Protection from lethal septic peritonitis by neutralizing the biological function of interleukin 27

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Sepsis exemplifies a complex clinical syndrome characterized by a severe infection in the body and bloodstream that most commonly originates in the lung, urinary tract, and abdomen (1, 2). Although sepsis is generally associated with a high mortality, patients with septic peritonitis have a particularly high mortality rate of 60–80%. Septic peritonitis is characterized by massive infiltration of neutrophils and macrophages into the peritoneum where these cells are the first line of defense for clearing invading microorganisms. However, once they fail to restrict microbes to the peritoneal cavity, microbes may reach the bloodstream, resulting in an overwhelming systemic immune response via production of proinflammatory mediators such as cytokines (1, 2). Such mediators in turn appear to play a key role in the pathogenesis of septic shock or multiorgan failure after bacterial infections.

Recently, IL-27 has been identified as a new bioactive member of the IL-12 cytokine family (3). It consists of an IL-12 p40–related polypeptide, denoted EBV-induced gene 3 (EBI3), and a novel p28 subunit with some similarities to IL-12 p35 and IL-23 p19, respectively (3, 4). The IL-27 heterodimer mediates its biological function via binding to a specific receptor on target cells consisting of the orphan receptor WSX-1/TCCR and the widely expressed gp130 protein (5). Over the last several years, IL-27 has emerged as a pivotal cytokine in the adaptive immune system by controlling T cell–dependent immune responses. Hereby, IL-27 activates STAT1 and STAT3 in naive CD4 T cells and NK cells. While STAT1 phosphorylation is required for IL-27–mediated activation of the Th1 master transcription factor T-bet (6), STAT3 is considered to be important for IL-27–induced T cell proliferation (7). DCs and macrophages have been identified as rapid producers of IL-27 subunits after...
Toll-like receptor (TLR) ligation (8), suggesting that IL-27 may act very early in Th1-mediated immunity. However, recent studies demonstrated that the biological function of IL-27/WSX-1 signaling is more complex, as it is also critically involved in the negative control of both Th1 and Th2 inflammatory responses (9–11). Finally, mice deficient for the EBI3 subunit of IL-27 showed reduced iNK T cell numbers and cytokine production, suggesting that EBI3 controls iNK T cell activity (12).

Because WSX-1 is highly expressed on naive T cells, IL-27–related research primarily concentrated on the biology of T cells; however, WSX-1 and gp130 are also expressed on B cells, DCs, macrophages, and mast cells, suggesting that IL-27 function is not restricted to T cells. In fact, stimulation of human mast cells and blood monocytes with rIL-27 led to the activation of STAT transcription factors and production of proinflammatory cytokines (5). However, IL-27 signaling has also been implicated in STAT3-dependent negative regulation of murine mast cells and activated macrophages (9, 13).

Cytokines have been shown to be critically involved in both protective and pathogenic antimicrobial immune responses. As an imbalance in cytokine responses may result in persistent infections or destructive systemic inflammatory response leading to multiorgan failure and death (1), a detailed understanding of local cytokine function during infections is of crucial importance for therapy of sepsis. However, the factors that determine the immune response during sepsis are still incompletely understood. Here, we demonstrate a crucial role of IL-27 in innate immunity and experimental septic peritonitis.

RESULTS AND DISCUSSION

Early local and systemic up-regulation of IL-27 expression after cecal ligation and puncture (CLP)

Septic peritonitis is characterized by an acute inflammatory response with a rapid production of cytokines. We and others have recently shown that microbial stimuli strongly induce IL-27 mRNA expression in vitro (3, 8), suggesting that this novel cytokine could be involved in the pathogenesis of septic peritonitis and shock. Therefore, we analyzed in an initial series of studies the expression of IL-27 in the murine CLP model of bacterial peritonitis. Accordingly, we analyzed the expression of the EBI3 and p28 subunits of IL-27 in organ lysates of septic animals and found that EBI3 and p28 mRNA levels in the lung and spleen were substantially increased 6 h after CLP, as determined by quantitative PCR (Fig. 1 A). Interestingly, IL-27 EBI3 and p28 mRNA levels during CLP were up-regulated earlier and stronger than mRNA levels of IL-12 and IL-23 subunits, consistent with a potential role of IL-27 in innate immunity and the early pathogenesis of septic peritonitis. However, although EBI3 mRNA levels went back to almost normal levels 20 h after CLP, p28 mRNA levels still remained up-regulated. To confirm the data.

Figure 1. Local and systemic accumulation of IL-27 protein in mice with septic peritonitis. 8-wk-old C57BL/6 mice (n = 5) were subjected to sham or CLP surgery. (A) Organs were removed for total RNA isolation at the indicated time points, and quantitative real-time PCR was performed. Mean values are shown as fold induction relative to transcript levels in mice 6 h after sham surgery. (B) 6 or 20 h later, blood was collected by cardiac puncture and peritoneal lavage fluid was obtained by washing the peritoneal cavity with 5 ml of sterile PBS. Samples were assayed for IL-27 content by specific sandwich ELISA. (C) Resident peritoneal macrophages and elicited neutrophils were isolated as described in Materials and methods and stimulated for 20 h with 100 ng LPS. Supernatants were assayed for IL-27 content by ELISA. One representative experiment out of three is shown.
described above at the protein level, we performed IL-27–specific ELISA and detected a strong up-regulation of IL-27 protein in the blood and peritoneal lavage fluid shortly after CLP (Fig. 1 B). In addition, experiments with resident peritoneal macrophages and elicited neutrophils demonstrated that these cells produce high amounts of IL-27 upon stimulation with LPS in wild-type mice (Fig. 1 C), whereas such cells from TLR4 signaling–deficient C3H/Hej mice did not up-regulate IL-27 expression in response to LPS (macrophages: C3H, 122 pg/ml vs. C3H/Hej, 0 pg/ml; neutrophils: C3H, 87.6 pg/ml vs. C3H/Hej, 0 pg/ml). Thus, our data suggested that these cells are an important source of IL-27 upon TLR ligation in wild-type mice.

IL-27 EBI3 deficiency protects from sepsis-induced lethality
To determine the contribution of IL-27 to the mortality in sepsis after CLP, we performed studies in mice lacking the EBI3 subunit of IL-27. As shown in Fig. 2 A, survival rates in EBI3−/− mice were significantly higher than in the control group. Although all wild-type mice died within 4 d after CLP, 15% of the EBI3−/− mice survived for more than 2 wk. In addition, i.p injection of 4 × 10⁸ live Escherichia coli led to high lethality in wild-type mice, whereas all EBI3−/− mice survived (Fig. 2 B). These data suggested that IL-27 EBI3−/− mice are more resistant to lethality induced by microbial infections.

Because IL-27 EBI3 deficiency was found to protect mice from CLP-associated lethality, we next examined whether administration of recombinant IL-27 would reverse this effect. Accordingly, we generated recombinant IL-27 (rIL-27) as specified below in Materials and methods. EBI3−/− mice were then subjected to CLP and injected i.p. with 10 μg rIL-27 or saline immediately after CLP. Treatment with rIL-27 markedly increased mortality of IL-27 EBI3–deficient mice,
suggesting that IL-27 plays an important regulatory role in septic peritonitis (Fig. 2 C).

**Increased early leukocyte influx and bacterial clearance in IL-27 EBI3−/− mice**

In an initial attempt to explore the functional role of IL-27 in septic peritonitis, we next examined whether decreased mortality in EBI3−/− mice is associated with changes in cell numbers of the peritoneal cavity. Therefore, we assessed the leukocyte influx into the peritoneum after CLP and found that the granulocyte infiltration at 4 h was significantly augmented in IL-27–deficient mice as compared with wild-type mice (Fig. 3 A). Similar results were obtained 4 h after i.p. challenge of wild-type and knockout mice with 1.5 ml of 3% thioglycollate (Fig. 3 A).

Because an early removal of bacteria is critical for the prevention of an overwhelming systemic immune response, we subsequently compared bacterial numbers in the serum and peritoneum of EBI3−/− mice and controls. Interestingly, EBI3−/− mice had significantly lower numbers of bacteria both in the blood and peritoneum than EBI3+/+ mice 24 h after CLP (Fig. 3 B) or i.p. administration of 2 × 10⁸ live *E. coli* (Fig. 3 C). Collectively, these data indicated that EBI3−/− mice have a higher capacity to eliminate microbial infections and thus to suppress septic peritonitis.

**IL-27 down-regulates protective innate immune responses of myeloid cells**

It has been recently shown that a variety of immune cells besides T and NK cells may express both IL-27 receptor chains (5). We thus evaluated whether murine macrophages and polymorphonuclear neutrophils express WSX-1. RT-PCR and Western blot analysis revealed that both cell types express WSX-1 (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20060471/DC1) and thus can potentially respond to IL-27 stimulation.

Because the findings described above indicated that the improved survival of EBI3−/− mice was related to a more effective bacterial clearance and local control of infection, we next compared the bactericidal activity of leukocytes between EBI3−/− and EBI3+/+ mice by analyzing reactive oxygen intermediates (ROI). Mice were injected with 1.5 ml of 3% thioglycollate for 4 and 24 h to generate an inflammatory stimulus, and their potency to produce ROI was determined using flow cytometry. Interestingly, granulocytes from EBI3−/− mice displayed a markedly enhanced ability to produce ROI as compared with EBI3+/+ mice, suggesting that IL-27 negatively regulates the biological functions of granulocytes (Fig. 4 A). To further verify the latter hypothesis, we next isolated elicited leukocytes from wild-type C57BL/6 mice and stimulated them for 12 h with LPS or LPS plus rIL-27. As shown in Fig. 4 B,
rIL-27 down-regulated LPS-induced activation and ROI production of both granulocytes and monocytes/macrophages. However, rIL-27 alone had no effect on ROI production, suggesting that this cytokine controls inducible ROI production upon TLR ligation. In a subsequent series of studies, we determined systemic cytokine levels using bead multiplexing technology. Although no differences in levels of IL-10, TNF, and IFN-γ were noted, levels of IL-1 and IL-6 were significantly reduced in EBI3−/− mice as compared with wild-type mice at 20 h after CLP, suggesting that the enhanced control of infection in EBI3−/− mice results in lower production of some proinflammatory cytokines as compared with wild-type mice (Fig. 4 C).

A soluble IL-27 receptor fusion protein protects mice from CLP-induced mortality

Because EBI3−/− mice were protected from septic peritonitis, we finally determined the potential beneficial effects of neutralizing the biological function of IL-27 in vivo. As soluble gp130 protein does not interfere with IL-27 signaling (14), we attempted to block the biologic activity of IL-27 by generating a soluble form of its high-affinity receptor WSX-1. Accordingly, we constructed a fusion protein of the extracellular part of WSX-1 with the Fc part of IgG and expressed the resulting designer fusion protein, denoted sIL-27R, in CHO cells (Fig. 5 A). In vitro studies showed that sIL-27R was able to inhibit IL-27–induced proliferation of splenic CD4+ T cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20060471/DC1).

Subsequently, we induced septic peritonitis in C57BL/6 mice by CLP followed by a single i.p treatment with the sIL-27R fusion protein immediately or 2 h after surgery. The survival rates in sIL-27R–treated mice were higher than in the saline-treated control group (Fig. 5 B), showing that blockade of IL-27 is an effective therapy for experimental septic peritonitis.

IL-27: a novel key player in septic peritonitis

Early immune responses to bacterial infections are primarily mediated by neutrophils and macrophages (1, 15–17) expressing pattern recognition receptors that bind to specific molecular structures of microorganisms. The triggering of pattern recognition receptors and TLR signaling then initiate the secretion of proinflammatory mediators, which promote the elimination of infectious agents. However, excessive inflammation in bacterial infections can lead to marked tissue damage and lethal septic shock (1, 2). Thus, the immune response induced by neutrophils and macrophages during bacterial infections needs to be tightly controlled. Here, we have identified the cytokine IL-27 as a novel regulator that controls the magnitude of the innate immune reaction during experimental septic peritonitis. These findings may also at least partially explain the antiinflammatory effects of IL-27/WSX-1 signaling observed in various parasitic infections, e.g. Toxoplasma gondii and Leishmania donovani (10, 18). Thus, in addition to negative regulatory functions of IL-27 on T lymphocytes (11), IL-27 exerts profound effects on the activation of myeloid cells during infections.
The regulation of neutrophil functions during septic peritonitis is still incompletely understood. However, C5a/C5aR and TREM signaling have been recently identified as important modulators of the biological function of these cells (19–22). Here, we found that IL-27 subunits were rapidly produced by both macrophages and neutrophils during septic peritonitis. This rapid production is presumably due to augmented EBI3 and p28 gene transcription, as both promoters contain TLR-responsive NF-κB binding elements (8).

Our data suggest a novel key role of IL-27 in regulating innate immunity and neutrophil function during septic peritonitis. Specifically, IL-27 modulates neutrophil influx/activation by an as yet unknown mechanism that presumably involves modulation of chemokines. Consistently, we recently found that IL-27 suppresses production of various chemokines in myeloid cells such as RANTES and I-TAC that control migration of immune cells (unpublished data). Furthermore, we identified IL-27 as a key negative regulator of oxidative burst by neutrophils. The clinical relevance of these observations was underlined in two models of experimental peritonitis.

First, we observed that EBI3Δ/Δ mice were protected from experimental septic peritonitis, and injection of recombinant IL-27 prevented such protection. Second, we found that EBI3Δ/Δ mice have significantly reduced mortality upon i.p. injection of live E. coli. In both models, IL-27 EBI3Δ/Δ mice had significantly reduced numbers of bacteria in the peritoneum and blood associated with an increased oxidative burst capacity of neutrophils. The fact that neutrophils produce a cytokine such as IL-27 that inhibits their influx/activation may represent an autoregulatory loop to limit neutrophil responses, or, more likely, may indicate that IL-27 has additional positive functional roles that remain to be elucidated.

Collectively, our data suggest a major role of IL-27 in modulating influx and oxidative burst of granulocytes and elimination of bacteria during peritonitis. Thus, early IL-27 production by myeloid cells may exert a negative feedback mechanism that limits protective innate immune responses in peritonitis. The therapeutic implication of these observations was finally highlighted by the finding that blockade of IL-27 function using a newly designed sIL-27R, fusion protein suppressed experimental septic peritonitis and reduced mortality in vivo. Thus, our data provide novel insights into the cytokine-driven regulation of neutrophil function during sepsis. Furthermore, blockade of IL-27 function by sIL-27R could be a novel treatment modality for patients with septic peritonitis.

MATERIALS AND METHODS

Animals and sepsis models. C57BL/6 mice were obtained from Charles River Laboratories. EBI3−/− mice have been described previously (12). Animals were bred and maintained under specific pathogen-free conditions in our animal facility. All experiments involving animals were performed under protocols approved by the Animal Ethics and Experimentation Committee of The Johannes Gutenberg University of Mainz. For CLP, mice were anesthetized by i.p. injection of ketamin/xylazin. A 1-cm abdominal midline incision was made, and the exposed cecum was ligated with a 5-0 silk suture below the ileocecal valve and punctured with an 18-gauge needle. Mice subjected to sham CLP underwent the same procedure, except for ligation and puncture of the cecum. In an additional series of experiments, mice were injected i.p. with live E. coli K12. For survival analysis, mice were monitored twice daily for 14 d.

Measurement of cytokines. For measurement of cytokines in peritoneal lavage fluid or serum/plasma, we used the mouse FlowCytomix kit ( Bender MedSystems) according to the manufacturer’s instructions using a FACSCalibur System (Becton Dickinson). IL-27 levels were determined by sandwich ELISA (R&D Systems).

Determination of CFUs. Serial dilutions of peripheral blood or peritoneal lavage fluid of CLP-treated or E. coli–injected mice were plated on Caso blood agar plates (HiMedia) at 37°C. CFUs were determined after 24 h.

Determination of leukocyte oxidative burst activity. Intracellular respiratory burst activity was measured separately in monocytes and neutrophils using the Phagoburst kit (Orpegen) according to the manufacturer’s instructions. In brief, 100 μl of heparinized peripheral blood was mixed with 20 μl of a substrate solution containing nonfluorescent dihydrodihromamine 123 (DHK) and incubated for 10 min at 37°C. Oxidation of DHK to the green fluorescent rhodamine 123 was used as an indicator of respiratory burst. The percentage of ROI-producing cells was counted in a flow cytometer (FACSCalibur, Becton Dickinson). To discriminate monocytes and granulocytes, gates were set by a linear FCS/SSC analysis.

Isolation of resident macrophages and elicited neutrophils. For isolation of resident macrophages, mice were injected with 5 ml PBS. Macrophages were isolated from peritoneal lavage by plastic adherence. For isolation of granulocytes, mice were injected i.p. with 1.5 ml sterile thioglycollate (3%). Elicited cells were harvested 4 h later by peritoneal lavage with 5 ml of cold PBS. In some experiments, neutrophils were further purified from peritoneal lavage by magnetic cell sorting. In brief, cells were labeled with an FITC-conjugated anti-neutrophil antibody (Caltag) and sorted to a purity of >95% with the FITC multisort kit (Miltenyi Biotec).

Quantitative analysis of gene expression. Total cellular RNA was extracted from cells and organs with RNeasy columns (QIAGEN), including DNase I digestion. Quantitative real-time PCR analysis for IL-27 EBI3 and p28, IL-12 p35, IL-23 p19, IL-12/IL-23 p40, and HPRT was performed using specific Quantitect Primer/Probe assays (QIAGEN).

scIL-27 and sIL-27R. For construction of bioactive murine IL-27, the cDNAs encoding for EBI3 and p28 were cloned from LPS-stimulated splenocytes by reverse transcriptase PCR. For cloning of single-chain IL-27 (scIL-27), fragments encoding the EBI3 part, followed by a Val4Gly4Pro4 linker and the mature coding sequence of p28, were generated by PCR and cloned into the p3XFlag expression vector (Sigma–Aldrich). To ensure efficient secretion, the EBI3 leader sequence was replaced by the signal peptide of IgG1.

The cDNA coding for a soluble WSX fusion protein was generated by ligation of the extracellular portion of WSX-1 to the Fc part of IgG2a. The extracellular part of WSX-1 was cloned into pCDNA3.1 TOPO expression vector by PCR using Phusion (Stratagene) and a primer introducing a factor Xa cleaving site followed by a XhoI site (pcDNA3.1–WSX). The Fc part of IgG2a was amplified from cDNA of the OKT3 hybridoma and cloned into the XhoI-cut pCDNA3.1–WSX vector. For protein expression, CHO cells were transfected with 5 μg of linearized plasmid DNA and stable transfectants were isolated by selection with 1 mg/ml G418. Culture supernatants of stable transfectants were harvested, and the fusion protein was purified from the supernatant with Fastflow protein G columns (GE Healthcare).

Statistical analysis. Data are expressed as mean values ± standard deviations. Statistical analysis was performed using the unpaired Student’s t test or with a log rank test for survival analysis (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Online supplemental material. Fig. S1 shows the expression of the IL-27 receptor on mouse-resident peritoneal macrophages and elicited neutrophils.
Fig. S2 shows the specific down-regulation of IL-27–induced proliferation of T cells by sIL-27R. Fig. S3 shows a comparison of HMGB1 expression in septic EB3+/− and EB3+/+ mice. Figs. S1–S3 are available at http://www.jem.org/cgi/content/full/jem.20060471/DC1.

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REFERENCES