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Septic syndrome is a consequence of innate immune failure. Recent studies showed that the CC chemokine CCL6 enhanced antimicrobial immunity during experimental sepsis through an unknown mechanism. The present study demonstrates that transgenic CCL6 expression abolishes mortality in a septic peritonitis model via the modulation of resident peritoneal cell activation and, more importantly, through the recruitment of IFN-producing NK cells and killer dendritic cells into the peritoneum. Thus, CCL6 attenuates the immune failure during sepsis, in part, through a protective type 1-cytokine mediated mechanism. The Journal of Immunology, 2007, 179: 5474–5482.

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evere sepsis, defined as sepsis associated with acute organ dysfunction, may result from exuberant innate immune and procoagulant responses to an infection (1). Despite important advances in antibiotic development and the improved effectiveness of critical care units with advanced ventilator support, the mortality due to sepsis has not dramatically changed over the past three decades (2). The present limitations for clinical intervention in sepsis reflect, in part, our limited understanding of the mechanisms involved in the regulation of the innate immune response during sepsis. It is now appreciated that the immune system during sepsis is precariously balanced between pro- and anti-inflammatory mediators. If the patient fails to mount an effective innate response, the clinical outcome could be an overwhelming infection. Conversely, should the immune system be improperly regulated, the patient may develop a systemic inflammatory response syndrome (SIRS), characterized by the high expression of several proinflammatory cytokines. SIRS may be just as lethal as an overwhelming infection, thus the immune system initiates a compensatory anti-inflammatory response (CARS), which can lead to a syndrome leaving the patient’s immune system in a state of immune suppression. CARS may increase the mortality of patients; this host response can lead to an immunocompromised patient and increase the susceptibility to secondary infection.

Chemokines play important roles in several activities such as angiogenesis/angiostasis, cellular differentiation and activation, wound healing, lymphocyte homing and development of lymphoid tissue, and influencing the overall type 1/type 2 balance of an immune response (3–5). Clinical studies have identified elevated levels of chemokines associated with human sepsis and acute lung injury (6–9). Because chemokines are essential to the innate immune response, they have been considered therapeutic targets during sepsis.

CCL6 is a CC chemokine initially isolated from mouse bone marrow (10). This chemokine is mostly a macrophage chemoattractant in vitro and in vivo (11, 12), but it can also attract B cells, CD4+ lymphocytes and eosinophils (11–13). CCL6 is a murine chemokine with several functional homologues in humans including macrophage inflammatory protein-1γ, CC chemokine F-18, hemofiltrate CC chemokine-1 and hemofiltrate CC-2. These chemokines have an unusual genomic structure, with a unique second exon and the ability to bind to and activate CC chemokine receptor 1 (14–19). Unlike all other chemokines, CCL6 is IL-4 inducible but not LPS inducible (13, 20). Several studies have shown that CCL6 is produced in large quantities during inflammatory and remodeling disorders, including those that involve alveolar remodeling, dermal wounding, allergic bronchopulmonary aspergillosis, bleomycin-induced pulmonary fibrosis, experimental demyelinating diseases, and acute and chronic peritonitis (11, 14, 16, 21–24). Recently, it was shown that rat microglia can express CCL6, and this study shows a possible role of this protein in cell-cell communication (22). CCL6 is also up-regulated in the peritoneal fluid of mice following cecal ligation and puncture (CLP) (18, 25, 26). In an experimental model of sepsis, recombinant CCL6 protected mice from CLP-induced lethality and this protection was associated with increased expression of TNF-α, IL-13, and CCL2 (18). Also, exogenous CCL6 enhanced bacterial clearance and the phagocytic capacity of peritoneal macrophages (18).

In the present study, we investigated the mechanism(s) through which this chemokine protects mice from severe sepsis. To this end, we used mice transgenically overexpressing CCL6 (CCL6 Tg) in the lung. Our data demonstrate that CCL6 Tg mice are extremely resistant to the lethality following CLP. These mice exhibited significantly increased levels of CCL2/MCP-1 at 4 h, and IFN-γ at 24 and 72 h, after CLP in the peritoneal lavage fluid. IFN-γ was identified as critical cytokine for the survival of CCL6 Tg mice, because anti-IFN-γ Ab abolished the protective effect of CCL6. Increased numbers of IFN-producing killer dendritic cells (IKDCs) and NK cells were observed in the peritoneal cavity of
A representative of three experiments.

The percentage of survival per day and each group has 10 mice. The data are analysis of the survival of these mice was followed until day 7. The results are expressed as a survival rate per day and per group. §, * p < 0.05 as compared with sham group.

CLP-CCL6 Tg mice, suggesting these cells were the source of the observed increases in IFN-γ. Thus, these data support the concept that CCL6 modulates endogenous macrophages activation possibly via CCL2 and, more importantly, facilitated the recruitment of effector immune cells with the enhanced ability to generate IFN-γ.

In vivo experimental protocol

IFN-γ neutralization. Wild-type (WT) and CCL6 Tg mice were treated i.p. with either control rabbit IgG (500 µg/mouse) or rabbit anti-murine IFN-γ-specific IgG (500 µg/mouse) 1 h before CLP surgery. The control IgG and anti-IFN-γ were purified from antisera using a Protein A column. Anti-IFN-γ antisera was raised by immunizing New Zealand white rabbits with murine IFN-γ (R&D Systems). The polyclonal Abs were titered by direct ELISA. The Abs did not cross-react with a number of other murine cytokines, including CXC and CC chemokines, as seen in the fact that IFN-γ ELISA established with the Abs did not detect any murine cytokine to a concentration as high as 100 ng/ml. Mouse survival was monitored in CLP groups containing ten mice for a total of 7 days. All mouse survival studies were conducted a maximum of three experiments.

NK cells depletion. WT and CCL6 Tg mice were treated i.p. with either isotype control (200 µg/mouse) or hamster anti-murine NK 1.1 (200 µg/mouse), provided by Dr. T. Moore (University of Michigan, Ann Arbor, MI), beginning at 24 h prior CLP and readministered at 48 h after CLP surgery. Mouse survival was monitored in CLP groups containing ten mice for a total of 7 days. All mouse survival studies were conducted a maximum of three experiments.

Clinical chemistry

Serum was separated from whole blood and the aspartate transaminase (AST) and alanine transaminase (ALT) levels were measured by Clinical Pathology at the University of Michigan Medical School using standardized techniques.

Peritoneal cells culture

Peritoneal cells were harvested from sham and CLP mice at 72 h after surgery and subjected in RPMI 1640 (BioWhittaker) containing 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were plated in plastic 24-wells plates (1 × 10^6 cells/well) and incubated at 37°C in 5% CO₂. After 24 h, supernatants were removed, clarified by centrifugation, and analyzed by ELISA for IFN-γ production.

Determination of CFU

Peritoneal lavages fluid and EDTA-treated blood from 72 h post-CLP were placed on ice and serially diluted in sterile saline. A 10-µl aliquot of each
dilution was spread on thymic-shared Ag agar plates (BD Diagnostic Systems) and incubated at 37°C overnight. Colonies were counted and expressed as CFU/10^9 H9262/l. Groups contained four to six mice and the experiments were repeated on three different occasions. Results were similar for each experiment and subsequent pooled.

**Measurement of cytokines and chemokines by ELISA**

Concentrations of murine IFN-γ, IL-12 (p70), IL-10, IL-13, TNF-α, CCL2, CCL3, CCL5, and CCL17 were measured in cell-free peritoneal lavage fluid and cell culture supernatants using a standardized sandwich ELISA previously described (31). In brief, 96-well microtiter plates (Nunc) were coated overnight at 4°C with mAb specific for the murine cytokine and chemokine being measured (R&D System). Wells were washed with PBS plus 0.05% Tween 20 and nonspecific sites were blocked with 2% BSA in PBS for 90 min at 37°C. Plates were washed and samples were loaded and incubated at 37°C for 1 h. After washing, a secondary, biotinylated, cytokine/chemokine-specific polyclonal Ab (R&D Systems) was added for 30 min at 37°C. Plates were washed again and peroxidase-conjugated streptavidin (Bio-Rad) was added. Plates were washed and a chromogenic substrate (Bio-Rad) was added and incubated at room temperature until fully developed. Reactions were stopped and read at 490 nm in an ELISA plate reader. Recombinant murine cytokines/chemokines (R&D System) were used to generate standard curves and concentrations were expressed as ng or pg/ml. Experimental groups consisted of triplicate samples in vitro from three independent experiments.

**Flow cytometry analysis**

Peritoneal cells were harvested from sham and CLP groups 72 h after surgery. RBC were lysed in lysis buffer and the remaining cells were washed in PBS and resuspended in PBS containing 1% BSA. Cells were Fc blocked and stained with various Abs. For cells staining, we used Abs labeled with PE, FITC, PeCy5, or PeCy7 direct against the following: CD11b, MHCI, CD19, IgM, CD3, CD4, CD8, NK 1.1 (all from BD Pharmingen), CD49b (eBioscience), or F4/80 (abCam). For intracellular staining, peritoneal cells were collected and placed in plastic 24-wells plates (2 x 10^6 cell/well) and incubated at 37°C in 5% CO2 with protein transporter inhibitor Golgi Plug (BD Pharmingen) to prevent the release of IFN-γ. After 6 h of incubation, cells were transferred to 5 ml tubes and staining first for the surface markers described above and then for IFN-γ intracellular (BD Pharmingen). Cells were analyzed with a Beckman Coulter Cytomics FC500.
ANOVA. When significance was detected, individual differences were analyzed using the Bonferroni’s t test for unpaired values. Statistical significance was set at \( p < 0.05 \). Survival rates were expressed as percentages, and a log rank test (\( \chi^2 \) test) was used to detect differences in mouse survival.

**Results**

**Expression and biologic activity of CCL6 during the evolution of experimental sepsis**

CLP-induced sepsis exhibits many of the features of clinical sepsis including SIRS and CARS. We previously demonstrated that exogenous CCL6 exerted a protective effect in CLP-induced sepsis, but the mechanism responsible for this protective effect was unclear. In an attempt to understand the protective role of this chemokine, CCL6 Tg and WT mice were subjected to experimental peritonitis. Fig. 1, A and B shows that the baseline and CLP-induced CCL6 levels were significantly higher in the CCL6 Tg group compared with the WT group. At baseline these values were 7-fold higher in the lung (Fig. 1A) and 9-fold higher in the peritoneal cavity (PC) (Fig. 1B). After CLP, these values were 6-fold higher in the lung (Fig. 1A) and 4-fold higher in the PC (Fig. 1B). To investigate the susceptibility of CCL6 Tg mice to CLP, WT, and CCL6 Tg mice were subjected to sham and CLP without antibiotic treatment. Fig. 1C shows mouse survival following sham and CLP. Ninety percent of the CCL6 Tg group was alive at day 7 after CLP, whereas only 20% of the WT group was alive at the same time. Together, these data suggest that increased levels of CCL6 dramatically protected mice from severe sepsis.

**CCL6 Tg mice had enhanced bacterial clearance and reduced bacteremia after CLP**

To determine whether the improved survival of CCL6 Tg mice was attributable to improved bacterial clearance, peritoneal, and blood samples were collected to determine local and systemic bacterial loads, respectively (Fig. 2). At 72 h post-CLP, the bacteria CFU cultured from plasma (Fig. 2A) and peritoneal wash samples (Fig. 2B) from CCL6 Tg mice were lower compared with WT mice. These results were statistically significant for the PC samples (\( p = 0.05 \)) but not for the blood samples despite a log difference in the means of these samples.

**CCL6 Tg mice have less liver injury induced by CLP**

One of the major complications of sepsis is multiple organ failure, which often leads to death (1). To determine the ability of CCL6 to prevent CLP-induced liver injury, the levels of liver enzymes in the serum of sham- and CLP-operated mice were measured in WT and CCL6 Tg groups (Fig. 3). The AST (Fig. 3A) and ALT (Fig. 3B) levels of both groups were similar in both sham- and CLP-operated mice at 4 h after surgery. At 24 h post-CLP, the serum of both CCL6 Tg and WT mice showed significantly higher levels of ALT and AST compared with sham-operated mice levels, but CCL6 Tg mice had less liver injury induced by CLP (Fig. 2). CCL6 Tg mice exhibited lower AST and ALT levels (550 ± 70 and 110 ± 10, respectively) than CLP-WT mice (780 ± 50 and 200 ± 32, respectively). At 72 h post-CLP, both AST and ALT returned to baseline levels in CLP-CCL6 Tg mice, whereas these values were 50 and 110, respectively, in CLP-WT mice. This difference was statistically significant (\( p < 0.05 \)) as compared with WT-CLP group.

**Statistical analysis**

All data are shown are means ± SE and are representative of three separate experiments. The means between different treatments were compared by ANOVA. When significance was detected, individual differences were analyzed using the Bonferroni’s t test for unpaired values. Statistical significance was set at \( p < 0.05 \). Survival rates were expressed as percentages, and a log rank test (\( \chi^2 \) test) was used to detect differences in mouse survival.
levels remained elevated in CLP-WT mice. Thus, these data suggest that the CCL6 Tg mice recovered faster from the liver injury caused by CLP.

**Time point differences in cytokine and chemokine levels in the peritoneal cavity of WT and CCL6 Tg mice: diminished early cytokine storm in CCL6 Tg group**

Previous studies have shown that the local and systemic levels of many cytokines and chemokines are markedly increased, following CLP surgery (32, 33). To establish the cytokine profile in CCL6 Tg mice, PC fluid from both sham and CLP groups was collected and analyzed in ELISA (Fig. 4). At 4 h after surgery, higher levels of IFN-γ and IL-10 were detected in CLP-WT mice compared with CLP-CCL6 Tg mice (Fig. 4, A and D). At this same time, only CCL2 levels were significantly increased in the CLP-CCL6 Tg group when compared with WT mice (Fig. 4D). Levels of CCL3, CCL17, CCL2 and IL-10 were elevated in CLP-WT group at 24 h post-CLP (Fig. 4, B and E). CCL2, CCL3, CCL17, and particularly, IL-10 levels were markedly higher in CLP-WT mice at 72 h after CLP (Fig. 4C). In contrast, at 72 h post-CLP, CLP-CCL6 Tg mice exhibited significantly higher levels of IFN-γ (0.459 ng/ml vs 0.055 ng/ml) and IL-12 (0.225 ng/ml vs 0.054 ng/ml) compared with CLP-WT group at this time after CLP (Fig. 4, C and F).

**Cells from the peritoneal cavity of CCL6 Tg mice produce high levels of IFN-γ**

Peritoneal cells from WT and CCL6 Tg mice harvested at 4, 24, and 72 h post-sham and post-CLP surgery were cultured in vitro for 24 h and IFN-γ and IL-10 levels were measured by ELISA. Confirming the trends showed in Fig. 4, cells isolated from the peritoneal cavity of CLP-WT mice generated higher levels of IFN-γ and IL-10. For NK cell depletion had no significant effect on CCL6 Tg mice survival following CLP. WT and CCL6 Tg mice received either control IgG (200 μg/mouse) or anti-NK 1.1 (200 μg/mouse) 24 h before and 48 h after CLP surgery. At 72 h after CLP, cells from peritoneal cavity were collected and stained with Abs for the following specific cell markers: NK cells, NK 1.1 CD49b (A); and IKDCs, NK 1.1 CD49b MHCII (B). The survival of WT (C) and CCL6 Tg (D) mice was followed until day 6. The results are expressed as percentage of survival per day and each group has ten mice. The data are representative of three experiments. * p < 0.05 as compared with IgG-CLP-operated mice.

**FIGURE 7.** Profile of peritoneal cell populations of WT and CCL6 Tg mice following sham and CLP surgery. Mice were killed 72 h after sham and CLP surgery and the cells were collected and processed for FACS analysis. The peritoneal cells were stained with Abs to specific cell markers following: macrophages, CD11b F4/80 MHCII (A); B1 lymphocytes, CD11b IgM CD19 (B); T lymphocytes, CD3 CD4+ (C) and CD3 CD8+ (D); NK cells, NK 1.1 CD49b (E); and NKT cells, CD3 NK 1.1 (F). The results are expressed as number of cells × 10⁶ per cavity and each group has three mice. The data are representative of three experiments. * p < 0.05 as compared with sham group. §, p < 0.05 as compared with WT-CLP group.

**FIGURE 8.** NK cell depletion had no significant effect on CCL6 Tg mice survival following CLP. WT and CCL6 Tg mice received either control IgG (200 μg/mouse) or anti-NK 1.1 (200 μg/mouse) 24 h before and 48 h after CLP surgery. At 72 h after CLP, cells from peritoneal cavity were collected and stained with Abs for the following specific cell markers: NK cells, NK 1.1 CD49b (A) and IKDCs, NK 1.1 CD49b MHCII (B). The survival of WT (C) and CCL6 Tg (D) mice was followed until day 6. The results are expressed as percentage of survival per day and each group has ten mice. The data are representative of three experiments. * p < 0.05 as compared with IgG-CLP-operated mice.
FIGURE 9. IKDCs population is increased in CCL6 Tg mice after CLP surgery. Mice were sacrificed 72 h after sham or CLP surgery and the peritoneal cells were collected and processed for FACS analysis. The peritoneal cells were stained for IKDCs specific markers NK1.1+CD49b+ MHCII+ . The results are expressed as number of cells × 10⁶ per cavity and each group has three mice. The data are representative of three experiments. *, p < 0.05 as compared with sham group. §, p < 0.05 as compared with WT-CLP group.

4 h after surgery compared with cells from CLP-CCL6 Tg mice (Fig. 5A). However, at later time points IFN-γ levels in cells from CLP-WT mice decreased until 72 h when they were comparable to values expressed in cells from sham-WT mice. In contrast, IFN-γ expression in cells from CLP-CCL6 Tg mice increased with time and was significantly higher than in all other experimental groups at 72 h (Fig. 5, A–C). In contrast, IL-10 expression showed a reverse trend (Fig. 5, D–F): In cells from WT-CLP mice, IL-10 significantly increased as a function of time between 4 and 72 h while significantly decreasing in CLP-CCL6 Tg mice. These data suggested that IFN-γ and IL-10 were expressed in a manner and at levels conducive for clearance and survival from SIRS.

Neutralization of IFN-γ caused CLP-induced lethality in CCL6 Tg mice

In an attempt to determine whether the delayed increase in IFN-γ levels in the peritoneal cavity of CCL6 Tg mice contributed to a protective role of CCL6 against sepsis-induced mortality, CCL6 Tg mice were pretreated with control rabbit IgG or affinity-purified rabbit IgG specific for murine IFN-γ 1 h before CLP surgery (Fig. 6). When CCL6 Tg mice received anti-IFN-γ before CLP, only 20% of these mice were alive at day 7 post-CLP compared with mice pretreated with control IgG (90% of survival). These data suggest a critical role for IFN-γ in the protective effect observed in CCL6 Tg mice in our model of severe sepsis.

Peritoneal cell population in WT and CCL6 Tg mice at 72 h after CLP surgery

To determine the type of cell contributing to increased IFN-γ during sepsis, peritoneal cells were isolated 72 h post-surgery from WT and CCL6 Tg mice subjected to either sham or CLP surgery and analyzed by flow cytometry. Cells from the peritoneal cavity of sham and CLP mice were stained with Abs to specific markers for macrophages (CD11b+F4/80+MHCII+), B1 lymphocytes (CD11b+IgM+CD19+), T lymphocytes (CD3+CD4+ and CD3+CD8+), NK cells (NK1.1+CD49b+), and NKT cells (CD3+NK1.1+) (Fig. 7). There was no significant difference in the numbers of macrophages, B1 or T cells in WT and CCL6 Tg mice after CLP surgery (Fig. 7, A–D). In contrast, there was a significant increase in the number of NK cells in CLP-CCL6 Tg mice compared with CLP-WT mice (Fig. 7E). Additionally, the number of NKT cells was significantly lower in both sham- and CLP-operated CCL6 Tg mice compared with WT mice (Fig. 7F).

Depletion of NK cells does not alter CCL6 Tg mice survival

Activated NK cells are a major source of IFN-γ during sepsis and are therefore involved in the pathogenesis of sepsis (34, 35). To determine whether NK cells were involved in the immunomodulatory role of CCL6 during severe sepsis, NK cells were depleted before CLP surgery. WT and CCL6 Tg mice received control IgG and anti-NK 1.1 Abs 24 h before and 48 h after CLP surgery. Fig. 8A shows that the treatment with anti-NK1.1 before CLP completely depleted NK cells in both groups while 97% and 92% of IKDCs were depleted in WT and CCL6 Tg mice, respectively (Fig. 8B). In WT mice (Fig. 8C), the NK cell depletion resulted in significant protection against CLP, while in CCL6 Tg mice (Fig. 8D) the NK depletion reduced survival, however these differences were not statistically significant.

IKDCs are increased in CLP-CCL6 Tg mice

NK cells and dendritic cells (DC) are central components of innate and adaptive immune responses. NK cells are believed to be the major producers of IFN-γ, which is secreted in response to IL-12 (36). Upon stimulation, DCs are also able to produce cytokines, such as IL-12 and TNF-α. Recently, DCs have also been shown to secrete IFN-γ in response to IL-12 alone or in combination with IL-18 (36, 37). Recent studies have characterized a new subset of DC that share phenotypic and functional properties of both NK cells and DCs. These cells express both NK cell (NK 1.1, CD49b)
and DC (CD11c, MHC class II (MHCII)) surface markers and produce significant amounts of IFN-γ and IL-12 in response to CpG (38). To determine whether IKDCs were present in the peritoneal cavity of WT and CCL6 Tg sham- and CLP-operated mice, cells from the peritoneal cavity of sham and CLP mice were stained for NK1.1, CD49b, and MHCII markers and analyzed by flow cytometry. The number of IKDCs in CCL6 Tg mice was higher than in WT mice regardless of the surgical intervention (sham and CLP) (Fig. 9). A 3-fold increase in the number of IKDCs was seen when CLP-CCL6 Tg mice were compared with CLP-WT mice. These data suggest that the protective role of CCL6 during severe sepsis may be related to the effect of this chemokine on IKDC recruitment into the peritoneal cavity after CLP surgery.

IKDCs are the main cell type producing IFN-γ in CCL6 Tg mice

To investigate whether the IKDCs were producing IFN-γ, peritoneal cells from WT and CCL6 Tg mice were harvested 72 h after CLP, stained for NK 1.1, CD49b, and MHCII markers and intracellular IFN-γ and then analyzed by flow cytometry. Fig. 10A shows that 63.3% of total IKDCs (NK1.1+CD49b+MHCII+) were positive for IFN-γ in CCL6 Tg mice. To investigate whether other cell types were producing IFN-γ, peritoneal cells were also stained for NK1.1/CD49b (NK cells) and CD3/CD8 (T lymphocytes) and IFN-γ. Only 1.6% of total NK cells and 1.8% of total CD8+ T lymphocytes were positive for IFN-γ in CCL6 Tg mice (Fig. 10, B and C). Other cell types such as macrophages, B1 cells, and T lymphocytes CD4+ were negative for IFN-γ in WT and CCL6 Tg mice at 72 h after CLP (data not shown). These data show that IKDCs are the key cells producing IFN-γ in CCL6 Tg mice at 72 h post-CLP surgery.

Discussion

Dysregulation of the immune system occurs during severe sepsis, leading to a rapid death due to the development of multiorgan failure and an increase in complications due to long-term immunosuppression (39–41). CCL6, a CC chemokine, is present in a variety of inflammatory and remodeling disorders (11, 14, 16, 18, 24). We previously showed that the immunoneutralization of CCL6 enhanced the sepsis-related mortality. In contrast, the administration of recombinant CCL6 to mice immediately after CLP surgery significantly improved the survival of these mice (18). In this study, we show that mice overexpressing CCL6 are protected from mortality following CLP. In our model of severe sepsis (with no antibiotic treatment), these mice presented an impressive 90% survival rate vs a 20% survival in WT mice. The CCL6 Tg mice exhibited higher levels of CCL6, in the lung and PC, at 24 h post-CLP (data not shown) and the levels remained elevated even at 72 h after CLP as shown in Fig. 1. These data agree with other studies demonstrating that the production of CCL6 remains elevated several days after septic peritonitis (23, 24). In our model, this sustained increase of CCL6 levels seemed to be important in regulating the deleterious effects induced by sepsis, because, in addition to improved survival, the CLP-CCL6 mice present an improved recovery from liver injury compared with CLP-WT mice. This protective effect is likely caused by an increase in CCL6-mediated cytokine cascades, which provides an apparent cytokine environment for both microorganism clearance and regulate the local inflammatory response.

Previous studies demonstrated that CCL2 has a protective role during septic peritonitis (32, 42). Mice treated with CCL2 were protected against a lethal dose of LPS or bacteria, and the mechanism of protection seems to be by way of increased IL-10 production concomitant with a decrease of IL-12 and TNF production (43). Several studies demonstrated that CCL2 is produced early in response to endotoxin challenge, returning to baselines levels after 48 h (18, 32, 42). In our model, CCL2 peaked at 4 h post-CLP in CCL6 Tg mice, which was coincident with low levels of IL-12 and IFN-γ. However, at 24 and 72 h, the CCL6 Tg mice presented very low levels of CCL2. Previous studies demonstrated that CCL6 neutralization decreased CCL2 production in the lung (16, 20). However, previous studies showed that the neutralization of CCL6 increased CCL2 levels in their model of acute peritonitis (23). In CLP-CCL6 Tg mice, CCL6 levels were elevated at 24 and 72 h post-CLP and very low levels of CCL2 were found at these time points, suggesting that CCL6 may inhibit CCL2 production. Previous studies demonstrated that CCL2 increases the production of IL-10 in experimental models of sepsis (42, 43). IL-10 has been identified as a crucial modulator of the inflammatory response in sepsis and it is an important cytokine mediator of sepsis-induced immunosuppression (44). Prolonged immune suppression is characterized by defects in Ag presentation, macrophage paralysis, T cell anergy, suppressed T cell proliferation, and increased T cell and B cell apoptosis, which may be partially attributable to the biological effects of IL-10. Indeed, in our model, the high and sustained levels of CCL2 and IL-10 in CLP-WT mice correlated with poor survival rates. Interestingly, we observed a gradual increase of IFN-γ and IL-12 in CLP-CCL6 Tg mice with time post-CLP. IFN-γ strongly stimulates monocytes/macrophages, increasing their microbicidal activity, Ag presentation function, and production of proinflammatory cytokines on contact with microbrial stimuli. Genetics defects in the IFN-γ receptor system have been described in patients with vaccine-associated bacterial infections, demonstrating the importance of IFN-γ-mediated immunity in human host defense against intracellular pathogens (45, 46). In addition, the important role of IFN-γ in the pathogenesis of LPS-induced shock was confirmed using mice deficient for the IFN-γ receptor (47, 48). In this study, we show the crucial role of IFN-γ in CCL6-Tg mice during severe sepsis. These mice expressed significantly elevated levels of IFN-γ at 72 h after CLP surgery and 90% survival was observed. This increase in IFN-γ was important for the survival of these mice because, 80% of CCL6 Tg mice died following anti-IFN-γ Ab treatment. In contrast, the neutralization of IFN-γ protected WT mice from sepsis-induced mortality suggesting that the rapid increase of IFN-γ levels observed at 4 h after CLP can be deleterious and increase the mortality of these mice (data not shown).

IFN-γ is produced primarily by NK cells and a certain subpopulation of T lymphocytes (49). In this study, we demonstrated that a NK cell population is increased in CLP-CCL6 Tg mice compared with CLP-WT mice. To determine whether NK cells were playing a role in the protective role of CCL6 during severe sepsis, we depleted NK cells in WT and CCL6 Tg mice. NK cells did not appear to provide a protective role, because we observed only a slight decrease (not statistically significant) in the survival rate of CCL6 Tg mice. However, the treatment with anti-NK1.1 Ab did not completely deplete IKDCs population, suggesting that there is still a small population of IKDCs that may be producing IFN-γ. This potentially could explain why there is not a significant difference in the CLP-CCL6 Tg survival rate. In contrast, the treatment with anti-NK Ab was protective for WT mice against sepsis-induced mortality. Some studies demonstrated a detrimental role for NK cells in experimental models of sepsis (50, 51). Activated NK cells are a main source of IFN-γ during sepsis and therefore likely are involved in the pathogenesis of sepsis (52). Indeed, depletion of NK cells in septic mice offers protection against cytokine- and LPS-induced shock (34, 52).
IKDCs are a new population of murine immune cells that are distinct from conventional DCs and plasmacytoid DCs because they express both NK cell (NK 1.1, CD49b) and DC (CD11c, B220; MHCII) markers (38, 53). It was demonstrated that IKDCs are present in the spleen, lymph node, thymus, and liver of normal mice. Upon activation with CpG, IKDCs secrete high levels of IFN-γ and this secretion depends on autocrine IL-12 (38). Also, these cells are able to directly lyse tumor cells, and present Ag to naïve T cells. In this study, we show that IKDCs were also present in the peritoneal cavity of both WT and CCL6 Tg mice. CCL6 Tg mice submited to sham and CLP surgery had higher numbers of IKDCs in the peritoneal cavity when compared with CLP-WT mice. These data suggest that IKDCs may function in a protective role in the CCL6 Tg mice and alter sepsis-induced mortality. A significant increase in total numbers and percent of IFN-γ positive IKDC were identified in CCL6 Tg mice post CLP, as compared with WT mice. This finding could aid in explaining the increase in IFN-γ associated with the CCL6 Tg mice.

Septic mortality is often depicted as being fueled by an imbalance in the proinflammatory and immunosuppressive homeostasis and in severe sepsis this imbalance is exemplified by SIRS and CARs. The present study demonstrated that transgenic CCL6 reduced mortality in a septic peritonitis model, via a mechanism that involved the recruitment and activation of IFN-γ producing killer DC into the peritoneum. In addition, CCL6 Tg mice expressed elevated levels of CCL2 early during the septic response and this chemokine may aid in preventing SIRS and enhancing survival. At later time points, when CARS is thought to exert an immunosuppressive environment, the increased levels of IFN-γ may have countered the severe immunosuppression and enhanced survival in the CCL6 Tg CLP mice. Our studies demonstrate the importance of CCL6 in attenuating immune failure during severe sepsis and future studies will continue to delve into the mechanisms and pathways underlying these protective events.

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