Netherlands). Next, the abdomen was opened and blood was drawn from the lower
caval vein into a sterile syringe, transferred to tubes containing heparin and immediately placed on ice. Plasma for these determinations was prepared by centrifugation at 1400 x g for 10 min at 4 °C, after which aliquots were stored at -20 °C.

**Determination of bacterial outgrowth**

Serial 10-fold dilutions of blood, homogenized liver and peritoneal lavage fluid were made in sterile saline and 50-μl volumes were plated onto blood agar plates. Plates were incubated at 37°C at 5% CO₂, and CFU’s were counted after 16h.

**Cell counts and differentials**

Cell counts were determined using a hemacytometer (Beckman Coulter, Fullerton, CA). Subsequently peritoneal fluid was centrifuged at 1400 x g for 10 min; the supernatant was collected in sterile tubes and stored at -20°C until determination of cytokines. The pellet was diluted with PBS until a final concentration of 10⁵ cells/ml and differential cell counts were done on cytospin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Dübening, Switzerland) according to the manufacturer’s instructions.

**Assays**

Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-12p40, IL-12p70, interferon (IFN)-γ, macrophage inflammatory protein (MIP)-2 and cytokine-induced neutrophil chemoattractant (KC) were measured by ELISA’s according to the instructions of the manufacturer (all R&D Systems, Abingdon, United Kingdom). Aspartate aminotranspherase (ASAT) and alanine aminotranspherase (ALAT) were determined with commercially available kits (Sigma, St. Louis, MO), using a Hitachi analyser (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions.

**Histology**

Directly after sacrifice, samples from liver and lung were fixed in 10% formaline, and embedded in paraffin for routine histology. Sections of 4 μm thickness were stained with haematoxylin and eosin. All slides were coded and scored by a pathologist without knowledge of the type of mice or treatment.

**Statistical analysis**

All data are expressed as mean ± SE. Differences between groups were analyzed by Mann-Whitney U test. Survival was analyzed with Kaplan-Meier. Values of P < 0.05 were considered to represent a statistically significant difference.
Results

Production of IL-12.

To determine whether IL-12 is produced during peritonitis, C57BL/6 WT mice received an i.p. injection with 200 μl NaCl containing $10^2$, $10^3$, or $10^4$ E.coli CFU’s or 200 μl NaCl as a control. Peritonitis was associated with elevated IL-12p40 and IL-12p70 concentrations in both peritoneal fluid and plasma at 6h and 20h after infection (Table I). IL-12 levels increased with increasing doses of E.coli. After inoculation with $10^2$ E.coli CFU, IL-12p40 peaked at 6h, while after infection with $10^4$ CFU E.coli, IL-12p40 and IL-12p70 levels peaked after 20h. Mice i.p. injected with sterile saline did not have detectable IL-12p70 and significantly lower levels of IL-12p40 in peritoneal lavage fluid or in plasma (data not shown). Subsequent experiments were done with $10^4$ CFU E.coli as inoculum.

<table>
<thead>
<tr>
<th>Peritoneal fluid (pg/ml)</th>
<th>$10^2$ CFU</th>
<th>$10^3$ CFU</th>
<th>$10^4$ CFU</th>
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<tr>
<td></td>
<td>T 6h</td>
<td>T 20h</td>
<td></td>
</tr>
<tr>
<td>IL-12 p40</td>
<td>350 ± 23</td>
<td>74 ± 9</td>
<td></td>
</tr>
<tr>
<td>IL-12 p70</td>
<td>56 ± 8</td>
<td>34 ± 9</td>
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<th>Plasma (pg/ml)</th>
<th>$10^2$ CFU</th>
<th>$10^3$ CFU</th>
<th>$10^4$ CFU</th>
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<tbody>
<tr>
<td></td>
<td>T 6h</td>
<td>T 20h</td>
<td></td>
</tr>
<tr>
<td>IL-12 p40</td>
<td>129 ± 78</td>
<td>60 ± 35</td>
<td></td>
</tr>
<tr>
<td>IL-12 p70</td>
<td>45 ± 3</td>
<td>21 ± 7</td>
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Normal wild type mice were injected i.p. with 200 μl containing $10^2$, $10^3$ and $10^4$ CFU E.coli and sacrificed after 6h and 20h. Results are expressed as mean ± SEM of 8 mice per group. Mice i.p injected with sterile saline did not have detectable IL-12p70 and significant lower levels of IL-12p40 in peritoneal lavage fluid or in plasma (data not shown).

Endogenous IL-12 limits bacterial outgrowth.

To determine the role of endogenously produced IL-12 in early host defense against peritonitis, we first compared the bacterial outgrowth after i.p. inoculation with E. coli in peritoneal lavage fluid (the site of the infection), blood (to evaluate to which extent
Figure 1. IL-12 KO mice demonstrate an enhanced bacterial outgrowth in blood and liver. E. coli CFU in peritoneal lavage fluid (upper panel), blood (middle panel) and liver (lower panel) in IL-12 KO and WT mice 6 and 20h after i.p. administration of 104 CFU E.coli. Data represent medians with interquartile ranges of 8-9 mice per strain at each time point. * P < 0.05 for the difference between groups.

the infection became systemic), and liver of WT compared to IL-12 KO mice (Figure 1). At 6h post-infection, no difference was found among WT mice and IL-12 KO mice. At 20h post-infection, IL-12 KO mice had more bacteria in the blood and liver compared to WT mice (P < 0.05). Also, the peritoneal lavage fluid of IL-12 KO mice contained more bacteria than WT mice, although the difference between the two strains did not reach statistical significance.

**IL-12 is not involved in cell recruitment to the peritoneal cavity.** Since leukocytes play an important role in the local host defense against invading bacteria, we next determined leukocyte counts and differentials in peritoneal lavage fluid during peritonitis. Peritonitis was associated with a profound recruitment of cells to the peritoneal cavity, which was mainly the result of neutrophil influx (Table II). No difference was seen in neutrophils influx in the peritoneal fluid between IL-12 KO and WT mice at either 6 or 20h postinfection.
Figure 2. IL-12 KO mice demonstrate increased liver and lung injury.
Representative view of the histological damage in the lungs (C and D) of WT mice (A and C) and IL-12 KO mice (B and D) 20h after infection. Liver necrosis was more extended in IL-12 KO mice compared to WT mice. The lungs of IL-12 KO mice were also more inflamed than those of WT mice. Numerous thrombi were observed (insert). Slides shown are representative of a total of eight mice per group. (haematoxylin and eosin staining, original magnification x20, insert panel D magnification x40).
Table II

<table>
<thead>
<tr>
<th></th>
<th>T 6h</th>
<th>T 20h</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>IL-12 KO</td>
</tr>
<tr>
<td>Total cells (x10⁶/ml)</td>
<td>2.44 ± 0.91</td>
<td>2.34 ± 0.94</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.66 ± 0.12</td>
<td>1.70 ± 0.99</td>
</tr>
<tr>
<td>Macrophages</td>
<td>0.66 ± 0.12</td>
<td>0.50 ± 0.25</td>
</tr>
<tr>
<td>Others</td>
<td>0.12 ± 0.05</td>
<td>0.14 ± 0.07</td>
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Data are mean ± SE (n = 8 mice per group for each time point) 6 and 20h after i.p. administration of E.coli (10⁴ CFU). WT = wild type mice. IL-12 KO = p35 gene deficient mice. Differences between strains were not significant.

IL-12 KO mice show elevated proinflammatory cytokine levels
To obtain insight into the role of IL-12 in the local production of cytokines and chemokines during septic peritonitis, the concentrations of IFN-γ, TNF-α, IL-1β, MIP-2 and KC were measured in peritoneal lavage fluid 20h postinfection (Table III). Mediator levels did not differ between IL-12 KO and WT mice, except for the proinflammatory cytokines TNF-α and IL-1β, which both were significantly higher in IL-12 KO mice.

IL-12 KO mice demonstrate enhanced lung and liver injury
To evaluate the role of endogenous IL-12 in organ injury during abdominal sepsis, we performed histological analysis of liver and lungs 20h after infection. As shown in figure 2, IL-12 KO mice displayed more severe liver and lung damage than WT mice.

Figure 3. IL-12 KO mice demonstrate enhanced hepato cellular injury. Plasma concentrations of ALAT and ASAT 20h after i.p. injection of E. coli (10⁴ CFU). Filled bars represent WT mice; open bars indicate IL-12 KO mice. Data are mean ± SE of eight mice for each mouse strain. Dotted lines represent the mean values obtained from normal
Table III

<table>
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<tr>
<th>Peritoneal Fluid</th>
<th>WT</th>
<th>IL-12 KO</th>
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<tbody>
<tr>
<td>IFN-γ</td>
<td>87.9 ± 20.1</td>
<td>125.1 ± 29.4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>30.1 ± 35.5</td>
<td>429.5 ± 35.5*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>525.7 ± 168.8</td>
<td>1456.1 ± 304.2*</td>
</tr>
<tr>
<td>MIP-2</td>
<td>1600 ± 333.3</td>
<td>1540 ± 270</td>
</tr>
<tr>
<td>KC</td>
<td>689.9 ± 280.1</td>
<td>1085 ± 410</td>
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Mice received an i.p. injection with $10^4$ CFU E.coli, and peritoneal fluid was harvested after 20h. Data are mean ± SE in pg/ml ($n = 8$ mice per group). WT = wild type mice. IL-12 KO = p35 gene deficient mice. *P < 0.05 vs. WT mice.

Although the lungs of both IL-12 KO and WT mice displayed congestion and interstitial inflammation, these changes were more severe in IL-12 KO mice (figure 2D), than in WT mice (figure 2C). Moreover, in IL-12 KO mice, small thrombi were seen (insert figure 2D), as well as foci of pleuritis (data not shown). The livers of IL-12 KO mice showed numerous thrombi (insert figure 2D) and small areas of necrosis (figure 2D). These pathological changes were more pronounced than in livers of WT mice (Figure 2C). The histopathological findings of the liver were confirmed by clinical chemistry, i.e. IL-12 KO mice had higher plasma concentrations of ALAT and ASAT 20h post infection than WT mice (both P < 0.05; Figure 3).

**Mortality is not influenced by IL-12 deficiency**

To determine the influence of endogenous IL-12 on survival, IL-12 KO and WT mice were infected with $10^4$ E.coli CFU, and followed for 4 days (Figure 4). Survival did not differ between the two mouse strains.

**Figure 4. IL-12 deficiency does not influence survival during murine peritonitis.** Survival of WT and IL-12 KO mice infected i.p. with $10^4$ CFU E.coli (n=16 per group). Mice that survived more than 4 days appeared to be long-term survivors. The difference between groups was not significant.
Discussion

Peritonitis is a serious clinical condition that frequently results in systemic dissemination of bacteria and septic shock. Early production of IL-12 is part of the host response to severe peritonitis (7, 13), and previous studies found that the administration of anti-IL-12 antiserum impairs host defense mechanisms in mice with Gram-negative abdominal infection (7, 13). However, these investigations left two questions unanswered, i.e. whether endogenous IL-12 was completely eliminated by the polyclonal antibody and whether the antibody also influenced the activity of IL-23, a heterodimeric cytokine that like IL-12 contains a p40 subunit. We here firmly establish, using p35 gene deficient mice, which completely and selectively lack IL-12, that IL-12 contributes to an effective early antibacterial host response in E. coli induced peritonitis. Indeed, IL-12 KO mice displayed higher bacterial counts in liver and blood, which was accompanied by an exaggerated inflammatory response as reflected by higher proinflammatory cytokine levels and increased lung and liver injury.

Our current data are in line with the study by Zisman et al. (7), although considerable differences with the present investigation can be pointed out. These authors induced peritonitis by i.p. injection of another E. coli strain (American Type Tissue Collection #33985) at a dose of 2 x 10^8 CFU. At 24h postinfection the median bacterial count in peritoneal lavage fluid was slightly above 10 CFU/mL in mice treated with preimmune serum versus 10^9 CFU/ml in animals treated with anti-IL-12 serum (7). In contrast, we injected 10^4 E. coli CFU (>4 logs less) and recovered approximately 10^9 CFU/ml peritoneal lavage fluid at 20h postinfection. Apparently, Zisman et al. used a model of peritonitis with a less virulent E. coli strain that was cleared by the host over time (7), whereas we used a highly virulent strain that was able to grow in infected mice. Nonetheless, both investigations point to a role for endogenous IL-12 in antibacterial defense: the data reported by Zisman et al. suggest that anti-IL-12 treatment delays the clearance of E. coli during peritonitis (7), whereas our study using IL-12 KO mice indicate that the complete lack of endogenous IL-12 facilitates the ongoing outgrowth of bacteria. Mortality was not influenced by inhibition or elimination of IL-12 in either model. The role of endogenous IL-12 has also been examined in the model of peritonitis induced by CLP. Like after i.p. injection of live E. coli, CLP results in a rise in IL-12 concentrations in peritoneal lavage fluid and plasma (13, 19). Treatment of mice with anti-IL-12 antiserum resulted in a diminished survival (13, 14), and an increased number of aerobic bacterial CFU in the peritoneal lavage fluid (19). Overall, the results obtained with anti-IL-12 antiserum resemble our present data obtained with IL-12 KO mice in murine peritonitis. This would argue against an additional role for IL-23 in the host response to E. coli peritonitis. In accordance, we found that mice deficient for p40 and therefore lacking both IL-12 and
IL-23, demonstrate a similarly enhanced bacterial outgrowth in this model as IL-12 KO mice (our own unpublished data).

IL-12 KO mice did not show an altered influx of hematopoietic cells into the peritoneal cavity, indicating that IL-12 is not involved in this inflammatory response. Remarkably, IFN-γ levels were not influenced by IL-12 deficiency, although IL-12 is considered to be the most potent inducer of IFN-γ (4, 5). While we do not have a firm explanation for this finding, it should be noted that IFN-γ concentrations were rather low in both mouse strains and that the bacterial load was higher in IL-12 KO mice, providing a more potent proinflammatory stimulus. This may also explain why the levels of TNF-α and IL-1β were higher in IL-12 KO than in WT mice. In the same line, we consider it likely that the enhanced lung and liver injury in IL-12 KO mice was related to the increased bacterial load in these animals. In this respect, our current findings strongly resemble our earlier observations in IL-18 KO mice in the same model (18). Indeed, like IL-12 KO mice, IL-18 KO mice also showed unaltered low IFN-γ levels (even though IL-18 is the most important cofactor for IL-12 induced IFN-γ production (4, 20)) in the presence of higher bacterial loads and increased lung and liver damage. Together these data support the concept that a number of proinflammatory cytokines act as double-edged swords in the early phase of bacterial infection. Indeed, whereas cytokines like IL-12 and IL-18 play an essential part in the systemic toxicity induced by high dose LPS challenges (7, 9, 10, 21, 22), the local production of these mediators apparently contributes to an effective antibacterial defense (7, 13, 18).

Recently, mice deficient for Signal transducer and activator of transcription (Stat) 4, which is essential for IL-12 signaling, were reported to demonstrate a reduced lethality after peritonitis induced by CLP (14, 19), which was associated with an unaltered bacterial outgrowth in one report (19), and reduced bacterial counts in another (14). These findings contrast with findings that anti-IL-12 treatment enhanced lethality after CLP together with increased bacterial loads (13, 14). Moreover, in contrast to IL-12 KO mice in the current study, Stat4 KO mice displayed less organ injury during CLP induced peritonitis (19). The disparate effects of IL-12 and Stat4 deficiency suggest that IL-12 activates pathways other than Stat4 to initiate the host response during abdominal sepsis.

We here demonstrate that IL-12 is produced at the site of the infection during experimentally induced *E. coli* peritonitis. Endogenous IL-12 contributes to an adequate host response by limiting the bacterial outgrowth and thereby reducing secondary tissue injury. Hence, IL-12 production is part of a protective early immune response to abdominal sepsis caused by *E. coli*.
References


