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IL-12, but Not IL-18, Is Critical to Neutrophil Activation and Resistance to Polymicrobial Sepsis Induced by Cecal Ligation and Puncture

Susana E. Moreno,* José C. Alves-Filho,* Thais M. Alfaya,* João S. da Silva,† Sergio H. Ferreira,* and Foo Y. Liew2‡

Sepsis is a systemic inflammatory response resulting from local infection due, at least in part, to impaired neutrophil migration. IL-12 and IL-18 play an important role in neutrophil migration. We have investigated the mechanism and relative role of IL-12 and IL-18 in polymicrobial sepsis induced by cecal ligation and puncture (CLP) in mice. Wild-type (WT) and IL-18−/− mice were resistant to sublethal CLP (SL-CLP) sepsis. In contrast, IL-12−/− mice were susceptible to SL-CLP sepsis with high bacteria load in peritoneal cavity and systemic inflammation (serum TNF-α and lung neutrophil infiltration). The magnitude of these events was similar to those observed in WT mice with lethal CLP sepsis. The inability of IL-12−/− mice to restrict the infection was not due to impairment of neutrophil migration, but correlated with decrease of phagocytosis, NO production, and microbicidal activities of their neutrophils, and with reduction of systemic IFN-γ synthesis. Consistent with this observation, IFN-γ−/− mice were as susceptible to SL-CLP as IL-12−/− mice. Moreover, addition of IFN-γ to cultures of neutrophils from IL-12−/− mice restored their phagocytic, microbicidal activities and NO production. Mortality of IL-12−/− mice to SL-CLP was prevented by treatment with IFN-γ. Thus we show that IL-12, but not IL-18, is critical to an efficient host defense in polymicrobial sepsis. IL-12 acts through induction of IFN-γ and stimulation of phagocytic and microbicidal activities of neutrophils, rather than neutrophil migration per se. Our data therefore provide further insight into the defense mechanism against this critical area of infectious disease. The Journal of Immunology, 2006, 177: 3218–3224.

Neutrophils migrate rapidly to the site of infection, where they are activated to release a range of microbial mediators (reviewed in Ref. 1). In addition, neutrophils also release cytokines and chemokines, which enhance their own recruitment and activation as well as inducing the migration of other immune cells (2). Our previous studies showed that lethal sepsis induced by cecal ligation and puncture (CLP)3 or Staphylococcus aureus inoculation is associated with reduction of neutrophil rolling, adhesion, and transmigration to sites of infection (3–8). The impairment of neutrophil migration was also associated with high mortality and increased numbers of bacteria in peritoneal exudates and blood. Conversely, in nonlethal sepsis, the bacterial infection was restricted to the peritoneal cavity, and neutrophil rolling, adhesion, and migration were not affected, and no significant mortality was observed (3–8). The mechanism involved in the paralysis of neutrophil migration function is still elusive. We and others have reported that IL-12 and IL-18 play an important role in neutrophil migration (9–11). We have now investigated the relative roles of IL-12 and IL-18 in an experimental polymicrobial sepsis in mice using the CLP models.

IL-12 is a heterodimeric proinflammatory cytokine (IL-12p70), consisting of a p35 and a p40 subunit, which is mainly produced by APCs (12, 13). It is a potent stimulator of T cell functions, driving the differentiation of naive CD4+ T cells into the Th1 lineage and the production of IFN-γ, a potent activator of antimicrobial functions of phagocytes against a range of pathogenic microorganisms (13, 14). IL-12 is also important in the pathogenesis of autoimmune inflammatory diseases (15, 16) but its role in sepsis remains controversial. It has been reported that administration of anti-IL-12 antiserum increased the bacteria load following CLP or i.p. administration of Escherichia coli, suggesting that IL-12 contributes to an effective antibacterial defense (17, 18). In contrast, it has also been shown that polyclonal IL-12 Ab decreased mortality of mice after CLP or i.p. injection of live E. coli (17–20).

IL-18 is produced by several cell types, including activated macrophages, dendritic cells, keratinocytes, osteoblasts, and intestinal epithelial cells (21–23). IL-18 is proinflammatory, inducing the release of TNF-α, IL-1β, and chemokines such as CXCL-8 (24–26). Recent reports indicate an important role of IL-18 in the pathogenesis of several inflammatory diseases, including rheumatoid arthritis, psoriasis, inflammatory bowel disease, and sarcoidosis (27–29). It was also demonstrated that IL-18, acting synergistically with IL-12, IL-15, or microbial agents, stimulates T cells and NK cells from mice and humans to produce IFN-γ (26, 30, 31). The role of IL-18 in sepsis remains controversial. It has been reported that IL-18-deficient mice exhibited reduced capacity to kill S. aureus, associated with increased severity of sepsis (32).
contrast, it was also reported that specific blockage of IL-18 protects mice from death induced by E. coli or Salmonella typhi-
murium administration (33) or endotoxemia (10, 34). Furthermore, patients with severe sepsis were reported to have elevated circu-
lating levels of IL-18 (35). A possible explanation to these appar-
ently contradictory results is that IL-18 and IL-12 produced in the infections site play a host defense role by inducing leukocyte mi-
gration and activation. However, these cytokines have host dam-
age effects when produced systemically, by inducing organ le-
sions (36, 37).

In the present study, we compared the role of IL-12 and IL-18 in resistance and neutrophil migration and activation (phagocytosis and microbicidal activity) in sublethal (SL) and lethal polymicro-
bial sepsis induced by CLP. We report that IL-18 deficiency did not affect the resistance to, and neutrophil migration and activation in, SL-CLP. In contrast, IL-12−/− mice were highly susceptible to SL-CLP. Furthermore, although neutrophils from IL-12−/− mice migrated normally during SL-CLP, they had markedly reduced phagocytosis, NO production, and microbicidal activity attribut-
able to decreased systemic IFN-γ production. Our data therefore provide direct evidence for a differential role of IL-12 and IL-18 in the peritoneal exudates and serum cytokine assays in the peritoneal exudates and serum of mice with cecal bacteria (106 CFU) at 37°C with gentle shaking. After 1.5 h, the cells were washed with PBS at 4°C and centrifuged at 200 × g (to remove extracellular bacteria), and then deposited onto microscope slides in a cytocentrifuge at 200 × g (Thermo Shandon), fixed with methanol, and stained with the May-Grünwald-Giemsa (Rosenfeld) method. The number of neutrophils that ingested bacteria and the number of ingested bacteria per neutrophil were counted under a phase contrast microscope. Neutrophil migration to infection focus, bacterial CFU and NO production by neutrophils


eaces of mice were sacrificed 6 h after sepsis induction. The survival rate of mice was determined daily for 5 days after surgery. Neutrophil migration to infection focus, bacterial CFU in the exudates, cytokine levels in the exudates and serum, and neutrophil sequestration in lung tissues were determined as previously described (38).

Neutrophil migration to the peritoneal cavity

Neutrophil migration was assessed 6 h after sepsis induction. The animals were sacrificed, and cells present in the peritoneal cavity were harvested by cecal lavage. The peritoneal exudates were collected, and cecal bacteria were cultured for 1 h at 37°C in antibiotic-free RPMI 1640, and the adherent cells were cultured in RPMI

expression of 0.2% NaCl solution followed 30 s later by the addition of an equal volume of a solution containing 1.6% NaCl and 5% glucose). After a further centrifugation, the pellet was resuspended in 200 μl of 50 mM NaPO4 buffer (pH 5.4), and centrifuged at 4°C for 15 min. The supernatant was then frozen and thawed twice and centrifuged again at 10,000 rpm for 15 min. The supernatants was determined using the Griess method (40). Briefly, 50 μl of reagent at room temperature. The absorbance was measured on a plate scanner (Spectra Max 250; Molecular Devices) at 540 nm. The NO3− concentration was determined using a standard curve for 1–200 μM NaNO2,
**IFN-γ activation of neutrophils**

Neutrophils (1 × 10⁶) were preincubated with IFN-γ (100 U/ml) for 1 h. At the end of the incubation period, the cells were incubated with cecal bacteria for phagocytosis (10 bacteria/neutrophil) and killing assays (1 bacterium/neutrophil) or stimulated with LPS (200 ng/ml) with or without amnioguanidine (50 μM) for nitrite production as described.

**IFN-γ treatment of IL-12−/− mice**

IL-12−/− mice were injected i.p. with IFN-γ (5000 U/0.2 ml; PeproTech) at 3, 12, 24, and 36 h after SL-CLP surgery and the survival of mice was recorded daily for 7 days.

**Statistical analysis**

The data (except for the survival curves) are reported as the mean ± SEM of values obtained from two different experiments. The mean of different treatments was compared by ANOVA. If significance was determined, individual comparisons were subsequently tested with Bonferroni's t test for unpaired values. Bacterial counts were analyzed by the Mann-Whitney U test. The survival rate was expressed as the percentage of live animals, and Fisher’s exact test was used to determine differences between survival curves. A value of p ≤ 0.05 was considered significant. The number (n) of individual mice for each experiment is represented.

**Results**

**IL-12, but not IL-18, deficiency decreases resistance to SL-CLP sepsis**

We examined the role of IL-12 and IL-18 in the outcome of polymicrobial septic peritonitis induced by CLP. IL-12 (p40)−/− and IL-18−/− mice and their WT controls (C57BL/6 and BALB/c mice, respectively) were exposed to SL-CLP or lethal CLP. IL-12−/− mice given SL-CLP showed 100% mortality by 96 h, whereas all WT mice survived (Fig. 1A). In contrast, all the WT and IL-18−/− mice given SL-CLP survived (Fig. 1B). The IL-12−/− mice with SL-CLP failed to control the infection with a high number of CFU in peritoneal cavity (Fig. 1C). IL-18−/− mice given SL-CLP controlled the infection as efficiently as WT mice (Fig. 1D). There was no difference between WT and IL-12−/− mice given lethal CLP, with 100% mortality and high CFU in the exudates (Fig. 1, A and C). WT and IL-18−/− mice subjected to lethal CLP also died within 72 h with high CFU in the exudates (Fig. 1, B and D).

To identify the mechanisms underlying the susceptibility of IL-12−/− mice to SL-CLP, we investigated the neutrophil recruitment toward peritoneal cavity after sepsis induction. Although IL-12−/− mice with SL-CLP failed to control the infection they showed a...
The susceptibility of SL-CLP IL-12−/− mice despite normal neutrophil migration, led us to investigate the phagocytic and microbicidal activities of the neutrophils from IL-12−/− and IL-18−/− mice. Neutrophils from IL-12−/− mice had significantly reduced phagocytosis and microbicidal activity compared with neutrophils from SL-CLP WT mice (Fig. 3A). In contrast, neutrophils from SL-CLP IL-18−/− mice had phagocytic and microbicidal activities similar to those of SL-CLP WT mice (Fig. 3B). Furthermore, neutrophils from SL-CLP IL-12−/− mice, but not SL-CLP IL-18−/− mice, produced little or no detectable NO when activated with LPS in vitro (Fig. 3, C and D). These data therefore suggest that the increase of bacterial load and mortality of SL-CLP IL-12−/− mice was likely associated with reduction of phagocytosis and bactericidal activity of neutrophils, which probably resulted from decreased NO production.

**IL-12-deficient mice produce less IFN-γ in SL-CLP**

There is clear evidence that IL-12-induced IFN-γ production is critical to the host antimicrobial defense. To determine whether the reduction of survival rate and neutrophil activation after SL-CLP...
in IL-12−/− mice was associated with a decrease in IFN-γ production, we measured the concentrations of IFN-γ in the peritoneal exudates and serum of IL-12−/− mice. Peritoneal exudates and serum from SL-CLP IL-12−/− mice contained significantly reduced concentrations of IFN-γ 6 h after surgery compared with similarly treated WT mice (Fig. 4). In contrast, peritoneal exudates (Fig. 4A) and serum (Fig. 4B) from SL-CLP IL-18−/− mice contained comparable levels of IFN-γ to those of the SL-CLP WT mice.

**SL-CLP IFN-γ−/− mice show reduced survival rate, neutrophil phagocytic and microbicidal activity, and NO production**

We then investigated the direct role of IFN-γ in SL-CLP. IFN-γ−/− mice exposed to SL-CLP had significantly reduced survival rates compared with WT mice (Fig. 5A), despite the fact that the neutrophil migration to the infectious focus was higher in the IFN-γ−/− mice than in the WT mice (Fig. 5B). Neutrophils from IFN-γ−/− mice had reduced phagocytic and microbicidal activity and LPS-stimulated NO production compared with cells from the WT mice (Fig. 5, C and D). These results indicate that IL-12 and IFN-γ deficiencies have a similar phenotype during SL-CLP.

**FIGURE 5.** Survival and neutrophil activities of IFN-γ-deficient mice exposed to polymicrobial sepsis. A, IFN-γ−/− and WT mice were exposed to CLP and mortality monitored at regular intervals. IFN-γ−/− were significantly more susceptible to SL-CLP than the WT mice (n = 10 mice). A value of p < 0.05 using Mantel-Cox log rank test was obtained. B, Neutrophil migration into peritoneal cavity in WT and IFN-γ−/− mice exposed to SL-CLP was determined 6 h after surgery. Results are mean ± SEM. *p < 0.05 compared with sham-operated animals and #, p < 0.05 compared with WT SL-CLP group. C, Neutrophils (1 × 10⁶) from WT or IFN-γ−/− mice were incubated in vitro with bacteria to evaluate phagocytosis and microbicidal activity as described in Materials and Methods. Results are the mean ± SEM (n = 5 mice). *p < 0.05 compared with WT mice. D, Neutrophils from WT or IFN-γ−/− mice were stimulated with medium alone (dash) with LPS (200 pg/ml) ± aminoguanidine (Amino, 50 μM), and the concentration of nitrite in the supernatants was determined 12 h after. Results are expressed as the mean ± SEM (n = 5 mice). *, p < 0.05 compared with LPS-stimulated neutrophils and #, p < 0.05 compared with untreated IL-12−/− mice. A value of p < 0.05 (n = 5) using the Mantel-Cox log rank test was obtained.

**IFN-γ restores phagocytic and microbicidal activity, NO production, and resistance to SL-CLP of IL-12−/− mice**

We then investigated whether IFN-γ is able to restore the phagocytic capacity and killing activity of neutrophils from IL-12−/− mice. Neutrophils from IL-12−/− or WT mice were cultured with IFN-γ for 1 h and the phagocytic and bacteria killing activity and NO production were determined. Treatment of neutrophils from IL-12−/− mice with IFN-γ (100 U/ml) restored the bacterial phagocytic activity (Fig. 6A), microbicidal activity (Fig. 6B), and NO production (Fig. 6C) to levels attained in neutrophils from WT mice. The treatment of neutrophils from WT mice with recombinant IFN-γ did not affect their bacterial phagocytic activity, microbicidal activity, or LPS-stimulated NO production. Furthermore, all the SL-CLP IL-12−/− mice treated with IFN-γ survived the infection as in the SL-CLP WT mice (Fig. 6D). These data therefore demonstrated that the high susceptibility of IL-12−/− mice to SL-CLP was due to the deficiency in IFN-γ production.

**FIGURE 6.** IFN-γ restored phagocytosis, microbicidal activity, and NO production of neutrophils and reversed susceptibility of IL-12-deficient mice. Neutrophils from WT or IL-12−/− mice were preincubated in vitro with medium (dash) or 100 IU/ml of the recombinant IFN-γ for 1 h, followed by incubation with bacteria. Phagocytosis (A) and microbicidal activity (B) of the neutrophils were determined as in Fig. 3. Results are the mean ± SEM (n = 4), *, p < 0.05 compared with neutrophils incubated without IFN-γ. C, Neutrophils from IL-12−/− or WT mice were cultured in medium (dash) with LPS (200 ng/ml) with or without aminoguanidine (Amino, 50 μM) or with or without IFN-γ (100 IU/ml), and NO production was determined as nitrite concentration in the culture supernatant 12 h later. Results are the mean ± SEM (n = 4 mice), *, p < 0.05 compared with respective nonstimulated neutrophils; #, p < 0.05 compared with neutrophils from WT mice incubated with LPS; and △, p < 0.05 compared with LPS-stimulated neutrophils from IL-12−/− mice. D, Effect of IFN-γ treatment on survival of IL-12−/− mice after SL-CLP. Mice were injected i.p. with IFN-γ (5000 IU) at 3, 12, 24, and 36 h after CLP surgery. The untreated IL-12−/− SL-CLP group was significantly different from WT SL-CLP mice or IFN-γ-treated IL-12−/− mice. A value of p < 0.05 (n = 5) using the Mantel-Cox log rank test was obtained.
Discussion

Neutrophil recruitment to the infection site is an essential step in the control of bacterial infections (2, 41). Recently, we showed that neutrophil migration is impaired in lethal sepsis induced by CLP or by *S. aureus* inoculation (3–7) and also in human sepsis (42). The impairment is associated with an ineffective bacterial clearance, leading to bacterial dissemination and high mortality. Neutrophil activation, including phagocytosis, microbicidal activity, and NO production, is also fundamental to limiting infections (43–45). In the present study, we investigated the relative roles of IL-12 and IL-18 in neutrophil migration and activation, and in disease outcome in sublethal or lethal polymicrobial sepsis induced by CLP. Data presented demonstrated that IL-12 but not IL-18 is key to the resistance of SL-CLP. Furthermore, the susceptibility of IL-12−/− mice to SL-CLP was not related to the ability of neutrophil migration to the infectious foci, but was due to a decrease in IFN-γ production, leading to reduced phagocytic and microbicidal activity and NO synthesis, which were completely restored by treatment with recombinant IFN-γ.

Systemic inflammatory response is considered a central deleterious pathogenic event in severe sepsis. High levels of serum inflammatory cytokines are involved in development of multiple organ failure and cardiovascular collapse (6, 46, 47). Consistent with the inability of SL-CLP IL-12−/− mice to control the infection, these mice showed elevated systemic inflammation, similar to that observed in WT mice given lethal CLP. It is important to note that neutrophil infiltration in distant organs of the infectious focus, such as lung, contributes to the tissue damage, multiple organ failure, and consequently to death of the host in the sepsis (48, 49). An earlier report showed that polyclonal anti-IL-12 Ab did not interfere with the mortality of mice given CLP or i.p. injection of live *E. coli* (17–20). However, in these studies, the animals were under severe sepsis, a situation similar to our lethal CLP reported in this study, in which IL-12 did not appear to play a detectable role.

IFN-γ can negatively regulate neutrophil migration to the inflammatory sites through the inhibition of the CXC chemokine synthesis and their receptors (46, 50) and also the expression of adhesion molecules, such as ICAM-1 (51). It is important to note that although IL-12−/− and IFN-γ−/− mice had a decreased survival rate following SL-CLP, the magnitudes of the response were different. Although IL-12−/− mice showed 100% mortality within 96 h after surgery, IFN-γ−/− mice exhibited only 35% mortality during this period. The increased neutrophil influx in the infection focus observed in IFN-γ−/− mice (Fig. 5B) may counterbalance the reduction of the phagocytic and microbicidal activity of the neutrophils. It is important also to note that IL-12−/− mice showed a residual production of IFN-γ (Fig. 4A) that may be mediated by other cytokines such IL-18 and TNF-α, which also can induce IFN-γ production (13, 14, 52).

Recently, studies have demonstrated that some effects originally ascribed to IL-12 may also be mediated by IL-23, which also induces IFN-γ and TNF-α production in several infections (13, 53). The p40 subunit associates not only with p35 to form IL-12p70, but also with p19, to form IL-23 (52, 54). The IL-12−/− mice used in our study are deficient in p40 and therefore were also IL-23 deficient. Thus, the reduced host defense observed in the IL-12−/− mice under SL-CLP may also be associated with reduction of IL-23. However, it was reported that mice deficient in the p35 subunit, which is present only in the IL-12 molecule, were also susceptible to bacteria infection (55). Moreover, the increase in survival rate of IL-12p40−/− mice infected with *Toxoplasmagondii* by treatment with IL-23 was not mediated by IFN-γ but were due to IL-17 produced by NK cells and neutrophils (55). Together, our results indicate that the host deficiency observed in IL-12p40−/− mice exposed to SL-CLP might be caused by the absence of IL-12 rather than IL-23.

There are contradictory reports on the role of IL-18 in sepsis. IL-18−/− mice injected with LPS had a higher survival rate than WT mice (22). IL-1β-converting enzyme-deficient mice, lacking the ability to process mature IL-18 and IL-1β, were completely resistant to lethal doses of LPS derived from either *E. coli* or *S. typhimurium*. In contrast, both WT and IL-1β−/− mice were equally susceptible to the lethal effects of LPS, implicating that absence of mature IL-18 but not IL-1β-converting enzyme-deficient mice is responsible for this resistance (33). Furthermore, the blockage of IL-18 also reduces the lethal effects of *E. coli* and *S. typhimurium* administration, which correlated with the reduction of systemic MIP-2 production and neutrophil lung infiltration (33). Our results suggest that IL-18 is not involved in the pathogenesis of SL-CLP and lethal CLP sepsis. Our results are consistent with an earlier report that endogenous IL-12 improved the early antimicrobial host response to murine *E. coli* (56), but are in contrast to another report that IL-18 facilitated the early antimicrobial host response to *E. coli* peritonitis (57). The use of different experimental models may explain the discrepancies. The CLP model used in our study is a polymicrobial model of sepsis and in the majority of the studies just described monomicrobial sepsis or endotoxemia was used.

In conclusion, the present study provides direct evidence that IL-12 and not IL-18 plays a fundamental role in both host defense in sublethal sepsis and the systemic deleterious inflammation in lethal sepsis. IL-12 acting through IFN-γ is essential for mounting an efficient defense response against polymicrobial infection via increased phagocytosis, microbicidal activity, and NO production. Our results therefore provide further insight into the role of various cytokines in sepsis and clarify a key controversy on the relative role of IL-12 and IL-18 in this field.

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Disclosures

The authors have no financial conflict of interest.

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