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Immunomodulatory Role of CXCR2 During Experimental Septic Peritonitis

Traci L. Ness,* Cory M. Hogaboam,* Robert M. Strieter,† and Steven L. Kunkel2**

The loss of CXCR2 expression by neutrophils is a well-described, but poorly understood, consequence of clinical sepsis. To address the potential impact of this CXCR2 deficit during the septic response, we examined the role of CXCR2 in a murine model of septic peritonitis provoked by cecal ligation and puncture (CLP). CLP-induced mouse mortality was significantly attenuated with i.v. or i.p. administration of an affinity-purified murine CXCR2-specific polyclonal Ab. Mouse survival required Ab administration before and every 2 days following CLP. Furthermore, mice deficient in CXCR2 (CXCR2−/−) were significantly protected against CLP-induced mortality compared with control (CXCR2+/+) mice. The anti-CXCR2 Ab treatment delayed, but did not completely inhibit, the recruitment of leukocytes, specifically neutrophils, into the peritoneal cavity. Peritoneal macrophages from anti-CXCR2 Ab-treated mice exhibited markedly increased RNA and protein levels of several key proinflammatory cytokines and chemokines. Specifically, isolated preparations of these cells released ~11-fold more CXCL10 protein compared with peritoneal macrophages from control-treated or naive mice. CXCR2−/− mice had higher resting and CLP-induced levels of peritoneal CXCL10 compared with CXCR2+/+ mice. Administration of a neutralizing, affinity-purified, murine CXCL10-specific polyclonal Ab before CLP in wild-type mice and every 2 days after surgery significantly increased mortality compared with control Ab-treated mice. Anti-CXCL10 treatment in CXCR2−/− mice negated the protective effect associated with the absence of CXCR2. In summary, these data demonstrate that the absence of CXCR2 protects mice from septic injury potentially by delaying inflammatory cell recruitment and enhancing CXCL10 expression in the peritoneum. The Journal of Immunology, 2003, 171: 3775–3784.

Sepsis and septic shock are clinical manifestations of an overzealous innate immune activation due to the presence of bacterial pathogens within infected tissues. At present, sepsis remains the most common cause of death in noncoronary critical care units in the United States. The Center for Disease Control estimated a total of 400,000 cases of sepsis/year in the 1980s (1), whereas more recent national estimates show a much higher incidence of 750,000 cases/year in the mid 1990s (2). The mortality rate among all patients with clinically defined sepsis or systemic inflammatory response syndrome remains unacceptably high at ~35%, but the presence of complicating factors such as disseminated intravascular coagulation, acute respiratory distress syndrome, and multiorgan failure propels the mortality rate to almost 60% (2). Unfortunately, these mortality rates have not changed appreciably over the last 2 decades, since the care of septic patients remains largely organ supportive (3). A number of novel therapeutics specifically developed to prevent sepsis-induced mortality have failed (4, 5), with the exception of recombinant activated protein C (6).

Chemokines, a superfamily of small, predominantly secreted proteins with unique cysteine amino acid motifs, are viewed as therapeutically important targets during inflammatory responses, including those invoked during sepsis (7, 8). The actions of chemokines, particularly those in the CC and CXC chemokine families (8, 9), in the septic response have traditionally been described in terms of their direct effects on the migration of inflammatory cells (8). Much less is known about the manner in which the specific G protein-coupled receptors that bind chemokines are regulated on various leukocyte populations during sepsis. To date, an interesting observation has emerged from clinical studies indicating that CXC receptor 2 (CXCR2), but not CXCR1, surface expression is down-regulated by 50% on neutrophils from septic patients compared with normal donors (10, 11). CXCR2 binds CXC chemokine ligand 1–3 (CXCL1–3) and CXCL5–8, whereas CXCR1 appears to be more selective in its binding profile, in that CXCL6 and CXCL8 are its only recognized ligands (12, 13). The decrease in CXCR2 expression has been shown to markedly impair the ability of neutrophils to respond to CXCR2, but not CXCR1 ligands (10, 11). The loss of CXCR2 function has raised concerns about the predisposition of septic patients to further infection or pneumonia (11), but the impact of CXCR2 loss during sepsis has not been previously explored in detail.

The present study addressed the overall impact of CXCR2 deficiency during experimental septic peritonitis in mice. Criticism has been directed at the relevancy of murine sepsis models given that many novel antisepsis therapies that were efficacious in these models proved ineffective in clinical sepsis trials (3, 14). However, this criticism is most appropriate for experimental models of direct LPS or endotoxin infusion, models that recapitulate the cardiovascular ramifications of sepsis but ignore the impact of pathogen

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Abbreviations used in this paper: CXCL, CXC chemokine ligand; ALT, alanine transaminase; AST, aspartate transaminase; CCL, CC chemokine ligand; CLP, cecal ligation and puncture; Cx32, threshold cycle; MIP-2, macrophage inflammatory protein 2.
infection typically observed in sepsis. The cecal ligation and puncture (CLP) surgery model addresses this concern, since it involves trauma (perforation) to the bowel, thereby permitting the introduction of multiple bacterial strains into the peritoneal cavity. This experimental scenario with fluid resuscitation more accurately reflects the pathophysiologic responses observed in human sepsis, which can be complicated by the presence of multiple bacterial strains (14). Herein, we demonstrate that the absence of CXCR2 (due to Ab neutralization or genetic deletion) during the systemic inflammatory response that follows CLP surgery provides a significant survival effect in this murine model. While the lack of functional CXCR2 has a temporal effect on the recruitment of neutrophils into the inflamed peritoneal cavity, this study shows that the lack of functional CXCR2 also leads to the augmented release of CXCL10 from peritoneal macrophages.

Materials and Methods

Mice

Specific pathogen-free female CD-1 mice (6–8 wk of age) were purchased from Charles River Laboratories (Portage, MI), and specific pathogen-free BALB/c mice (6–8 wk of age) were purchased from The Jackson Laboratory (Bar Harbor, ME). CXCR2−/− mice (on a BALB/c background) were bred and maintained by the University Laboratory of Animal Medicine at University of Michigan. All mice were housed five to a cage in filter-topped cages and had free access to food and water before their use in an experiment. The animal use committee at University of Michigan approved all experimental procedures involving mice.

CLP surgery

CLP surgery was performed on all mice as described previously in detail (15). Briefly, mice were anesthetized with an i.p. injection of 2.25 mg of ketamine HCl (Abbott Laboratories, Chicago, IL) and 150 μg of xylazine (Lloyd Laboratories, Shenandoah, IA). Under sterile conditions, a 1- to 2-cm incision was made on the lower left abdomen, and the cecum was exposed. The distal portion of the cecum was ligated with 3.0 silk suture and punctured through and through (two punctures to the cecal wall) with a 21- or 26-gauge needle. The cecum was returned to the peritoneal cavity, and the incision was closed with surgical staples. Sham-operated mice were subjected to a similar laparotomy without ligation or puncture. Mice were rehydrated with 1 ml of saline s.c. and placed on a heating pad until they recovered from the anesthetic.

In vivo experimental protocols

CXCR2 immunoneutralization. CD-1 mice were treated i.p. or i.v. with either 0.5 mg of control goat IgG (Sigma-Aldrich, St. Louis, MO) or goat anti-murine CXCR2-specific IgG purified from antiserum described previously (16) 2 h before 21-gauge CLP and every 2 days postsurgery. The CXCR2-specific IgG was purified from antiserum using a protein G column (Pierce, Rockford, IL). Mouse survival was monitored in CLP groups containing 9–11 mice for a total of 7 or 8 days. All mouse survival studies were conducted a maximum of four times. In a second set of experiments, the timing of Ab administration was varied. The first dose of Ab was given i.p. either 4 h or 1 day after CLP surgery, and anti-CXCR2 was readministered every 48 h thereafter. Alternatively, Ab treatment was given only as a pretreatment, 2 h before surgery. In a third set of experiments, 0.5 mg of Ab (anti-CXCR2 or control IgG) was administered i.p. 2 h before CLP surgery. Before CLP but 2 h after Ab, and at 4, 8, and 24 h after CLP surgery, 0.5 mg of Ab (anti-CXCR2 or control IgG) was administered (i.p.) or through a 21-gauge needle. Peritoneal lavages were performed in each mouse using 2 ml of sterile saline, and lavage fluids were collected for cell and protein analyses (see below).

CXCR2-deficient mice. Groups of 8–12 wild type BALB/c (CXCR2+/+) controls and CXCR2−/− mice were subjected to 21-gauge CLP, and survival was monitored for up to 7 days. All mouse survival studies were conducted a maximum of four times.

CXCL10 immunoneutralization. CD-1 mice were treated i.p. with 0.5 mg of anti-murine CXCL10 (Lloyd Laboratories, Shenandoah, IA) or control rabbit IgG 2 h before 21-gauge CLP and every 48 h after surgery. Both rabbit IgGs were purified from antiserum using protein A columns (Pierce). Control and anti-CXCL10-treated mice (n = 10/group) were monitored for mortality up to 7 days after CLP. The effect of CXCL10 neutralization was also examined in CXCR2-deficient mice (CXCR2−/−). Briefly, anti-CXCL10 or control IgG (n = 9/group) was administered 2 h before CLP surgery. Due to the enhanced CLP susceptibility of these mice (BALB/c background), a more mild CLP surgery was performed using a 26-gauge needle. Survival was followed for 7 days after CLP.

Clinical chemistry

Serum was separated from whole blood, and the clinical pathology laboratory at University of Michigan Medical School (Ann Arbor, MI) measured levels of aspartate transaminase (AST) and alanine transaminase (ALT) using standard procedures.

Peritoneal leukocyte totals and cell differentials

An aliquot of peritoneal lavage fluid was diluted, and the number of cells was counted in a hemocytometer. Results are presented as leukocytes per milliliter peritoneal lavage fluid. Cytospins were prepared from peritoneal lavage fluid and stained with Diff-Quik solutions (Dade Behring, Dudingen, Switzerland). The leukocyte composition was analyzed, and the percentage for each population was determined after counting a total of 300 cells from several high powered fields. The percentage of each cell type was multiplied by the total cell count to derive a population number for each cell type.

Measurement of cytokines and chemokines by ELISA

Peritoneal fluids were clarified by centrifugation. The resulting supernatants were stored at −20°C until ready for cytokine analysis. Murine IL-12 (p70), IL-10, CCL2, CCL3, CCL6, CCL22, and CXCL10 concentrations were measured using a standardized sandwich ELISA as previously described in detail (17). Briefly, 96-well immunoplates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with mAb specific for the murine cytokine/chemokine being measured (R&D Systems, Rochester, MN). Wells were washed with PBS and 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 adding (peroxidase-conjugated) 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, Richmond, CA) 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. Each ELISA was screened for specificity, and standard curves were generated using recombinant mouse cytokines and chemokines (R&D Systems). Limits of detection were consistently ~50 pg/ml. Cytokine/chemokine levels were expressed as nanograms per milliliter of peritoneal fluid. There were five mice in each group, and ELISAs were run on groups of mice from three separate experiments.

Isolation of peritoneal macrophages

For maximal recovery of peritoneal cells, peritoneal lavages were performed using 10 ml of DMEM (BioWhittaker, Walkersville, MD) containing 5% FCS for each mouse. Lavages containing peritoneal cells from individual mice were maintained separately. RBC were lysed in ammonium chloride lysis buffer, and the remaining cells were thoroughly washed in medium before performing a total cell count and differential analysis. Cells were pelleted and resuspended in a concentration of 106 macrophages/ml in RPMI 1640 (BioWhittaker) containing 5% FCS, 5 mM HEPES, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Macrophages were plated in plastic 24-well plates (5 × 105 macrophages/well) and incubated at 37°C in 5% CO2 for 1 h. Cells were washed three times with RPMI medium. Total RNA was isolated from selected wells using the TRIzol (Invitrogen, Carlsbad, CA) method for nucleic acid isolation according to the manufacturer’s instructions. RNA concentrations were determined by spectrometric analysis at the 260 nm wavelength. The remaining wells were incubated with 0.5 ml of RPMI medium overnight at 37°C in 5% CO2. After 24 h, supernatants were removed, clarified by centrifugation, and analyzed by ELISA for cytokine and chemokine production.

TagMan real-time RT-PCR analysis

Total RNA (1 μg) was reverse transcribed in a reaction mixture containing 1X First Strand Buffer (Life Technologies, Gaithersburg, MD), 250 ng of oligo(dt)20, primer, 1.6 mM dNTPs (Invitrogen), 5 μl of RNase inhibitor (Invitrogen), and 100 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) at 38°C for 60 min, followed by 90°C for 10 min. All TaqMan reagents were obtained from PE Applied Biosystems (Foster City, CA). Custom primers and probes were designed using Primer Express (PE Applied Biosystems). The amplification mixture consisted of 1X TaqMan Universal PCR Master Mix, 1X target primers and probe (FAM or MGB), 1X TaqMan, and 1X sample or control cDNA. PCR amplification was performed using TaqMan 7700 or Sequence Detection Systems (PE Applied Biosystems) in a 96-well format. The expression of each cytokine was normalized to the levels of 18S ribosomal RNA to account for any variation in the amount of RNA per sample.
1× control GAPDH primers and probe (VIC), and 1–2 μl of cDNA/reaction. Negative controls (no cDNA added) were included for each primer/probe set. PCR reaction conditions were as follows: 2 min at 50°C, 10-min denaturation at 95°C, and then cycled 40 times for 15-s annealing at 95°C and 1-min extension at 60°C. Data were analyzed using SDS software (PE Applied Biosystems). The threshold cycle (CT) is the number of cycles required for the reporter dye fluorescence released from the target-specific probe to reach the threshold level (set 10 SD above the mean baseline fluorescence). The ΔCT value was determined for each sample as the CT value for the gene of interest – the CT for the housekeeping gene GAPDH. Expression in each treatment group was converted to fold expression (increase or decrease) over the average basal expression level seen in macrophages from untreated mice, which was arbitrarily set at a value of 1.

Statistics

In survival studies a log-rank test was used to test for significance. For all other studies results are presented as the mean ± SEM, and unpaired Student’s t test was applied to evaluate significance. A p < 0.05 was considered statistically significant.

Results

Neutralization of CXCR2 protects against CLP-induced mortality

To determine the role of CXCR2 in the CLP model of experimental sepsis, CXCR2 was neutralized with specific anti-murine CXCR2 IgG 2 h before CLP surgery and every 2 days thereafter. Control mice received equal doses of purified goat IgG. As shown in Fig. 1, neutralization of CXCR2 significantly protected mice against CLP-induced mortality. When CXCR2 Ab was administered i.p. (Fig. 1A), lethality was reduced by 50% in the anti-CXCR2 treatment group (20% mortality) compared with the control group (40% mortality) at 2 days post-CLP surgery. This level of protection was maintained throughout the course of the study. At 7 days post-CLP surgery, the mortality rate in control mice was 61% (25 of 41 mice) compared with 36% in the anti-CXCR2-treated mice (14 of 39 mice).

In other in vivo CLP studies involving the immunoneutralization of chemokines during CLP-induced sepsis (18), the route of administration was found to be a major factor in the effect of the Ab. To address whether the route of administration affected the protective effects of CXCR2 neutralization, a survival study was conducted in which purified Abs were administered i.v. (Fig. 1B). Similar results were observed, in that a significant protective effect from the anti-CXCR2 Ab treatments was seen as early as 2 days post-CLP. At this time, 20% of the control mice were deceased (4 of 20 mice), whereas no deaths were observed in the anti-CXCR2 group. Significant enhancement of mouse survival was apparent from 3–7 days following CLP surgery. The mortality rate of control mice was 35% (7 of 20 mice) at 3 days post-CLP and increased to 55% (11 of 20 mice) by day 7, whereas the mortality rates for anti-CXCR2 Ab-treated mice progressed from 5% (1 of 21 mice) to 24% (5 of 21 mice), respectively, at days 3 and 7 after CLP surgery.

While the anti-CXCR2 Ab therapy worked regardless of its route of administration, it was not apparent whether this treatment could be administered after CLP surgery was initiated or CLP-induced sepsis was established. When the first dose of CXCR2 Ab was delayed until 4 h or 1 day post-CLP and was followed every 48 h with subsequent treatments, no survival advantage was observed (data not shown). In another experiment the purified Abs were only administered 2 h before CLP, and once again no survival advantage was observed in the anti-CXCR2-treated group (data not shown). Together, these data suggest that the immunoneutralization of CXCR2 before CLP and throughout the septic insult are important to provide protection against lethality. In subsequent experiments, anti-CXCR2 Ab treatments were administered through the i.p. route.

CXCR2−/− mice were significantly protected against mortality following CLP surgery

A second, independent analysis to elucidate the role of CXCR2 in the CLP model was initiated using CXCR2-deficient (CXCR2−/−) mice and wild-type BALB/c mice as CXCR2+/+ controls (Fig. 1C). The difference in survival between these two groups of mice became apparent on day 3 post-CLP when only 21% of the CXCR2+/+ mice (8 of 38 mice) compared with 47% of the CXCR2−/− mice (20 of 42 mice) were still alive. After 7 days, 4 of 37 CXCR2+/+ mice (11% survival) were alive, while almost 3 times as many CXCR2−/− mice remained (11 of 38 mice; 29% survival). These studies independently provide evidence that the absence of CXCR2 does not lead to increased susceptibility following CLP-induced septic peritonitis.

FIGURE 1. Neutralization or genetic deletion of CXCR2 improved mouse survival following CLP. A and B, CLP (21-gauge) mice received 0.5 mg of control goat IgG or goat anti-murine CXCR2 IgG i.p. (A) or i.v. (B) 2 h before and every 48 h after CLP. C, Wild-type BALB/c (CXCR2+/+) and CXCR2−/− mice were subjected to 21-gauge CLP. In all studies survival was followed for 7 days following surgery. The graphs represent survival data pooled from two to four different experiments. Individual experiments contained 8–12 mice/group and showed similar results.
CLP-induced liver injury was significantly decreased in anti-CXCR2 Ab-treated mice

One of the major complications of sepsis is that it can often result in multiple organ injury or failure, which can contribute to death (19). The levels of liver enzymes in the serum were measured to determine the effect of anti-CXCR2 Ab treatment on CLP-induced liver injury. In this mild model of sepsis, AST and ALT levels were significantly increased in the serum of CLP mice (indicating liver injury, but not liver failure) compared with sham surgery controls at 24 h (Fig. 2). At 24 h post-CLP, the serum of anti-CXCR2-treatment CLP mice showed significantly lower levels of AST (309 ± 40) and ALT (116 ± 14) enzymes compared with the serum of control IgG-treated CLP mice (503 ± 32 and 195 ± 16, respectively). These data suggest that neutralizing CXCR2 ameliorates hepatic injury in CLP mice. The level of myeloperoxidase (MPO) is often used as an indirect measure of neutrophil accumulation in various tissues. When MPO levels were measured in the liver and lung of control IgG- and anti-CXCR2 IgG-treated CLP mice, no differences in MPO levels in either organ were detected between the two groups (data not shown).

Neutralization of CXCR2 before CLP delays neutrophil migration into the peritoneum, but has no significant effect on bacterial clearance

In an attempt to determine the role of CXCR2 on leukocyte infiltration during septic peritonitis, mice were treated with anti-CXCR2 Ab or control IgG 2 h before CLP. Peritoneal lavage was performed just before CLP (2 h after IgG administration) and at 4, 8, and 24 h after CLP surgery. Total leukocyte numbers were assessed for each mouse. Leukocyte infiltration was initiated by administration of control goat IgG (without CLP; time zero), but not anti-CXCR2-IgG treatment (Fig. 3A). Four hours after CLP, leukocyte numbers had significantly increased in control IgG-treated mice, whereas no differences could be seen in the number of peritoneal cells in anti-CXCR2-treated mice. In fact, increases in the cell population of anti-CXCR2-animals were not seen until much later (between 8 and 24 h post-CLP). Differential analysis of the peritoneal leukocyte populations showed a significantly delayed neutrophil recruitment in anti-CXCR2-treated mice compared with control mice (Fig. 3B). At 4 h post-CLP, anti-CXCR2 treatment reduced neutrophil recruitment by 76%. Nonetheless, this effect was transient and by 24 h post-CLP, control and anti-CXCR2-treated mice had similar numbers of neutrophils in the peritoneal cavity. No differences were observed in numbers of mononuclear cells (Fig. 3C), eosinophils, or mast cells (data not shown). Therefore, immunoneutralization of CXCR2 did not prevent neutrophil recruitment, but this immunotherapy significantly delayed the neutrophil recruitment response, albeit modestly. To examine the effect of delayed neutrophil recruitment on bacterial clearance or spread, the bacterial load of peritoneal fluids and sera from CLP mice treated with anti-CXCR2 or control IgG was examined. Although marginally lower numbers of bacteria were detected locally and systemically in anti-CXCR2-treated mice 24 h post-CLP, no significant differences could be observed, indicating that the delayed leukocyte recruitment in these mice was sufficient for containment and clearance of the peritoneal infection (data not shown).

Treatment with anti-CXCR2 Ab does not significantly change cytokine levels in the peritoneum at various times after CLP surgery

To examine the effects of blocking CXCR2 on the production of cytokines and chemokines following CLP, peritoneal lavages were collected from CLP-mice pretreated with anti-CXCR2 or control IgG and measured using specific ELISAs. Most cytokines and chemokines examined in this experiment were not detectable in untreated mice (sham; time zero), but levels of several were significantly increased following CLP surgery (Fig. 4, A and B). IL-12 levels in the peritoneum continued to rise until 24 h post-CLP, while much higher levels of IL-10 were produced as early as 4 h and maintained until 24 h (Fig. 4A). Systemic IL-12 was detected in the serum as early as 4 h after CLP and continued to increase until 24 h. IL-10 was not detected in circulation. No significant differences were seen between IgG groups; however, there was a trend toward lower local and systemic levels of IL-12 at all times and lower peritoneal levels of IL-10 at 24 h in anti-CXCR2-treated mice compared with controls. The chemokines CCL2, CCL22, and CCL6 were induced both locally in the peritoneum and systemically following CLP, with overall peak expression at 8 and 24 h, respectively (Fig. 4B). The anti-CXCR2 treatment had no significant effect on the overall chemokine balance. At 8 h post-CLP, anti-CXCR2-treated mice had increased expression of CCL2, and at all times CCL22 expression was slightly lower in the peritoneum compared with control mice. Macrophage inflammatory protein 2 (MIP-2) and keratinocyte-derived chemokine levels were elevated in anti-CXCR2-treated mice at 4 and 8 h post-CLP, presumably because they were unable to bind their receptor.

Anti-CXCR2 Ab treatment augmented the RNA and protein levels of several proinflammatory cytokines and chemokines, most notably CXCL10, in isolated and purified peritoneal macrophages

The analysis of peritoneal leukocytes indicated that neutrophil migration into the peritoneum was delayed in anti-CXCR2 mice following CLP surgery. While neutrophils are considered essential to the host defense response during sepsis, the peritoneal macrophage has also been shown to play an important role in host defense within the peritoneal cavity (18, 20, 21). In an attempt to identify any specific effects that the anti-CXCR2 Ab exerted on peritoneal

![FIGURE 2](http://www.jimmunol.org)
peritoneal macrophages (Fig. 5). Neutralization of CXCR2 increased both IL-12 and IL-10 transcription compared with naive control IgG increased IL-12 (7.7 ± 0.15-fold), while anti-CXCR2 treatment induced by both IgGs, while CCL3 and CCL2.2 production were equally induced by both IgGs, while CCL2 production was suppressed in macrophages from control-treated mice (Fig. 6B). Most strikingly, an 11-fold increase in CXCL10 secretion from peritoneal macrophages was observed when these cells were isolated from anti-CXCR2-treated mice compared with naive, untreated mice.

**CXCR2−/− mice express significantly higher levels of endogenous and CLP-induced CXCL10 in the peritoneal cavity**

Since increased CXCL10 expression was observed from peritoneal macrophages of anti-CXCR2 Ab-treated mice, we sought to determine whether the same response was present in CXCR2-deficient mice. CXCR2−/− and CXCR2+/+ mice were left untreated or were subjected to CLP surgery. Peritoneal lavages were subsequently collected from both groups of mice, specifically at 8 h after surgery for the CLP group. Constitutive levels of CXCL10 were higher in untreated CXCR2−/− mice compared with untreated CXCR2+/+ mice (Fig. 7). Furthermore, levels of CXCL10 were significantly increased at 8 h after CLP surgery in CXCR2−/− mice compared with CXCR2+/+ mice. Thus, these data indicated that enhanced CXCL10 levels were present in the peritoneal cavities of CXCR2−/− mice.

**Neutralization of CXCL10 increases CLP-induced lethality**

Since the anti-CXCR2 Ab treatment appeared to promote CLP10 production by isolated peritoneal macrophages, and CXCL10 levels were enhanced in CXCR2−/− mice, we next investigated the role of CXCL10 in the CLP surgery model. A specific rabbit anti-murine CXCL10 Ab was used to neutralize CXCL10−2 h before CLP and every 2 days thereafter. Control mice received equal doses of purified rabbit Ab. In these studies the mortality rate induced by CLP was <30% at 8 days after CLP surgery (in contrast to the ~60% mortality rate observed in the previous experiments described above). CLP-induced mortality was almost 2-fold greater in anti-CXCL10-treated mice (52%; 15 of 29 mice) compared with the control group (27% 8 of 30 mice; Fig. 8A). These data indicated that CXCL10 protected mice from sepsis-induced lethality, and therefore we hypothesized that increased levels of this chemokine may have been partly responsible for the protection observed in the absence of CXCR2.

**Neutralization of CXCL10 in CXCR2-deficient mice increases CLP-induced lethality**

In an attempt to determine the role of enhanced CXCL10 expression in the absence of CXCR2 in vivo following CLP surgery, CXCR2−/− mice were treated with purified anti-CXCL10 2 h before surgery. Control CXCR2−/− mice received an equal dose of purified rabbit IgG. Since CXCR2−/− mice, which are on a BALB/c background, are more susceptible to the lethal effects of CLP compared with CD-1 mice, these groups of CXCR2−/− mice were subjected to CLP with a 26-gauge needle. In this model we have previously shown that mortality is much lower than that produced in the 21-gauge model (22). As shown in Fig. 8B, neutralizing CXCL10 before surgery increased CLP-induced lethality in CXCR2−/− mice. This was most apparent on day 4 after CLP surgery.
when the mortality rate of anti-CXCL10-treated mice (56% mortality; five of nine mice) was 5-fold greater than that of control IgG-treated mice (11% mortality; one of nine mice). Thus, these data provide evidence showing that enhanced CXCL10 expression is at least partially responsible for the protection against CLP-induced injury seen in the absence of CXCR2.

Discussion
CXCL8 plays a critical role in recruiting neutrophils to sites of infection, inflammation, or injury (23). While these cells are critical for the host to mount an effective response to bacterial pathogens, excessive neutrophil recruitment and activation have been implicated in several inflammatory conditions, including sepsis (24). In septic patients, elevated plasma and bronchoalveolar lavage levels of CXCL8 are often associated with adult respiratory distress syndrome and multiple organ failure (25, 26), major complications associated with sepsis. The expression of the murine analog of CXCL8, namely MIP-2, has also been directly correlated with the severity of the septic response that follows CLP surgery in mice (27). Immunoneutralization of MIP-2 in this CLP surgery model resulted in decreased mortality due to significantly reduced neutrophil recruitment to the peritoneal cavity (27). Because MIP-2 was shown to play such a crucial role in CLP-related organ injury and mortality, we sought to investigate the role of its receptor, CXCR2, in this murine CLP surgery model. While previous clinical studies have documented that CXCR2 expression is decreased on neutrophils in septic patients (10, 11, 28), the overall significance of the loss of this chemokine receptor has not been explored. The present study demonstrated that the absence of CXCR2 in the context of experimental septic peritonitis conveys a protective effect that is related to its known effects on neutrophil recruitment and due to its previously unknown regulatory effect on the synthesis of CXCL10 by peritoneal macrophages. In fact, CXCL10 was required for the enhanced survival effect observed in
the context of anti-CXCR2 Ab treatment and genetic deletion (i.e., CXCR2−/−) in mice subjected to CLP-induced sepsis.

Although humans and rabbits possess two CXCL8 receptors (i.e., CXCR1 and CXCR2), CXCR2 is the only CXCL8 receptor homologue in mice (29, 30). Naive mice deficient for CXCR2 (CXCR2−/−) appear normal and healthy, but have lymphadenopathy, splenomegaly, and significantly increased numbers of circulating neutrophils (29). However, these mice exhibited a complete inhibition of MIP-2 responsiveness and an 80% reduction in thioglycolate-induced neutrophil recruitment (29). Several subsequent studies with CXCR2−/− mice have revealed a critical role for this receptor in neutrophil-mediated pathology. For example, in a murine model of helminth-mediated keratitis or river blindness, corneal neutrophil recruitment in CXCR2−/− mice was significantly abrogated compared with that in CXCR2+/+ mice (31). Other investigators have used a specific anti-CXCR2 Ab to show a critical role for CXCR2 in neutrophil recruitment during innate immune responses. With this unique anti-chemokine receptor Ab, it was shown that functional CXCR2 is needed for protective immunity against bacterial (Nocardia asteroides (32) and Pseudomonas aeruginosa (33)) and fungal (Candida albicans (34) and Aspergillus fumigatus (16)) pathogens. In these disease models, increased mortality rates were directly associated with reduced neutrophil recruitment and decreased pathogen clearance. In the present study neutrophil recruitment to the peritoneum was inhibited up to 8 h after CLP surgery in mice that received anti-CXCR2 Ab. However, at later times after CLP surgery, neutrophil numbers in the peritoneum were similar in both treatment groups (IgG vs anti-CXCR2 Ab). An explanation for this observation is not forthcoming at present, but it is plausible that CCR1, which appears to have evolved in the mouse to compensate for the lack of the CXCR1 gene in mice (35–37), is responsible for the neutrophil trafficking observed when CXCR2 is blocked or absent. A marked neutrophil recruitment defect has been observed in the CCR1−/− mouse (35), and immunoneutralization of CCL3 has been shown to inhibit i.p. neutrophil recruitment after infection with Mycobacterium bovis (36) or endotoxin challenge (37). We are presently exploring the role of CCR1 in our CLP surgery model.

Survival following a septic insult requires a host immune response that is a complex balance of pro- and anti-inflammatory cytokines and chemokines in the local (peritoneum) and systemic compartments. A local Th1-type cytokine response (i.e., IL-12 and...
IFN-γ is required for host defense against bacterial infection (38–42) and during sepsis (15), while a systemic Th2-type cytokine response (i.e., IL-10 and IL-13) has an important immunoregulatory role that protects the host against renal, liver, and lung injury (43, 44). When CXCR2 was immunoneutralized in our CLP surgery model there was a trend toward decreased levels of IL-12 in both the peritoneum and serum, with little effect on IL-10 levels. These data suggested that CXCR2-expressing cells were not involved in the overall balance of Th1/Th2-type cytokine responses during CLP-induced sepsis.

It has also been shown that several CC and CXC chemokines are elevated following CLP surgery, and these factors exert both pro- and anti-inflammatory effects during this septic response (18, 20, 22, 27, 45). Examples include CCL2 (an indirect mediator involved in recruitment of inflammatory leukocytes to the peritoneum via production of leukotriene B₄) (20), CCL6 (a chemokine that augments phagocytic activity of peritoneal macrophages and promotes containment of the infection by reducing bacterial leak from the septic gut) (45), and CCL22 (a major macrophage activation factor) (18). In the present study anti-CXCR2 treatment did not significantly alter levels of these chemokines in the peritoneum or serum of mice at any time after CLP surgery. Nevertheless, a closer examination of peritoneal macrophages revealed that the prior in vivo instillation of CXCR2-neutralizing Ab resulted in a significant up-regulation of transcript and protein levels of

![Figure 6](http://www.jimmunol.org/)

**Figure 6.** In vivo treatment with anti-CXCR2 IgG significantly increased CXCL10 secretion from peritoneal macrophages. Mice were treated with 0.5 mg of control goat or anti-CXCR2 IgG i.p. Two hours later peritoneal cells were collected, and macrophages were plated overnight. Twenty-four hours later supernatants were harvested and clarified, and cytokine/chemokine levels were measured by specific sandwich ELISA (n = 5/group; except n = 3 for control IgG group for IL-10 and CCL2). Results are presented as the average for the group ± SEM. ***p < 0.01 (compared with control IgG-treated mice).

![Figure 7](http://www.jimmunol.org/)

**Figure 7.** CXCR2⁻/⁻ mice had greater CXCL10 expression before and after CLP than CXCR2⁺/⁺ controls. Wild-type BALB/c (CXCR2⁺/⁺) and CXCR2⁻/⁻ mice were subjected to 21-gauge CLP. Peritoneal lavages (2 ml) were collected from untreated naive mice and CLP mice 8 h after surgery (n = 4/group). The concentration of CXCL10 was measured in lavage fluid for each mouse using a CXCL10-specific sandwich ELISA. Results are presented as the average for the group ± SEM. **p < 0.05 (compared with CXCR2⁺/⁺ CLP mice).
CXCL10 in these cells when they were examined in vitro. CXCR2−/− mice similarly expressed higher basal and CLP-induced CXCL10 levels in the peritoneum compared with control mice. CXCL10 expression has been observed previously in macrophages following their exposure to IFN-γ (46), endotoxin, or Gram-positive and Gram-negative bacteria (47, 48), indicating that CXCL10 is part of the host innate immune response to bacterial infection. CXCL10 also appears to be an important regulator that functions in translating innate immunity into acquired Th1-type immunity (48, 49). When immunoneutralized in CD-1 mice, CXCL10 was found to be necessary for mouse survival after CLP surgery. In fact, the increased protection against CLP-induced lethality seen in CXCR2−/− mice was negated by anti-CXCL10 Ab treatment before CLP surgery. Increased CXCL10 production in the peritoneum may play a role in enhancing the host Th1-type response in the absence of functional CXCR2. In addition, recent data have emerged that link CXCL10 to neutrophil migration (50-52), and these cells have been shown to produce CXCL10 (53). CXCL10 production may be the mediator responsible for CXCR2-independent neutrophil recruitment observed 8 h after CLP surgery.

To summarize, we have shown that when CXCR2 is immunoneutralized or absent due to gene deletion, mice were significantly protected from the tissue injury and mortality that follow CLP surgery. Surprisingly, the blockade or absence of CXCR2 did not ablate leukocyte recruitment in this model. In addition, the blockade or absence of CXCR2 appeared to favor the production of CXCL10 by peritoneal macrophages, an effect that is significant because we show that this CXC chemokine is an important protective mediator in this septic peritonitis model.

References