Interaction between inflammation, coagulation and fibrinolysis during infection

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Chapter 3

Interleukin-18 Improves the Early Antimicrobial Host Response to *Escherichia coli* Peritonitis

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Abstract

To determine the role of endogenous IL-18 during peritonitis, IL-18 gene-deficient (IL-18 KO) mice and wild-type (WT) mice were intraperitoneally (i.p.) infected with *Escherichia coli*, the most common causative agent found in septic peritonitis. Peritonitis was associated with a bacterial-dose dependent increase in IL-18 concentrations in peritoneal fluid and plasma. After infection, IL-18 KO mice had significantly more bacteria in the peritoneal lavage fluid and were more susceptible for progressing to systemic infection at 6 and 20h post-inoculation. The relative inability of IL-18 KO mice to clear *E. coli* from the abdominal cavity was not due to an intrinsic defect in the phagocytosing capacity of their peritoneal macrophages or neutrophils. IL-18 KO mice displayed an increased neutrophil influx into the peritoneal cavity, but these migrated neutrophils were less activated as reflected by a reduced CD11b surface expression. These data suggest that endogenous IL-18 plays an important role in the early antibacterial host response during *E.coli* induced peritonitis.

Introduction

Despite advances in diagnosis, surgery, anti-microbial therapy and intensive care support, the mortality rate associated with severe peritonitis remains high (5, 6). In particular sepsis that originates from the peritoneal cavity has a grim prognosis with mortality rates up to 80% (13). *Escherichia coli* (*E.coli*) is one of the most common causative pathogens (up to 60%) in intraperitoneal (i.p.) infections (18).

Cytokines play an important role in the pathogenesis of bacterial infections (32). The local activity of proinflammatory cytokines is required for an adequate antimicrobial defense against localized bacterial infections. On the other hand, systemic activity of proinflammatory cytokines such as observed during fulminant sepsis, can be toxic to the host and contribute to multiple organ failure and death. Our laboratory recently provided evidence for this dual effect of cytokine activity in a murine model of *E. coli* peritonitis (28). Indeed, mice deficient for the anti-inflammatory cytokine interleukin (IL)-10 (IL-10 knockout or KO mice) demonstrated an enhanced bacterial clearance from the abdominal cavity and a diminished dissemination of the infection to distant organs after i.p. injection of live *E. coli*. In spite of these findings, systemic inflammation and multiple organ failure were more prominent in IL-10 KO mice, and lethality was increased.

IL-18 was originally identified as an interferon (IFN)-γ inducing factor (IGIF), but now is generally recognized as a proinflammatory cytokine (1, 7). IL-18 can induce a wide array of inflammatory responses in different cell types, including activation of nuclear factor-κB, Fas ligand expression and induction of chemokines. IL-18 plays an important role in the host response to lipopolysaccharide (LPS), the toxic component of the Gram-negative bacterial cell membrane. IL-18 KO mice tolerated a 50% higher LPS dose than wild type (WT) mice (12), and treatment with an anti-IL-18 antiserum 34
protected mice against the lethal effects of both *E. coli* and *Salmonella* LPS (21). Moreover, treatment of mice with a fusion construct consisting of recombinant human IL-18 binding protein and human IgG1 Fc also conferred a strong protective effect against lethality after administration of LPS (9). Only few investigations have addressed the role of IL-18 in host defense against Gram-negative infection in vivo, demonstrating that passive immunization of mice against IL-18 impaired host defense against *Salmonella typhimurium* or *Yersinia enterocolitica* (4, 8, 19). The role of endogenous IL-18 in host defense against peritonitis is unknown. Therefore, in the present study we sought to determine the role of IL-18 in the local and systemic host response to abdominal sepsis caused by *E. coli*, making use of IL-18 KO mice.

**Material and methods**

**Animals**

The Institutional Animal Care and Use Committee approved all experiments. IL-18 KO mice were generated as described previously (30). IL-18 KO mice were on the C57BL/6 background. Normal C57BL/6 WT mice were obtained from Harlan Sprague-Dawley (Horst, The Netherlands). Sex- and age-matched (8- to 12-wk old) mice were used in all experiments.

**Induction of peritonitis**

Peritonitis was induced as described previously (28). 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.

**Reagents**

Rabbit anti-murine IL-18 antiserum, kindly donated by Dr. C. Dinarello, was prepared as described previously (10). The anti-IL-18 serum contained <10 pg/ml endotoxin as determined by *Limulus* assay. Anti-IL-18 antiserum (200 μl) was given i.p. 1 h before intraperitoneal administration of bacteria. This dose significantly reduced endotoxin-induced IFN-7 release and lethality in mice (21). Rabbit serum (Sigma-Aldrich, St. Louis, MO) was used as control. In other experiments, recombinant murine IL-18 (MBL, Naka-ku Nagoya, Japan) in a dose of 0.1μg/200μl was given i.p. 1 h before i.p. administration of bacteria. Saline was used as a control.

**RT-PCR**

Livers were harvested at 20h after administration of *E. coli* or sterile saline (controls), snap-frozen in liquid nitrogen, and stored at -70°C. To extract total cellular RNA,
lungs from three mice per group were pooled and homogenized in 1 ml of TRIzol Reagent (Life Technologies, Grand Island, NY). Then total RNA was isolated using chloroform extraction and isopropanol precipitation. The RNA pellet was dissolved in 100 μl of diethylpyrocarbonate-treated water and quantified by spectrophotometry. Reverse transcription was performed by mixing 2 μg of total cellular RNA with 0.5 μg of oligo(dT) (Life Technologies) in a total volume of 12 μl. The mixture was incubated at 72°C for 10 min. Thereafter, 8 μl of a solution containing 4 μl of 5x First Strand buffer (Life Technologies), 10 mM DTT (Life Technologies), 1.25 mM dNTPs (Amersham Pharmacia Biotech, Little Chalfont, U.K.), and 100 U of Superscript Reverse Transcriptase (Life Technologies) was added and the mixture was incubated at 42°C for 1 h. Finally, the tubes were heated to 72°C for 10 min after which 180 μl of H₂O was added to the reaction mixture. Samples were stored at -20°C until further use. For PCR, 5 μl of cDNA solution was mixed with 20 μl of a solution containing 1x PCR buffer (67 mM Tris-Cl (pH 8.8), 6.7 mM MgCl₂, 10 mM 2-ME, 0.67 μM of EDTA, 16.6 mM (NH₄)₂SO₄, 2% DMSO (Merck, München, Germany), 1.25 μg of BSA (New England Biolabs, Beverly, MA), 0.5 U of AmpliTaq DNA polymerase (PerkinElmer, Branchburg, NJ), and 75 ng of sense- and antisense oligonucleotide primers specific for IL-18 and β-actin (internal standard). The PCR were performed in a thermocycler (Gene Amp. PCR System 9700; PerkinElmer) using the following conditions: 94°C for 5 min (1 cycle) followed immediately by 95°C for 1 min, 58°C for 1 min, 72°C for 1 min (with variable numbers of cycles), and a final extension phase of 72°C for 10 min. For semiquantitative assessment of IL-18 mRNA, variable numbers of cycles were used to ensure that amplification occurred in the linear phase. To exclude the possibility of finding differences between tubes due to unequal concentrations of cDNA in the PCR, a PCR using β-actin as the internal standard was performed on each sample. β-actin was found to be linear at 27 amplification cycles, and IL-18 was found to be linear at 29 amplification cycles. The primers used for IL-18 (433 bp) were 5'-ACTGTACAACCGCAGTAATACGG-3' (sense) and 5'-AGTGAACATTACAGATTTATCCC-3' (antisense), and for β-actin (617 bp) 5'-GTCAGAAGGACTCCTATGTG-3' (sense) and 5'-GCTCGTTGCCAATAGTGATG-3' (antisense). PCR products were visualized by agarose gel electrophoresis.

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 (28); therefore, mortality was assessed every hour in this time period, and with 6-h intervals thereafter. Mice surviving more than 3 days appeared to be permanent survivors. At the time of sacrifice, mice were first anesthetized by inhalation of isoflurane (Abbott Laboratories Ltd., Kent, UK) / O₂ (2% / 21). 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 cytopsin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland) according to the manufacturer's instructions.

**FACS analysis**

FACS analysis was used to measure granulocyte activation in peritoneal lavage. FACS analysis was done by using a FACScan (Becton Dickinson). At least 5000 cells were analyzed in each sample. Erythrocytes were lysed with ice-cold isotonic NH₄CL solution (155 mMol/l NH₄CL, 10 mMol/l KHCO₃, 0.1 mMol/l EDTA, pH 7.4) for 10 minutes. Incubations for FACSScan analysis were performed in 96-well V-shaped micro plates (Greiner B.V., Alphen a/d Rijn, the Netherlands). For staining, 5x10⁵ cells/well were incubated with the following rat anti–mouse monoclonal antibodies: FITC-labelled anti-Ly-6G (Gr-1) and PE labelled anti-CD11b (1:100; all Pharmingen, San Diego, CA). The appropriate isotype controls (Pharmingen) were included in all experiments. Cells were incubated on ice for 30 min, and washed twice with cold FACS buffer (PBS supplemented with 0.01%NaN₃, 0.5 % Bovine Serum Albumen (BSA) and 0.3 mM EDTA) and re-suspended in FACS buffer. Granulocyte CD11b expression was determined by forward scatter and side scatter gating and by gating Ly-6G positive cells. Results are expressed in mean fluorescence of Ly-6G positive cells.

**Phagocytosis**

The analysis of uptake of *E.coli* by peritoneal macrophages and granulocytes was performed essentially as described (36). Macrophages were isolated from the
peritoneal cavity of untreated mice and 0.5x10^6 cells/well were cultured overnight in RPMI containing 10% fetal calf serum at 37°C to allow adherence. Macrophages were washed twice with Hanks’ balanced salt solution (HBSS) prior to the addition of labeled bacteria. Peritoneal granulocytes were harvested 5 hours after i.p. injection of 10% (w/v) proteose peptone (Becton Dickinson, Sparks, MD) (34). Peritoneal exudate cells were treated with 160 mM NH₄Cl and 10 mM KHCO₃ to lyse erythrocytes, washed with HBSS and plated at 0.5x10^6 cells/well. E.coli was heat-killed by incubation at 65°C for 1 hour and labeled with 0.2 mg/ml FITC (Sigma-Aldrich, St. Louis, MO) in 0.1 M NaHCO₃ pH 9.0 for 1 hour at 37°C. FITC-labeled E.coli (equivalent to 50x10^6 CFU) were added to the cells (bacteria : cell ratio of 100:1) and incubated for 1 hour (granulocytes) or 2 hours (macrophages) at 37°C or 4°C. Phagocytosis was stopped by immediate transfer of the cells to 4°C and washing with ice-cold FACS buffer (PBS containing 0.5% bovine serum albumin, 1.5 mM NaN₃ and 0.35 mM EDTA). Cells were treated with vital blue stain (Orpegen, Heidelberg, Germany) to quench extra cellular fluorescence, washed with FACS buffer and analyzed using a flowcytometer (Becton Dickinson FACScalibur). Macrophages and granulocytes were gated based on forward and side light scatter. Based on this selection criterion, the purity of the macrophages and neutrophils yielded more than 95%. Results are expressed as phagocytosis index, quantified as the percentage of cells with internalized E.coli times the mean fluorescence intensity.

Assays
IL-18, IL-12, IFN-γ, macrophage inflammatory protein-2 (MIP-2), and 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-18.

To determine whether IL-18 is produced during peritonitis, WT mice received an i.p. injection with 200 μl NaCl containing $10^2$, $10^3$, or $10^4$ E.coli CFU or 200 μl NaCl as a control. Peritonitis was associated with elevated IL-18 concentrations in both peritoneal fluid and plasma at 6h and 20h after infection (Figure 1A). IL-18 levels increased with increasing doses of E.coli and were higher in peritoneal fluid than in plasma. After inoculation with $10^2$ E.coli CFU, IL-18 peaked at 6h, while after infection with $10^3$ CFU E.coli, IL-18 levels peaked after 20h. Mice i.p. injected with sterile saline did not have detectable IL-18 in peritoneal lavage fluid or in plasma (data not shown). In IL-18 KO mice IL-18 was undetectable at all time points. To determine whether IL-18 mRNA is also produced during E.coli peritonitis; RT-PCR was performed on liver samples obtained from mice 20h after i.p. administration of saline or E.coli. A faint band of IL-18 mRNA was found in livers of mice that received saline, indicating that some IL-18 mRNA is constitutively expressed (Figure 1B). I.p. infection with E.coli was associated with enhanced expression of IL-18 mRNA, as indicated by equal intensity of β-actin bands and clear differences in band intensity between control and peritonitis samples for IL-18 RT-PCR products.

Figure 1A. Enhanced IL-18 production during peritonitis. IL-18 levels, measured by ELISA, in peritoneal fluid and plasma. WT 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. Filled bars represent IL-18 concentrations in peritoneal lavage fluid (PLF); open bars indicate IL-18 concentrations in plasma.
IL-18 KO mice show an increased bacterial outgrowth.

To determine the role of IL-18 in early host defense against peritonitis, we compared the bacterial outgrowth after i.p. inoculation with $10^4$ CFU of *E. coli* in peritoneal lavage fluid (the site of the infection), blood (to evaluate to which extent the infection became systemic), and in liver of WT and IL-18 KO mice (Figure 2). At both 6h and 20h post-infection, IL-18 KO mice had more bacteria in the peritoneal lavage fluid.
and blood than WT mice (P < 0.05). In addition, livers of IL-18 KO mice contained more bacteria compared to WT mice, although at 20 h post infection the difference between the two strains did not reach statistical significance. Hence, IL-18 KO mice demonstrated a reduced capacity to clear *E. coli* from the primary infectious site in association with an enhanced dissemination of the infection.

**Treatment of WT mice with anti-IL-18 and recombinant murine IL-18.**
Compensatory immune mechanisms may develop in mice that genetically lack the IL-18 signaling pathway. To determine whether the differences between IL-18 KO and WT mice were caused solely by the absence of IL-18, we injected WT mice intraperitoneal (i.p) with *E.coli* 1 h after i.p injection of 200 µl anti-IL-18 antibodies.

**Figure 3. Anti-IL-18 increases and rIL-18 decreases bacterial outgrowth.** WT mice were treated with 200-µl anti-IL-18 (A), or with 0.1 µg r-IL-18 (B). As control, normal rabbit serum and NaCl resp. were used. *E.coli* CFU in peritoneal lavage fluid (left panel) and blood (right panel) in WT mice 20h after i.p. administration of 10^4 CFU *E.coli*. 1h prior to infection, mice received either anti-IL-18 antibodies/r-IL-18 (filled symbols) or control serum/saline (open symbols). Horizontal lines represent medians. * P < 0.05 for the difference between groups.
The results of the experiments with IL-18 KO mice could be replicated in this experiment, i.e., treatment with anti-IL-18 antibodies in WT mice increased the number of CFU recovered from peritoneal lavage fluid and blood at 20h post infection relative to that observed in WT mice after treatment with control serum (Figure 3A). To obtain further evidence for an antibacterial effect of IL-18 during E. coli peritonitis, we injected mice i.p. with r-IL-18 (0.1 µg) 1h prior to infection, and determined the number of CFU 20h after the bacterial challenge (Figure 3B). IL-18 treated mice displayed a decreased outgrowth in both peritoneal lavage fluid and blood (P < 0.05 versus controls).

**IL-18 KO mice have an increased neutrophil 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 influx of cells into the peritoneal cavity, which was mainly the result of neutrophil influx (Table 1). IL-18 KO mice had more neutrophils in their peritoneal fluid than WT mice at both 6 and 20h postinfection (P < 0.05).

Table I

<table>
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<tr>
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<th>6h</th>
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<tr>
<td>IL-18 WT</td>
<td>IL-18 KO</td>
<td>IL-18 WT</td>
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<tr>
<td>Total cells (x10^6/ml)</td>
<td>7.6 ± 1.4</td>
<td>11.2 ± 2.4*</td>
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<tr>
<td>Neutrophils</td>
<td>5.8 ± 1.5</td>
<td>7.9 ± 1.4*</td>
</tr>
<tr>
<td>Macrophages</td>
<td>1.3 ± 0.6</td>
<td>2.6 ± 0.7</td>
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<tr>
<td>Lymphocytes</td>
<td>0.5 ± 0.3</td>
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**Table I.** IL-18 KO mice have an increased granulocyte influx into the peritoneal fluid during peritonitis Mice were sacrificed at 0h, 6h and 20h after i.p. administration of 10^4 CFU E.coli to obtain peritoneal lavage fluid. Data are mean ± SEM. Each group consisted of 8 mice for each time point, except the T=0 group (5 mice). * p<0.05 vs. WT mice.

**IL-18 KO mice have a reduced CD11b expression on peritoneal neutrophils** The extent of CD11b expression at the surface of neutrophils has been used as an activation marker of this cell type (17, 25, 33). Since IL-18 is able to activate neutrophils in vitro, which includes upregulation of CD11b (17), we considered it of interest to compare neutrophil CD11b expression at the site of the infection in IL-18 KO and WT mice (Figure 4). Neutrophils derived from peritoneal fluid of IL-18 KO mice obtained 20h after infection expressed less CD11b at their surface than peritoneal neutrophils from WT mice (P < 0.05).
Figure 4. IL-18 KO mice have a decreased CD11b expression on peritoneal neutrophils. CD11b expression was determined by FACS analysis on neutrophils harvested from peritoneal fluid at 20h post infection as described in the Methods section. Filled symbols represent WT mice; open symbols indicate IL-18 KO mice. Horizontal lines represent medians. Data in mean cellular fluorescence (MCF) * P < 0.05 for the difference between groups.

* * *

Unchanged phagocytosis of E.coli by IL-18 deficient peritoneal neutrophils and macrophages.

The increased bacterial load in IL-18 KO mice could be caused by an intrinsic defect of IL-18 deficient cells to phagocytose E.coli. To investigate this possibility we harvested neutrophils and macrophages from uninfected IL-18 KO and WT mice, and compared their capacity to phagocytose FITC labelled E.coli (Figure 5). Both peritoneal neutrophils and macrophages displayed a normal ability to phagocytose E.coli.

Figure 5. Phagocytosis of E.coli by peritoneal macrophages and neutrophils of IL-18 KO mice is unchanged. Phagocytosis of FITC-labeled E.coli by macrophages and granulocytes harvested from peritoneal fluid from uninfected mice was determined by FACS analysis as described in the Methods section. Filled bars represent WT mice; open bars represent IL-18 KO mice. Results (means ± SE) are expressed as phagocytosis index, quantified as the percentage of cells with internalized E.coli times the mean fluorescence intensity.

Chemokine and cytokine response

The mouse CXC chemokines MIP-2 and KC have been implicated in the attraction of neutrophils to the site of an infection (23, 35). Therefore, the concentrations of these mediators were measured in peritoneal fluid (Figure 6).
IL-18 KO mice had higher concentrations of both MIP-2 and KC at the site of the infection than WT mice (P < 0.05). In addition, since IL-18 together with IL-12 is required for optimal induction of IFN-γ (1, 7), the levels of IL-12 and IFN-γ were measured in peritoneal fluid (Figure 6). Whereas IL-12 concentrations were higher in IL-18 KO mice (P < 0.05), IFN-γ levels did not differ between IL-18 KO and WT mice.

**IL-18 KO mice demonstrate enhanced lung and liver injury**

To evaluate the role of endogenous IL-18 in organ injury during abdominal sepsis, we performed histological analysis of liver and lungs stained 20h after infection. As shown in figure 7, IL-18 KO mice displayed more severe liver and lung damage than WT mice (figure 7). The lungs of both mouse strains displayed congestion and interstitial inflammation. These changes were more severe in IL-18 KO mice (figure 7D), than in WT mice (figure 7C). Moreover, in IL-18 KO mice, small thrombi were seen (insert figure 7D) as well as foci of pleuritis (data not shown). In the livers of IL-18 KO mice, numerous thrombi were observed (figure 7B). These changes were more pronounced in livers of IL-18 KO mice, compared to WT mice (figure 7A). The histopathological findings of the liver were confirmed by clinical chemistry, i.e. IL-18 KO mice had higher plasma concentrations of ALAT and ASAT 20h post infection than WT mice (both P < 0.05; Figure 8).
Figure 7. **IL-18 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-18 KO mice (B and D) 20h after infection. Liver necrosis was more extended in IL-18 KO mice compared to WT mice. The lungs of IL-18 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. (hematoxylin and eosin staining, original magnification x20, insert panel D magnification x40).

**Survival**

To determine the influence of IL-18 on survival, IL-18 KO and WT mice were infected with $10^4$ *E.coli* CFU, and followed for 4 days (Figure 9). Survival did not differ between the two mouse strains.
IIL-18WT  IL-18 KO

Figure 8. IL-18 KO mice demonstrate enhanced hepatocellular 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-18 KO mice. Data are mean ± SE of eight mice for each mouse strain. Dotted lines represent the mean values obtained from normal plasma of mice that were i.p. injected with sterile saline (six mice). Data in units/liter (U/L). * P < 0.05 vs. WT mice.

Figure 9. IL-18 deficiency does not influence survival during murine peritonitis. Survival of WT and IL-18 KO mice infected i.p. with E.coli (10⁴ CFU; n=10 per group). No difference was found between the lethality in the two mouse strains. Mice that survived 3 days were permanent survivors.

Discussion

Severe bacterial infection is associated with enhanced production of IL-18, as reflected by elevated circulating levels of this proinflammatory cytokine in patients with sepsis (11, 16, 22). In experimental animals, several organs have been found to express IL-18 constitutively, especially liver and lung, and an increase in the plasma concentrations of IL-18 was detected after administration of high doses of LPS (3, 14, 21). In the present investigation we sought to determine the extent of IL-18 production during abdominal sepsis caused by E. coli, and to determine the role of this endogenously produced IL-18 in the host response to this infection. We here confirm constitutive expression of IL-18 mRNA in liver, and demonstrate bacterial dose dependent release of IL-18 in peritoneal lavage fluid and plasma. In addition, IL-18 KO mice were less able to clear bacteria from the site of infection and showed an increased dissemination of the infection to the blood compartment, indicating that IL-18 plays an important regulatory role in the early local antimicrobial host defense against E. coli. This was in accordance with the results obtained by treating WT mice with anti-IL-18 or IL-18 before infecting the mice with E.coli.
Peritonitis is characterized by the recruitment of phagocytic cells, especially neutrophils, to the site of infection (28, 35). Neutrophil influx into the peritoneal cavity was markedly increased in IL-18 KO mice 6h and 20h after *E.coli* administration. This finding was unexpected in light of a previous investigation that showed enhanced influx of neutrophils into peritoneal fluid of mice i.p. injected with recombinant IL-18 (17). The most likely explanation for the increased peritoneal neutrophil numbers in IL-18 KO mice is that they are the consequence of the increased proinflammatory stimulus provided by the higher bacterial load. Theoretically, the increased recruitment of neutrophils to the peritoneal fluid of IL-18 KO mice may in part also have been mediated by the elevated local concentrations of the CXC chemokines MIP-2 and KC, which are known to contribute to neutrophil attraction to sites of the bacterial infection (23, 35). Likely, the elevated MIP-2 and KC concentrations in peritoneal fluid of IL-18 KO mice were also the consequence of the enhanced outgrowth of *E. coli*, especially considering that in contrast to our study with living bacteria, treatment of mice with anti-IL-18 Abs before challenge with *E.coli* LPS was accompanied by a significant decrease in MIP-2 levels, and a diminished neutrophil accumulation in lungs and liver (21). Furthermore, IL-18 has been reported to increase rather than to inhibit the production of IL-8, the prototypic human CXC chemokine, in vitro (17, 24).

A recent study identified a role for IL-18 in the activation of neutrophils (17). IL-18 induced cytokine and chemokine release from human peripheral blood derived neutrophils, induced degranulation, enhanced respiratory burst upon stimulation with FMLP and up-regulated the surface expression of the activation marker CD11b. We here demonstrate that endogenous IL-18 plays a role in the activation of neutrophils recruited to the peritoneal cavity during *E. coli* peritonitis. Indeed, IL-18 KO mice displayed a reduced expression of CD11b at the surface of neutrophils recovered from their peritoneal fluid. These data are the first to indicate that IL-18 may contribute to neutrophil activation during infection in vivo.

IL-18 has been implicated, together with IL-12, in optimal production of IFN-γ (1, 7). IL-18 in particular seems important for IFN-γ production induced by a Gram-negative stimulus such as LPS. Indeed, elimination of endogenous IL-18 decreased IFN-γ release in mice during endotoxemia (9, 12, 21), whereas IL-18 did not contribute to IFN-γ release after administration of the superantigen staphylococcal enterotoxin B to mice (12, 15). Although IFN-γ concentrations in peritoneal fluid did not differ between IL-18 KO and WT mice, it is possible that the higher bacterial load in the former mouse strain compensated for the IL-18 deficiency, and that IL-18 does play a role in IFN-γ production during Gram-negative infection. IL-18 KO mice displayed more signs of lung and liver injury. Likely, the increased bacterial loads in these mice played an important role herein. Indeed, passive immunization against IL-18 diminished systemic inflammation elicited by LPS (21). Moreover, *Proprionibacterium acnes*-primed IL-18 KO mice have been found to be resistant against LPS-induced liver injury (26).
IL-18 exerts cellular effects by a specific interaction with the IL-18 receptor complex, consisting of a high affinity ligand binding chain (IL-18Rα or IL-18Rα) and a signal-transducing element (IL-18R accessory protein or IL-18Rβ) (29). The intracellular signaling cascade induced after triggering of the IL-18R complex is highly similar to the signaling cascades induced after stimulation of the Toll-like receptor (TLR) family. Indeed, these distinct receptors all use the same intracellular adapter molecules (MyD88, IRAK and TRAF6) and elicit similar responses (activation of NFκB, JNK and p38 MAP kinase (2, 29, 31). In this respect it is interesting to note that MyD88 gene deficient mice were reported to have a normal capacity to clear bacteria from their peritoneal cavity after induction of peritonitis induced by placing a stent in the colon ascendens (37). Yet, MyD88 KO mice displayed an enhanced survival in this abdominal sepsis model, presumably due to a relatively attenuated systemic inflammatory response. In contrast, mice lacking TLR4, the receptor considered to be essential for the recognition of LPS and Gram-negative bacteria, did not demonstrate an altered survival (37). Several mutually nonexclusive possibilities may explain these observations on the role of IL-18, TLR4 and MyD88 in abdominal sepsis, including differences in the models used, in the extent and localization of IL-18 production, and in the cellular and tissue distribution of the IL-18R, TLR4 and MyD88.

Previous investigations have addressed the role of IL-18 in host defense against Gram-negative bacterial infection in vivo. Administration anti-IL-18 to mice intravenously infected with *S. typhimurium* was associated with a relatively enhanced outgrowth of bacteria in liver and spleen seven days after the infection (19). In line with these findings, anti-IL-18 treatment increased bacterial growth in spleens of mice intravenously infected with *Yersinia enterocolitica* and IL-18 KO mice demonstrated a higher bacterial load in their lungs after intranasal infection with *Shigella flexneri* (4, 27). IL-18 also contributes to an effective host defense against Gram-positive infection, including systemic infection with *Listeria monocytogenes* and pneumonia caused by *Streptococcus pneumoniae* (14, 20). The present investigation provides the first evidence for a role of IL-18 in the early antibacterial defense against i.p. infection, as indicated by the fact that IL-18 KO mice had a reduced ability to clear *E. coli* from their peritoneal cavity and were less capable of preventing dissemination of the infection. This finding was supported by the fact that exogenous treatment with recombinant IL-18 facilitated antibacterial defense in this model. Notably, similar and even higher doses of recombinant IL-18 did not influence the outgrowth of *Yersinia enterocolitica* in an intravenous infection model (4), whereas daily treatment with IL-18 starting 2 days for intravenous infection with *Salmonella typhimurium* did reduce bacterial counts in liver and spleen (19). In our study IL-18 deficiency did not impact on survival. We recently reported similar findings in pneumococcal pneumonia, i.e. in that model IL-18 KO mice also demonstrated an increased bacterial outgrowth, yet did not have a reduced survival (14). Together these data suggest that IL-18 in particular contributes to the early host response to bacterial infection.
The current study indicates that IL-18 is produced at the site of the infection during experimentally induced *E. coli* peritonitis, where it facilitates an optimal host response by limiting the bacterial outgrowth and thereby reducing secondary tissue injury. Thus, IL-18 production is part of a protective early immune response to abdominal sepsis caused by *E. coli*.

**References**

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