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Multiple Roles for IL-12 in a Model of Acute Septic Peritonitis

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The present study addressed the role of IL-12 in a murine model of septic peritonitis, induced by cecal ligation and puncture (CLP). Although CLP surgery induced IL-12 production at 6 and 24 h after surgery, IL-12 immunoneutralization was clearly deleterious in this model: 54% of CLP mice receiving preimmune serum survived, whereas mice administered IL-12 antisera prior to CLP experienced a 25% survival rate. IL-12 immunoneutralization not only led to increased mortality, but also appeared to promote a shift away from IL-12 and IFN-γ, in favor of IL-10. This cytokine shift corresponded to changes in bacterial load, as CLP mice receiving IL-12 antisera yielded more CFUs from the peritoneal cavity at 24 h after CLP. To address the role of bacterial infection in IL-12 antisera-induced mortality following CLP, antibiotics were administered for 4 days after surgery. Despite regular antibiotic administration, IL-12 immunoneutralization still reduced survival in CLP mice. Furthermore, histology of the ceca revealed that mice administered IL-12 antisera failed to show typical organization of the damaged cecum wall. Accordingly, Gram staining revealed bacteria within peritoneal fluids from these mice, while peritoneal fluids from CLP mice that received preimmune serum and antibiotics were free of bacteria. Altogether, these data suggested multiple important roles for IL-12 in the evolution of murine septic peritonitis. The Journal of Immunology, 1999, 162: 5437–5443.

Sepsis is characterized by an acute, systemic immune response to a variety of noxious insults, particularly bacterial infections. The principle physiological symptoms often associated with sepsis syndrome are shock, coagulopathy, fever or hypothermia, tachycardia, tachypnea, and multorgan dysfunction. Ultimately, 25–35% of all septic episodes end in death (1, 2), while those patients suffering from septic peritonitis experience a much higher mortality rate of 60–80% (3). Bacterial sepsis commences with the initiation of the host’s innate immune response to bacterial infection. The Gram-negative bacterial cell wall component, LPS, mediates many of the pathophysiological symptoms of sepsis by inducing the release of TNF-α and IL-1. These host-derived, early response cytokines initiate a cascade of inflammatory cytokines, which together mediate the hemodynamic changes and inflammatory events that typify sepsis (4). IL-12 plays a critical role in this early inflammatory process by augmenting the production of IFN-γ and other cytokines from NK and T cells, by enhancing the cytolytic activity of CTLs and NK cells, and by stimulating the proliferation of activated T and NK cells (5). Furthermore, IL-12 appears to be a vital component of the host defense against both Gram-positive and Gram-negative bacterial organisms, as evidenced by the heightened host resistance conferred by IL-12 administration in several bacterial infection models (6–10). However, the proinflammatory effects of IL-12 can become detrimental during sepsis. The exaggerated proinflammatory response often associated with IL-12 production during sepsis may result in many of the injurious and sometimes fatal physiological symptoms of the disease (11, 12).

Host-derived, anti-inflammatory mediators like IL-10 counterbalance the IL-12-mediated inflammatory responses to bacterial pathogens. For example, IL-10 exerts potent in vitro and in vivo antiinflammatory effects, in part, by suppressing the production of proinflammatory cytokines, including TNF-α, IL-1, and IFN-γ, and by repressing macrophage and neutrophil phagocytic and bactericidal activities (13–15). Various studies have shown that a septic response dominated by proinflammatory cytokines is toxic. Conversely, tipping the cytokine balance in favor of an anti-inflammatory response with either IL-10 treatment or IL-12 neutralization has proved beneficial in various sepsis models (13, 16–18); however, in a murine model of neonatal, bacterial sepsis, IL-12 neutralization was clearly detrimental, suggesting that the severe suppression of proinflammatory mediators may also harm septic humans (19).

In this study, we explored the role of IL-12 in a clinically relevant model of bacterial sepsis, namely cecal ligation and puncture (CLP).3 The CLP mouse serves as a clinically relevant disease model, because it mimics the septic response sometimes associated with postsurgical or accidental trauma. Furthermore, this model meets the two general diagnostic criteria for sepsis, as the CLP model, because it mimics the septic response sometimes associated with postsurgical or accidental trauma. Furthermore, this model meets the two general diagnostic criteria for sepsis, as the CLP model, because it mimics the septic response sometimes associated with postsurgical or accidental trauma. Furthermore, this model meets the two general diagnostic criteria for sepsis, as the CLP model, because it mimics the septic response sometimes associated with postsurgical or accidental trauma. Furthermore, this model meets the two general diagnostic criteria for sepsis, as the CLP model, because it mimics the septic response sometimes associated with postsurgical or accidental trauma. Furthermore, this model meets the two general diagnostic criteria for sepsis, as the CLP model, because it mimics the septic response sometimes associated with postsurgical or accidental trauma. Furthermore, this model meets the two general diagnostic criteria for sepsis, as the CLP model, because it mimics the septic response sometimes associated with postsurgical or accidental trauma. Furthermore, this model meets the two general diagnostic criteria for sepsis, as the CLP model, because it mimics the septic response sometimes associated with postsurgical or accidental trauma. Furthermore, this model meets the two general diagnostic criteria for sepsis, as the CLP model, because it mimics the septic response sometimes associated with postsurgical or accidental trauma. Furthermore, this model meets the two general diagnostic criteria for sepsis, as the CLP model, because it mimics the septic response sometimes associated with postsurgical or accidental trauma. Furthermore, this model meets the two general diagnostic criteria for sepsis, as the CLP model, because it mimics the septic response...
produces a cascade of events that ultimately blocks bacterial clearance and tissue repair. These studies support the vital role of IL-12 in the host response during experimental sepsis in which the host faces the dual threat of pathophysiological imbalances and bacterial colonization.

Materials and Methods

Animals

Specific pathogen-free CD-1 mice (6- to 12-wk-old females, Charles River Breeding Laboratories, Wilmington, MA) were used in all experiments. All mice were housed in specific pathogen-free conditions within the animal care facility at the University of Michigan Unit for Laboratory Animal Medicine (ULAM, Ann Arbor, MI) until the day of sacrifice.

Animal model

The CLP model was used as previously described (17). The mice were anesthetized by i.p. injection with 3–3.5 mg of ketamine HCl (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA) followed by inhaled methoxyflurane (Metafane, Pitman-Moore, Mundelein, IL) as needed. The mice were given a 1- to 2-cm longitudinal incision to the lower left quadrant of the abdomen. The cecum was opened and the distal one-third was ligated with 3-0 silk suture, and punctured through and through with a 21-gauge needle. The cecum was then replaced in the peritoneal cavity and the incision was closed with surgical staples. In sham controls, the cecum was not ligated or punctured, but returned to the abdominal cavity. All mice were administered 1 ml of sterile saline s.c. as a fluid resuscitation measure immediately following surgery.

Murine cytokine ELISA

Murine IFN-γ, IL-12, and IL-10 were quantitated using a modification of a double-ligand method as previously described (22). Briefly, flat-bottom 96-well microtiter plates (Immu-Plate I 96-F; Nunc, Glostrup, Denmark) were coated with 50 μl/well of rabbit Ab against the various cytokines (1 μg/ml in 0.6 M NaCl, 0.26 M H3BO3, and 0.08 M NaOH, pH 9.6) for 16 h at 4°C and then washed with PBS, pH 7.5, 0.05% Tween-20 (wash buffer). Microtiter plate nonspecific-binding sites were blocked with 2% BSA in PBS and incubated for 90 min at 37°C. Plates were rinsed four times with wash buffer and diluted (neat and 1:10) cell-free supernatants (50 μl) in duplicate were added, followed by incubation for 1 h at 37°C. Plates were washed four times, followed by the addition of 50 μl/well biotinylated rabbit Ab against the specific cytokines (3.5 μg/ml in PBS (pH 7.5), 0.05% Tween-20, and 2% FCS), and plates incubated for 30 min at 37°C. Plates were washed four times. Streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) was added, and the plates were incubated for 30 min at 37°C. Plates were washed again four times and chromogen substrate (Bio-Rad Laboratories) was added. The plates were incubated at room temperature to the desired extinction, and the reaction was terminated with 50 μl/well of 3 M H2SO4 solution. Plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant murine cytokines from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected murine cytokine concentrations above 25 pg/ml, and recombinant murine cytokines from 1 pg/ml to 100 ng/ml. This ELISA method was terminated with 50 μl/well of rabbit Ab against the various cytokines (1 μg/ml in PBS, pH 7.5, 0.05% Tween-20, and 2% FCS), and plates incubated for 30 min at 37°C. Plates were washed four times. Streptavidin-peroxidase conjugate (Bio-Rad Laboratories) was added, and the plates were incubated for 30 min at 37°C. Plates were washed again four times and chromogen substrate (Bio-Rad Laboratories) was added. The plates were incubated at room temperature to the desired extinction, and the reaction was terminated with 50 μl/well of 3 M H2SO4 solution. Plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant murine cytokines from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected murine cytokine concentrations above 25 pg/ml, and recombinant murine cytokines from 1 pg/ml to 100 ng/ml. This ELISA method was utilized to compare bacterial CFU scattergrams. All calculations were performed on a Power Macintosh 7200 computer using Prism 2.0 (Graphpad Software, San Diego, CA). Significance was assigned for p values < 0.05.

Results

CLP induces IL-12 production

IL-12 production in CLP animals was initially examined because of the well-documented proinflammatory properties of this cytokine and its previously identified role in various other models of sepsis (5, 19, 25–27). Mice underwent CLP or sham surgery, and peritoneal and plasma IL-12 concentrations were analyzed by ELISA at 6 and 24 h after surgery (Fig. 1). IL-12 levels were undetectable in samples from the peritoneum and plasma of sham-operated mice at 6 and 24 h. However, animals experiencing CLP-induced sepsis produced significant levels of immunoreactive IL-12 at both time points.

FIGURE 1. CLP induces IL-12 production. CLP or sham surgeries were performed. Peritoneal lavage and plasma samples were collected at 6 h (A) and 24 h (B) after surgery. IL-12 concentrations were evaluated by ELISA. Experimental n = 6–8 per group.
IL-12 neutralization does not improve survival in antibiotic-treated CLP mice

We next explored the specific role of IL-12 in the CLP model by coupling the administration of IL-12-specific Abs with a full course of antibiotic therapy. As previously described, mice were administered IL-12-specific antisera or preimmune serum i.p. 2 h prior to CLP surgery, after which the mice were dosed with Primaxin (200 μg/dose) at the time of surgery and every 8 h thereafter. In CLP mice administered preimmune serum, the antibiotic treatment improved survival from 46 to 66%, suggesting that Primaxin partially prevented the detrimental effects of bacterial leakage into the peritoneum (Fig. 5). Nonetheless, IL-12 neutralization remained deleterious in the context of antibiotic therapy, as the survival rate decreased below 50% in CLP mice treated with both anti-IL-12 serum and antibiotics. These results suggested that the role of IL-12 during CLP-induced sepsis may extend beyond the activation of the innate immune response.

Passive immunization of IL-12 in the CLP model impairs the fibrotic wound-healing response in and around the injured cecum

To examine why IL-12 neutralization continued to be detrimental in the context of antibiotic treatment, Gram stains were performed on cytospins from peritoneal lavages of CLP mice treated with antibiotics and anti-IL-12 or preimmune serum. At 4 days after CLP surgery, bacteria were present in peritoneal fluids from mice administered both IL-12 antisera and antibiotics (Fig. 6B). In contrast, Gram stains of peritoneal fluids from control mice receiving preimmune serum and antibiotics revealed no evidence of bacteria (Fig. 6A). Upon gross morphological examination of the abdominal cavities of CLP mice, those mice administered preimmune serum and antibiotics displayed extensive fibrotic adhesions around the cecum, whereas CLP mice given IL-12 antisera and antibiotics showed no similar response. Furthermore, histological examination of cross-sections of the cecum revealed the formation of a fibrotic membrane or “pseudomembrane” on the lumen side of the cecum in control mice treated with preimmune serum and antibiotics, whereas this response was not evident in CLP mice treated with IL-12 Abs (Fig. 7). This pseudomembrane appeared to be composed of cellular debris, inflammatory cells, and extracellular matrix. Although differences in cellular recruitment were not directly assessed, at 4 days after CLP surgery ELISAs revealed no differences in several important chemotactic cytokines. However, increased levels of IFN-γ in the peritoneum 4 days after CLP surgery corresponded with the lack of pseudomembrane formation in the anti-IL-12 Ab-treated mice (data not shown). Increased IFN-γ production may have contributed to the delay in pseudomembrane formation or the fibrotic response, and as a consequence may have
contributed to the increased mortality observed in CLP mice passively immunized to IL-12 and treated with antibiotics.

Discussion
The present study queried the following possibilities. 1) Could IL-12 neutralization render a cytokine profile that was less favorable to the innate immune response during sepsis? 2) Since Th cell-mediated responses have been shown to be reciprocally modulated by IL-12 and IL-10 (20, 21), could IL-10 and IL-12 antagonistically regulate each other in an acute inflammatory disease, such as CLP-induced murine sepsis? Our observation that CLP induced IL-12 production provided the impetus to assess the contribution of this cytokine by systemically neutralizing IL-12 in the context of CLP-induced sepsis. The administration of

FIGURE 3. Immunoneutralization of IL-12 in CLP mice results in reduced IFN-γ concomitant with increased IL-10 production at 6 h and 24 h. IL-12 antisera or preimmune serum was administered at 2 h prior to CLP surgery. Peritoneal and plasma samples were collected at 6 h and 24 h after CLP surgery. Cytokine concentrations were measured by ELISA. Experimental n = 6–9 per group. *p < 0.05 as compared with preimmune serum control.
anti-IL-12 polyclonal Abs 2 h prior to CLP surgery resulted in a
significant decrease in survival. One possible reason for this de-
creased survival could have been some treatment-induced physi-
ological imbalance. However, this explanation seemed unlikely as
IL-12 has been identified as a deleterious byproduct in various
physiological models of septic shock, and IL-12 neutralization has
proved beneficial in an endotoxin-induced inflammatory response
(26, 30). Conversely, it seemed probable that CLP mice required
IL-12 production to contain the bacteria, as evidenced by several
studies with various infection models, which have shown that an
IL-12-mediated innate immune response is vital for the resolution
of infection (6, 8, 9, 31). Accordingly, we quantitated total bacte-
rrial CFU in the peritoneal cavities of CLP mice treated with pre-
immune or anti-IL-12 serum, and found that the immunoneu-
tralization of IL-12 in this model reduced bacterial clearance in the
peritoneum. The results from the present study agree with previous
observations that used a neonatal, murine sepsis model caused by
group B streptococci in which IL-12 neutralization induced lethal-
ity by facilitating bacterial infection (19). We subsequently sought
to examine how IL-12 contributed to the containment of the bac-
terial peritonitis associated with the CLP model.

While it has previously been shown that recurring peritoneal infe-
don is not directly responsible for the poor outcome associ-
ated with peritonitis (32), the manipulation of the balance between pro-
and anti-inflammatory cytokines following the immunoneu-
tralization of IL-12 may permit lethal bacterial colonization of the

FIGURE 4. The immunoneutralization of IL-12 reduces peritoneal bac-
terial containment. Mice were treated with either IL-12-specific antisera
(aIL-12) or preimmune serum (control) 2 h prior to CLP surgery, and
peritoneal lavages were performed at 6 (A) or 24 h (B) after surgery. Ex-
perimental n = 9–21. Line represents median CFU count, *, p < 0.05
as compared with preimmune serum control.

FIGURE 5. The immunoneutralization of IL-12 in antibiotic-treated
CLP mice reduces survival compared with CLP mice treated with preim-
mune serum and antibiotics. IL-12-specific antisera or control serum was
administered 2 h prior to CLP surgery. Antibiotic treatments (Primaxin:
200 μg/dose) were administered immediately after CLP surgery and every
8 h thereafter. Experimental n = 15–47 per group.

FIGURE 6. Gram staining of peritoneal wash cyto-
spins at 4 days after CLP surgery. Control (A): pretreat-
ment with preimmune serum followed by full course of
antibiotic therapy. Experimental (B): pretreatment with
IL-12-specific antisera followed by full course of antibi-
otic therapy. IL-12-specific antisera or control serum
was administered 2 h prior to CLP surgery. Antibiotic
treatments (Primaxin: 200 μg/dose) were administered
immediately after CLP surgery and every 8 h thereafter.
Representative of 7–10 samples per group.
peritoneum. An examination of cytokine concentrations substantiated this hypothesis, as the diminished presence of immunoreactive IL-12 resulted in decreased IFN-γ in favor of elevated IL-10. Although IL-12 was classically described as an important mediator of Th-1 phenotypic cell-mediated immune responses, recently more diverse roles have been ascribed to this cytokine. The contribution of IL-12 to innate immune activation appears especially relevant in the context of an active bacterial infection, as host responses to a gamut of intracellular and extracellular pathogens necessitates IL-12 activity. Furthermore, monocyte-derived IL-12 production has been shown to be vital in human patients suffering from postoperative sepsis (33). Although, IL-12 can directly augment host innate immunity by increasing cytolytic activity of CD8+ and NK cell populations, its most important contribution to host defense in the CLP model may be indirect inflammatory cell activation via up-regulation of IFN-γ production by NK and T cells (5, 11, 34). As a hallmark cytokine of the innate immune response, IFN-γ up-regulates HLA-DR expression, primes macrophages for enhanced TNF and IL-1β synthesis, and functions to stimulate PMN and macrophage microbicidal activity. Conversely, IL-10 serves an important immunoregulatory role during an inflammatory response by modulating potent inflammatory processes and by preventing tissue injury and/or shock. In addition, there is well-documented evidence for the detrimental effects of IL-10 neutralization in various sepsis and infection models, including CLP (17, 35, 36). It has been shown in various infection models that too much IL-10 can detrimentally hinder host defense, in part by suppressing the production of important activating and/or chemotactic cytokines and by inhibiting neutrophil and macrophage phagocytic and bactericidal activity (14, 37–41). The CLP model mirrors many of the common clinical symptoms of a septic response to polymicrobial infection. Thus, it is likely that excessive IL-10 production without a sufficient IL-12- and IFN-γ-mediated response may prevent disease resolution. It appears that in the CLP model, IL-12 neutralization effects an imbalanced cytokine profile in the peritoneal cavity, resulting in profound, early suppression of the innate immune response leading to increased lethality in this bacterial sepsis model.

To further determine whether bacterial colonization associated with IL-12 neutralization increased sepsis-induced lethality, anti-IL-12 polyclonal Abs were administered, followed by antibiotic treatment. CLP mice receiving preimmune serum or IL-12 benefited from antibiotic therapy, yet IL-12 immunoneutralization resulted in a worse prognosis for CLP mice treated with antibiotics. Despite aggressive antibiotic treatments in the anti-IL-12 Ab treatment group, we explored the possibility that the septic mice undergoing IL-12 immunoneutralization still suffered from bacterial contamination. In order to explore this hypothesis, mice were sacrificed at 4 days after CLP surgery. Gram stains of peritoneal wash cytospins revealed no obvious bacterial presence in those mice administered antibiotics in addition to preimmune serum, whereas CLP mice treated with anti-IL-12 serum and antibiotics showed clear evidence of bacteria in the peritoneum. This positive identification of bacteria in the peritoneum at 4 days after CLP suggested either 1) a persistent bacterial colonization within the peritoneal cavity or 2) some deficiency in the containment of intestinal microbes within the bowel. An examination of the injured ceca suggested the latter, as CLP mice receiving preimmune serum and antibiotic therapy displayed fibrotic encasement of the cecum, with fibrous adhesions to other portions of the bowel. Histologic examination of cross-sections of the damaged cecae revealed that CLP mice undergoing antibiotic treatment had developed a protective membrane on the lumen side of the cecum wall. In contrast, CLP mice administered IL-12-specific Abs and antibiotics were deficient in both the fibrotic response around the cecum and lacked evidence of pseudomembrane formation inside the cecum wall. While inflammation of the intestinal wall leading to fibrosis is a well-recognized response in chronic inflammatory bowel diseases (42), the present study suggests that the fibrotic containment of the damaged cecum is an important protective mechanism in the acute response to CLP induced sepsis, serving to separate the lumen and its contents from the damaged cecum wall. These data suggest that the immunoneutralization of IL-12 either prevented or delayed the development of a protective barrier to the further infiltration of the peritoneum by intestinal microbes. Without this mechanism of containing the damaged cecum from the rest of the body, neither the bacterial killing response of the host nor frequent antibiotic treatments quelled the bacterial infection that appeared to contribute to the observed lethality. While the lack of a healing or fibrotic response in the anti-IL-12 Ab-treated mice is currently being explored, it is postulated that the repair of the damaged cecum is analogous to wound repair in other parts of the body. This process

FIGURE 7. Representative composite cecum histologic sections from CLP control (preimmune serum + antibiotics) or experimental (IL-12 antisera + antibiotics) mice. IL-12-specific antisera or control serum was administered 2 h prior to CLP surgery. Antibiotic treatments (Primaxin: 200 μg/dose) were administered immediately after CLP surgery and every 8 h thereafter. Cecum samples were collected 4 days after CLP surgery. A and B represent the preimmune/antibiotics group at ×100 and ×400 respective magnifications. C and D represent the IL-12 antisera/antibiotics group at ×100 and ×400 respective magnifications. L = lumen; M = membrane. Representative of 7–10 samples per group.
requires a sequence of events that begins with inflammation and is followed by tissue restoration and resolution (43). The early damping of the inflammatory response by IL-12 immunoneutralization may prevent normal progression to subsequent stages of repair. The twofold increase in IFN-γ production at 96 h by CLP mice administered both IL-12 antisera and antibodies is suggestive of prolonged inflammation, without progress to tissue restoration and resolution. While increased IFN-γ production may be the natural effect of a continued cellular response to uncontrolled bacterial infection, this cytokine also functions as a potent inhibitor of wound repair and fibrotic processes (44-46).

In the CLP model of bacterial sepsis, IL-12 function is complex, and includes the following aspects: 1) IL-12 is a vital cofactor in the innate immunity against bacteria contained in spilled fecal matter and 2) IL-12 is involved in the containment of bacteria within the damaged cecum by directly (or indirectly) stimulating the fibrotic organization of the cecum wall. Furthermore, the latter roles for IL-12 in the septic response appeared to be critical to the survival of CLP mice, as antibiotic treatment failed to spare anti-IL-12 Ab-treated mice. Treatment of sepsis in humans has proved highly ineffective in preventing mortality, possibly casting importance upon the concept of therapeutic manipulation of cytokine cascades in septic patients. Accordingly, the present study warrants additional investigation into the role of cytokine balance during sepsis, while perhaps validating a reexamination of the clinical treatment avenue of single cytokine modulation.

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