STAT4 Is Required for Antibacterial Defense but Enhances Mortality during Polymicrobial Sepsis

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The signal transducer and activator of transcription factor 4 (STAT4) pathway mediates the intracellular effects of interleukin-12 (IL-12), leading to the production of gamma interferon, induction of a T helper type 1 response, and increased natural killer cell cytotoxicity. The purpose of this study was to determine the role of the STAT4 pathway during polymicrobial peritonitis in the cecal ligation and puncture (CLP) model. CLP was performed on STAT4-deficient (STAT4−/−) and wild-type control (BALB/c) mice. At 4 h after CLP, STAT4−/− mice had significantly higher bacterial counts in the peritoneal lavage fluid, liver, and blood. This difference persisted for 18 h in the peritoneal lavage fluid and blood. Neutrophil migration to the site of infection and into remote tissues was unaffected. Despite higher bacterial counts locally and systemically, STAT4−/− mice had a lower mortality rate than BALB/c controls. In contrast, blockade of IL-12 in BALB/c mice was detrimental to host survival. A blunted serum IL-12 response at 18 h after CLP was exhibited in STAT4−/− mice. These results suggest several critical roles for the STAT4 pathway in the resolution of polymicrobial infections. Additionally, the disparate effects observed with IL-12 blockade and STAT4 deficiency on host survival suggest that IL-12 may activate alternate pathways promoting survival.

Signal transducers and activators of transcription (STAT) transmit cytokine signals to the nucleus, facilitating gene transcription (19). Six different STAT proteins have been identified, each with its own array of cytokine specificities and resultant gene activations (19). The STAT factor 4 (STAT4) protein was originally cloned through its homology with previously described STAT proteins (43, 45) and is activated in T cells, natural killer cells, testis, and thymus in response to interleukin-12 (IL-12) (42). Interaction of IL-12 with its cell surface receptor leads to phosphorylation of STAT4 by receptor-associated Janus kinases. Once phosphorylated, STAT4 dimerizes and translocates to the nucleus and promotes gene transcription yielding production of gamma interferon (IFN-γ), development of the T helper type 1 response, and increased natural killer cell cytotoxicity (25, 39). The host immune response to infections therefore relies to some extent on IL-12 and STAT4.

Sepsis is characterized by an inflammatory cytokine response, including production of IL-12 (22). Supplemental IL-12 can improve host bacterial resistance, and deficiency of IL-12 predisposes patients to bacterial infections and sepsis (11, 17). When combined with IL-18 or IL-2, administration of IL-12 can induce a fatal response in mice similar to septic shock (3, 4). Additionally, IL-12 administration in humans has resulted in organ damage, hemodynamic instability, and death (7). These studies suggest an important role for IL-12 (and STAT4 by association) in survival, bacterial clearance, and organ failure during bacterial sepsis.

The aim of the present study was to examine the role of the STAT4 pathway during sepsis by using the clinically relevant cecal ligation and puncture (CLP) model (31, 33). We demonstrate a survival benefit and impaired bacterial clearance when the host lacks STAT4. Parallel studies of IL-12 neutralization and STAT4 deficiency suggest that the antibacterial effects of IL-12 are mediated by STAT4. However, these data also suggest that despite its antibacterial effects, STAT4 activation is detrimental to host survival.

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MATERIALS AND METHODS

Animals. Six- to 8-week-old STAT4−/− mice (BALB/c-Stat4+/- Gru mice deficient in the STAT4 protein) and their wild-type controls (BALB/c mice) were used (Jackson Laboratories, Bar Harbor, Maine). Animals were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care and were provided food and water ad libitum. Studies were conducted in accordance with the guidelines of the National Institutes of Health and under the supervision of a veterinarian.

CLP. Mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg) by intramuscular injection. CLP was performed as follows. The cecum was exposed through a midline laparotomy incision and ligated just below the ileocecal junction with 4-0 silk suture. For survival and 18-h harvest experiments, a single 23-gauge puncture was made in the cecum. For 4-h harvest experiments to yield higher bacterial counts, two 18-gauge punctures were made in the cecum. The cecum was then returned into the peritoneal cavity, and the abdominal incision was closed in layers.

Survival. Mice were injected with 1 ml of normal saline subcutaneously for volume resuscitation at the time of CLP. Cefoxitin (100 mg/kg) was administered subcutaneously every 12 h. For IL-12 immunoneutralization experiments, 200 µg of either IL-12 antibody or control immunoglobulin G (R&D Systems, Minneapolis, Minn.) was injected into the tail vein immediately prior to CLP.

Timed harvests. Anesthetized mice were killed at 4 or 18 h after CLP. Peritoneal lavage fluid was obtained for determination of bacterial counts, cytokine levels, neutrophil counts, and myeloperoxidase levels. Liver, lung, and spleen tissues were collected for determination of bacterial counts and myeloperoxidase levels. Blood was collected by cardiac puncture for evaluation of bacterial and leukocyte counts.

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Peritoneal lavage. Peritoneal exudate cells were recovered by peritoneal lavage with 4 ml of ice-cold heparinized RPMI 1640 medium (Gibco-BRL, Bethesda, Md.) and were counted manually by hemacytometer.

Myeloperoxidase assay. Myeloperoxidase was used as a measure of neutrophil accumulation. Liver, lung, and spleen tissues were homogenized in dilute phosphate buffer and then centrifuged at 10,000 rpm for 15 min. Peritoneal cells were collected through centrifugation of the peritoneal lavage collection. The pellets of the tissues and peritoneal cells were solubilized in a phosphate buffer containing a mild detergent. After freezing, thawing, and sonication, the solubilized pellets were heated for 2 h at 60°C. The myeloperoxidase level was determined spectrophotometrically by using tetramethylbenzidine as the color reagent.

Cytokine assays. Concentration of IL-6, IL-12, and IFN-γ in the serum and peritoneal lavage fluid was determined by enzyme-linked immunosorbent assays (Biosource International, Camarillo, Calif.) performed according to the manufacturer’s instructions.

Bacterial counts. Homogenized liver, lung, and spleen tissues (150 to 250 mg in 2 ml of sterile saline), whole blood, and peritoneal lavage fluid were plated in serial log dilutions on tryptic soy or brain heart infusion agar plates. After plating, tryptic soy agar plates were incubated at 37°C aerobically for 24 h, and brain heart infusion agar plates were incubated anaerobically for 48 h. Results are expressed as CFU per milliliter for blood, CFU per gram for tissues, and CFU per mouse for the peritoneal lavage fluid.

Statistical analysis. Concentrations of cytokines, neutrophil counts, and myeloperoxidase levels were compared by analysis of variance followed by the Tukey-Kramer HSD test. The Fisher exact test was used for differences in survival. Bacterial counts were compared nonparametrically using the Mann-Whitney U test. Cytokine and myeloperoxidase levels are expressed as means ± standard error of the mean (SEM). A P value of 0.05 or less was considered significant.

RESULTS

Disparate effects on survival after CLP with STAT4 deficiency and IL-12 neutralization. To assess the participation of the STAT4 pathway in the pathophysiological effects of polymicrobial sepsis, STAT4−/− and matched BALB/c control mice were subjected to CLP. Among the BALB/c controls, 90% of all mice were dead within 4 days (Fig. 1). STAT4 nullizygous mice displayed a significant increase in survival after CLP (P = 0.02), with only 50% dead by 6 days.

The activation of STAT4 is closely associated with IL-12 (19, 42), and blockade of IL-12 during CLP is detrimental to survival in pathogen-free CD-1 mice (36). Conversely, blockade of IL-12 improves survival after endotoxin challenge (46). To determine a more directly comparable effect of passive IL-12 immunization, we performed CLP on matched groups of BALB/c mice with and without IL-12 antibody pretreatment. Immunoneutralization of IL-12 was detrimental to host survival (P = 0.02) (Fig. 2).

Higher bacterial counts after CLP in STAT4−/− mice. To quantify the ability of the host to clear the bacterial challenge, bacterial counts were determined at the site of the infection, in the blood, and in remote tissues. At 4 h after CLP, aerobic counts were significantly higher in STAT4−/− mice in the peritoneal lavage fluid, liver, and blood (P < 0.05) (Fig. 3A). Anaerobic counts were significantly higher in the liver tissue (P < 0.05) (Fig. 3B). The liver was the only tissue sampled that had reliably detectable counts, which is consistent with observations that bacteria in the bloodstream ultimately accumulate in the liver (15, 35).

Bacterial counts were also ascertained at 18 h after CLP to examine the effect of the STAT4 pathway closer to the time of death. STAT4−/− mice had significantly higher aerobic bacterial counts in the peritoneal lavage fluid, blood, and liver tissue (P = 0.05) (Fig. 4A). Anaerobic counts were significantly higher in the peritoneal lavage fluid and blood (P = 0.05) (Fig. 4B), whereas liver anaerobic counts were below the limit of detection in both groups (not shown). At both 4 and 18 h, all of the colonies were morphologically similar. The species types have been described previously by McMasters et al. (28).

Neutrophil migration patterns are similar in STAT4−/− and BALB/c control mice. We evaluated myeloperoxidase levels in the lung, liver, spleen, and peritoneal exudate cells and determined microscopic neutrophil counts in the blood and peritoneal lavage fluid to detect changes in neutrophil trafficking. There were no significant differences in neutrophil accumulation to account for the differences in survival or bacterial counts (Table 1). Concurrently, cell counts in the peritoneal lavage fluid and blood were not significantly different (data not shown).

Cytokine determinations. Dysregulation of IL-12 has been associated with injury, and supplemental IL-12 in this setting has been beneficial (11). To assess whether changes in IL-12 levels accounted for differences in survival or bacterial counts, IL-12 levels were measured in the serum and peritoneal lavage fluid.
fluid. At 18 h, BALB/c control mice showed a significant increase in serum IL-12 levels compared with baseline ($P < 0.05$) (Fig. 5). Unexpectedly, no increase was detected in the serum from STAT4$^{-/-}$ mice. Surprisingly, not only did the STAT4$^{-/-}$ mice have improved survival after CLP, where IL-12 might be expected to be protective, these mice also had higher bacterial levels that have been correlated with higher IL-12 levels (6, 8, 32). Peritoneal IL-12 levels did not differ from those in control mice.

In IL-6 levels at 4 or 18 h after CLP, there were no significant differences in serum or peritoneal specimens when STAT4$^{-/-}$ mice were compared with BALB/c mice (data not shown). IL-6 is a cytokine previously found to be predictive of outcome after shock (30). At 18 h after CLP in three wild-type control mice, IFN-$\gamma$ levels in the peritoneal lavage fluid and serum were 65$\pm$21 pg/ml and 80$\pm$16 pg/ml, respectively. These levels of IFN-$\gamma$ approach the limit of detection of the assay, limiting the utility for comparing experimental groups, a finding similar to previous observations (34).

### DISCUSSION

IL-12 is the principal activator of STAT4 (19, 42). Deficiency of STAT4 and immunoneutralization of IL-12 would therefore be expected to have the same effects. Quite unexpectedly, we observed opposite effects on survival with IL-12 blockade and

### TABLE 1. Impact of STAT4 deficiency on myeloperoxidase levels after CLP

| Harvest time and mouse group | Peritoneal cells$^a$ | Liver$^b$ | Lung$^b$ |
|-----------------------------|----------------------|-----------|---------|
| 4 h BALB/c                  | 0.8$\pm$0.3          | 4.0$\pm$0.7 | 104$\pm$30 |
| 4 h STAT4$^{-/-}$           | 0.4$\pm$0.1          | 5.8$\pm$1.3 | 112$\pm$20 |
| 18 h BALB/c                 | 7.4$\pm$1.3          | 6.3$\pm$1.7 | 66$\pm$19 |
| 18 h STAT4$^{-/-}$          | 4.7$\pm$2.0          | 3.3$\pm$0.9 | 33$\pm$12 |

$^a$ Units per mouse.

$^b$ Units per gram of tissue.
STAT4 deficiency after CLP. Alternate pathways activated by IL-12 and for other activators of STAT4 may explain these effects. IFN-α was one of the first described alternative activators of STAT4 in a human cell line (5). Since then, IL-2 has been shown to activate STAT4 in natural killer cells but not T cells (41), and IL-17 can activate STAT4 in human leukemia cells (37). Additionally, translocation of STAT4 to the nucleus has been observed in vascular smooth muscle cells in response to activation of urokinase-type plasminogen activator receptor (9). All of these mediators could function during a septic challenge in the face of IL-12 blockade, but their effects may be abrogated in STAT4−/− mice.

IL-12 also has the potential to exert effects during sepsis when STAT4 is not present. Activation of STAT1, STAT3, and STAT5 can occur in response to IL-12 in Th1-differentiated lymphocytes, providing alternate STAT pathways for IL-12 (12, 20). Nuclear factor kappa B (NF-κB) translocates to the nucleus in response to IL-12 in dendritic cells and is associated with a structurally different IL-12 receptor than in T cells (16). It is not clear to what extent each of these alternate pathways function during polymicrobial sepsis, or whether they account for the effects observed in the present study. It is apparent from our data, however, that IL-12 may not signal solely through STAT4 and that STAT4 has divergent effects itself, promoting antibacterial defense while increasing the rate of mortality.

The effects of IL-12 blockade and STAT4 deficiency on bacterial clearance in polymicrobial peritonitis are more congruent. IL-12 is known to be important in host defense against mono- and polymicrobial infections (10, 14, 29), and immunoneutralization of IL-12 prior to CLP impairs peritoneal bacterial clearance (36). Given the close association of IL-12 and STAT4 activation, the impaired clearance of bacteria observed in STAT4 deficiency is not surprising. Others have shown that STAT4−/− mice exhibit increased parasitemia when infected with Trypanosoma cruzi (38). However, a patient with intact STAT4 activation but impaired STAT1, STAT3, and STAT5 activation in response to IL-12 with recent Staphylococcus aureus and Mycobacterium avium infections has been described (13). This raises the possibility that the antibacterial effects of IL-12 are elicited primarily through pathways not involving STAT4.

Surprisingly, survival after CLP in STAT4−/− mice did not correlate with the higher observed bacterial counts. A similar combination of worsened bacterial clearance and improved bacterial tolerance was noted with IL-12 immunoneutralization prior to intraperitoneal Escherichia coli administration (46). Although there is a suggested increase in bacterial tolerance in STAT4−/− mice during CLP, we cannot exclude the possibility of altered bacterial composition or virulence even though the morphology of the colonies was similar in both groups. Our data confirm that signaling via STAT4 is an important aspect of the host’s mechanism in clearing bacteria.

Bacterial products, including lipopolysaccharide, lipoteichoic acid, and bacterial DNA, induce IL-12 production (6, 8, 32). Because of higher bacterial counts, mice deficient in STAT4 would be expected to have higher IL-12 levels. Instead, we observed an attenuated serum IL-12 response after CLP in STAT4−/− mice. IFN-γ is produced in response to IL-12 and STAT4 activation and can further induce IL-12 production (18, 27). Interference with this positive feedback loop is one explanation of the apparently counterintuitive effect of the muted IL-12 response demonstrated in STAT4−/− mice. Spleen cells from STAT4−/− mice are unable to generate IFN-γ in response to IL-12 in vitro, lending further support to the proposed mechanism (39). Confirmation of an attenuated IFN-γ response during polymicrobial peritonitis in STAT4−/− mice is limited, however, because measurable levels of IFN-γ in vivo approximate the limit of detection of the assay (34).

Neutrophil migration and activation are implicated in the pathogenesis of organ failure during sepsis, and therefore alterations in neutrophil trafficking are pivotal (1, 21, 26, 40). Th1 cells, induced mainly but not exclusively through a STAT4-dependent process (23, 24, 39), express chemokine receptors differently from Th2 cells (2). The secretion of chemokines also differs between Th1 cells and Th2 cells (44), suggesting a possible role for a STAT4-dependent mechanism of neutrophil recruitment. We observed no differences in neutrophil migration to account for the observed effects on bacterial counts or survival. This observation is corroborated by in vitro experiments demonstrating intact chemokine secretion in Th1 cells from STAT4−/− mice (44).

In summary, STAT4 participates in several important ways during polymicrobial sepsis, including eradicating bacteria, regulating IL-12 levels, and affecting survival. Based on our data, it appears that IL-12 mediates bacterial clearance via activation of STAT4 during CLP. However, blockade of IL-12 results in decreased survival, whereas STAT4−/− mice display increased survival. These findings suggest that STAT4 activation is detrimental to host survival and that IL-12 may activate other signaling pathways that promote survival. Further investigation into the nuances of STAT4 and IL-12 function and application of this knowledge to sepsis may ultimately reveal an intervention allowing increased survival without adversely affecting bacterial clearance.
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