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Absence of CC chemokine receptor 8 enhances innate immunity during septic peritonitis

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ABSTRACT

An effective clearance of microbes is crucial in host defense during infection. In the present study, we demonstrate that CC chemokine receptor 8 (CCR8) skews innate immune response during septic peritonitis induced by cecal ligation and puncture (CLP). CCR8 was expressed in resident peritoneal macrophages and elicited leukocytes during CLP in the wild-type $CCR8^{+/+}$ mice. $CCR8^{-/-}$ mice were resistant to CLP-induced lethality relative to $CCR8^{+/+}$ mice, and this resistance was associated with an augmented bacterial clearance in $CCR8^{-/-}$ mice. In vitro, peritoneal macrophages from $CCR8^{-/-}$ mice, but not neutrophils, exhibited enhanced bactericidal activities relative to those from $CCR8^{+/+}$ mice. Upon stimulation with the bacterial component LPS, elevated levels of superoxide generation, lysosomal enzyme release, and nitric oxide generation, effector molecules for bacterial killing were detected in $CCR8^{-/-}$ macrophages relative to $CCR8^{+/+}$ macrophages. In addition, $CCR8^{-/-}$ macrophages produced significantly higher levels than $CCR8^{+/+}$ macrophages of several cytokines and chemokines known to augment bactericidal activities of leukocytes that include TNF-α, IL-12, macrophage-derived chemokine (MDC/CCL22), macrophage inflammatory protein (MIP)-2, and KC. Altogether, these results indicate that CCR8 may have a negative impact on host defense during septic peritonitis, providing a new paradigm for the role of CCR8 in innate immunity.

Key words: sepsis • macrophages • cytokines • inflammation

Recruitment of leukocytes into inflammatory foci is the first-line of host defense in infection, enabling the host to achieve effective and efficient removal of microbes (1). Chemokines are crucial in this process, attracting and activating leukocytes that bear specific chemokine receptors. Chemokine receptors constitute a large superfamily and are classified into four subtypes, depending on which chemokine subfamily is recognized (2, 3). Recent studies have demonstrated that chemokine receptors are preferentially expressed on

specific leukocyte subpopulations. For example, Th1 cells express CC chemokine receptor (CCR)5 and CXC chemokine receptor (CXCR)3, whereas Th2 cells are associated with CCR3, CCR4, and CCR8 (4).

In addition to being expressed on in vitro polarized Th2 cells, CCR8 has been detected in T cells from asthma patients, a Th2-dominant disorder (5). $CCR8^{-/-}$ mice demonstrate impaired Th2 response in animal models of granuloma and allergic airway inflammation where Th2 cells play a central role (6), albeit the role of CCR8 in allergic airway disease is controversial (7, 8). Thus, studies to date in the context of CCR8 have focused attention on adaptive immunity. However, CCR8 was originally identified on monocytes (9), and T cell activation-specific gene 3 (TCA3)/CCL1, a ligand for CCR8, is chemotactic for monocytes/macrophages and neutrophils (10, 11). Because infiltrating macrophages and neutrophils are essential for bacterial killing in infection (12), including septic peritonitis (13, 14), it seemed possible that CCR8 may play a role in the innate immunity.

To identify the functional role of CCR8 in bacterial infection, we used $CCR8^{-/-}$ mice and examined the innate immune response during septic peritonitis induced by cecal ligation and puncture (CLP). CLP is a clinically relevant model of intra-abdominal sepsis, which develops slowly and simulates a polymicrobial enteric insult similar to that seen in patients with colonic perforations (15). In the present study, we demonstrate for the first time that mice with CCR8 deficiency are resistant to CLP-induced lethality, resulting from augmented innate immune responses. These results provide a new paradigm for the role of CCR8 in host defense during sepsis.

MATERIALS AND METHODS

Mice

$CCR8^{-/-}$ mice (6) were backcrossed 10 generations onto the C57BL/6 mice (Charles River Laboratories, Wilmington, MA). Age- and sex-matched C57BL/6 mice were used as wild-type $CCR8^{+/+}$ mice. All chemokine receptors examined (CCR1-11, CXCR1-6, XCR1, CX3CR1, DARC, and D6), but CCR8 were similarly expressed in $CCR8^{-/-}$ mice relative to $CCR8^{+/+}$ mice as assessed by quantitative PCR using specific primers for each chemokine receptor (data not shown). Cell populations in the resident peritoneal cells were similar between $CCR8^{+/+}$ and $CCR8^{-/-}$ mice as estimated by flow cytometry (F4/80 positive cells, 36–40%; B 220 positive cells, 20–24%; Thy1.2 positive cells, 11–16%). Mice were used in all experiments under specific pathogen-free conditions.

In vivo experimental protocol

CLP surgery was performed as described elsewhere (13, 15). In brief, mice were anesthetized and the cecum was exposed, ligated with a 3–0 silk suture below the ileocecal valve, and punctured through and through once with a 21-gauge needle. To determine mice survival, CLP-mice were monitored for 7 days after CLP. In the different set of experiments, CLP mice were anesthetized, bled, and killed at appropriate intervals after CLP. The peritoneal cavities were washed with 2 ml of sterile saline, and the lavage fluids were harvested under sterile conditions. Peritoneal lavage fluids and peripheral blood (10 μl) from each mouse were serially diluted with

sterile saline, and 5 μl of each dilution was plated on trypticase soy agar (TSA) plates with 5% sheep blood and incubated overnight at 37°C, after which the number of aerobic bacteria colonies was counted. The remaining lavage fluids were centrifuged at 6000 g for 1 min at 4°C, and cell-free peritoneal fluids were stored at –80°C. Cell pellets were resuspended in saline, and the cell numbers were counted in a hemocytometer. Differential cell analyses were made by Diff-Quik staining of the smear slides. Smear slides were also used for immunocytochemistry. In other experiments, mice were intraperitoneal inoculated with live bacteria (1×10^8 CFU/mouse) recovered from $CCR8^{+/+}$ mice undergone CLP. The peritoneal lavage fluids at 24 h after the inoculation were serially diluted with sterile saline, and 5 μl of each dilution was plated on TSA-blood plates, incubated overnight at 37°C, and the CFU was counted.

Quantitative PCR

Chemokine receptor expression was quantitated by quantitative RT-PCR. Briefly, total RNA was isolated from resident macrophages, treated with DNase I and reverse-transcribed using Random Hexamer as primers and reverse transcriptase. cDNA (25 ng) was amplified in a mixture of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and primers using GeneAmp 7700 (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min; Applied Biosystems). CCR8 primers are sense, 5′-TGACCGACTACTACCCTGATTTCTT-3′ and antisense, 5′-GCTGCCCCTGAGGAGGAA-3′. Target gene expression was shown as relative values compared with the amount of ubiquitin cDNA in samples. Ubiquitin primers are sense, 5′-TGGCTATTAATTATTCGGTCTGCAT-3′ and antisense, 5′-GCAAGTGGCTAGAGTGCAGAGTAA-3′.

Western blotting

Cells were dissolved in Laemmli buffer (1×10^6/50 μl), sonicated, boiled, fractionated on SDS-polyacrylamide gel (10 μl), and transferred to a nitrocellulose membrane. After blocking with TBS-T (Tris-buffered saline+0.1% Tween-20) containing 5% skim milk for 1 h at room temperature, the membrane was incubated with goat anti-murine CCR8 IgG (Alexis Biochemicals, San Diego, CA) overnight at 4°C. After washing with TBS-T, the membrane was incubated with rabbit anti-goat Fab' conjugated to peroxidase-labeled dextran polymer (Nichirei Co., Tokyo, Japan) for 1 h at room temperature and visualized with an enhanced chemiluminescence system (Cell Signaling Inc., Beverly, MA).

Immunocytochemistry

Cytospin preparations were fixed immediately in 100% methanol. After blocking endogenous peroxidase using 0.3% H_2O_2 in methanol, we rehydrated the slides in Tris-buffered saline (TBS) and blocked with 10% normal goat serum for 1 h at room temperature. The slides were incubated with goat anti-murine CCR8 IgG (Alexis Biochemicals) or control goat IgG at 5 μg/ml in TBS-1% BSA overnight at 4°C. After being washed with TBS-tween 20 (0.1%), the slides were incubated with rabbit anti-goat IgG conjugated to horseradish peroxidase (HRP)-labeled dextran polymer (EnVision plus, Peroxidase: DakoCytomation, Carpinteria, CA) for 30 min at room temperature. After washing, the reaction was developed with diaminobenzidine (Sigma, St. Louis, MO). Counter-staining was done with hematoxylin.

In vitro phagocytic and killing activities of leukocytes

Peritoneal cells were harvested from non-treated mice, suspended in antibiotic-free RPMI medium containing 10% FCS, and cultured for 1 h at 37°C in two 24-well culture dishes (1.5×10^6/well). Non-adherent cells were removed, and the adherent macrophages were infected with 1×10^6 CFU of live bacteria, which were recovered from the peritoneum of $CCR8^{+/+}$ mice at 24 h after CLP. After 1 h-incubation, the wells were washed out to remove unphagocytized bacteria, and the cells in one plate were lysed with sterile 0.5% Triton X-100 for bacterial phagocytosis assay. Wells in the other plate were replaced with pre-warmed fresh medium and incubated for an additional 2 h, after which the cells were lysed with 0.5% Triton X-100 for bacterial killing assay (14). The lysates were serially diluted, plated on TSA-blood plates, and incubated overnight at 37°C, and the numbers of aerobic colonies were counted. In other experiments, infiltrating leukocytes were harvested at 6 h after intraperitoneal injection of 1 ml of 4% thioglycollate (Difco Laboratories, Detroit, MI). Neutrophils were isolated from the elicited leukocytes by Ficoll gradient centrifugation (neutrophil purity >94%). To determine in vitro bactericidal activities of neutrophils, a classical CFU assay was used with minor modifications (16). In brief, cells were infected with 1×10^6 CFU of live bacteria recovered from CLP-mice. Control wells contained only bacteria. After being cultured for 4 h in a 5% CO_2 incubator, plates were placed at –80°C for 30 min and cells were lysed by thawing. This did not affect bacteria viability, as determined in control experiments. The lysates were serially diluted, plated on TSA-blood plates, and incubated overnight at 37°C, and the number of aerobic colonies was counted. Bactericidal activity was expressed as % bacteria death = [CFU from control wells (without cells) – CFU from experimental wells]/[CFU from control wells (without cells)] × 100.

Cell culture

Adherent macrophages from resident peritoneal cells (1.5×10^6/well) were cultured in RPMI 1640 supplemented with 10% FCS, glutamine, and antibiotics in a 5% CO_2 incubator with or without the bacterial component LPS (1 μg/ml, *E. coli* 0111, B4: Difco Laboratories, Detroit, MI). After 24 h, the culture supernatants were harvested and centrifuged at 6000 *g* for 1 min at 4°C, and cell-free supernatants were stored at –80°C. The culture supernatants were used for measurements of cytokines, lysosomal enzyme release, and nitric oxide (NO) production. The lysosomal enzyme release was determined by β-glucuronidase activity, according to the methods described previously (17). NO level was determined by measuring nitrite and nitrate, stable end products of nitric oxide metabolism, using Colorimetric enzymatic NO assay kit (Oxford Biomedical Research, Oxford, MI).

The superoxide production from cells was measured using the reduction of ferricytochrome c (14, 18). In brief, adherent peritoneal macrophages or peripheral neutrophils purified by Ficoll gradient centrifugation (1×10^6/well) were cultured in phenol red-free RPMI 1640 containing cytochrome *c* (1.3 mg/ml, Sigma), and stimulated with LPS (1 μg/ml) for 30 min, and the supernatants were measured spectrophotometrically at 550 nm as a function of ferricytochrome *c* reduction.

Cytokine ELISAs

Murine cytokines were measured using a standard method of sandwich ELISA, as described (19, 20). The captured antibodies, detection antibodies, and recombinant cytokines were purchased from R&D Systems (Minneapolis, MN). The ELISAs used in this study did not cross-react with other murine cytokines available, and they consistently detected murine cytokine concentrations above 30 pg/ml.

Statistics

Statistical significance was evaluated by ANOVA. In case of survival curve and CFU count, the data were analyzed by the log-rank test and Mann-Whitney test, respectively. A $P < 0.05$ value was regarded as statistically significant. All data were expressed as mean ± SEM.

RESULTS

Expression of CCR8 in peritoneal leukocytes during CLP

In our initial experiments, we examined whether CCR8 was expressed in peritoneal leukocytes during CLP. Freshly isolated peripheral blood mononuclear cells, purified by Ficoll gradient centrifugation, showed negligible level of CCR8 expression as estimated by RT-PCR and Western blot (data not shown). The CCR8 mRNA expression was detected in resident peritoneal macrophages from $CCR8^{+/+}$ mice (Fig. 1*A*). Immunocytochemistry revealed that CCR8 protein was present on the resident macrophages (Fig. 1*B*). CCR8 was also detected in elicited leukocytes obtained from $CCR8^{+/+}$ mice that underwent CLP as examined by Western blot (Fig. 1*C*). At this time point, infiltrating macrophages and neutrophils were stained with anti-CCR8 IgG, and the overall staining intensity was stronger than resident macrophages (Fig. 1*B*). Thus, CCR8 was expressed in both resident macrophages and elicited leukocytes (macrophages and neutrophils) during CLP, but not in peripheral blood mononuclear cells, in $CCR8^{+/+}$ mice. In contrast, resident peritoneal macrophages as well as elicited leukocytes during CLP from $CCR8^{-/-}$ mice did not express CCR8 (Fig. 1*A*, *C*). No positive staining was observed in $CCR8^{-/-}$ macrophages (not shown).

$CCR8^{-/-}$ mice were resistant to CLP-induced lethality

To determine the functional role of CCR8 in host defense during CLP, survival rates in $CCR8^{+/+}$ and $CCR8^{-/-}$ mice were monitored after CLP. The data in Fig. 2 demonstrated that all $CCR8^{-/-}$ mice (23/23) survived for 2 days after CLP, whereas 12 out of 21 $CCR8^{+/+}$ mice were dead on day 2. Of the 23 $CCR8^{-/-}$ mice, 21 survived for 7 days; whereas only 5 $CCR8^{+/+}$ mice (5/21) survived for 7 days ($P<0.0001$, Fig. 2). These data clearly demonstrate that $CCR8^{-/-}$ mice are resistant to the lethality induced by CLP.

Increased bacterial clearance in $CCR8^{-/-}$ mice

Bacterial burden is tightly associated with the mortality in the CLP model where infiltrating leukocytes play a central role (13, 14). To identify the basis whereby $CCR8^{-/-}$ mice were resistant to CLP, bacterial burden and leukocyte infiltration in CLP-mice were investigated.

Although bacterial burden at 6 h post CLP was unchanged between $CCR8^{+/+}$ and $CCR8^{-/-}$ mice, $CCR8^{-/-}$ mice displayed an increased bacterial clearance at 24 h post CLP, as indicated by smaller numbers of recovered CFU counts in the peritoneum (Fig. 3*A*). The mean peritoneal CFU count in $CCR8^{-/-}$ mice was a 10^3-fold lower than that in $CCR8^{+/+}$ mice. At this time point, bacteremia was observed in 4/16 of $CCR8^{+/+}$ mice, but not in $CCR8^{-/-}$ mice (0/13) (Fig. 3*B*). Enhanced bacterial clearance in $CCR8^{-/-}$ mice was also observed when mice were intraperitoneally received live bacteria (1×10^8 CFU/mouse) recovered from $CCR8^{+/+}$ mice undergone CLP (Fig. 3*C*). On the other hand, no statistical differences were found in the number of infiltrating neutrophils and macrophages at 6 and 24 h after CLP between $CCR8^{+/+}$ and $CCR8^{-/-}$ mice (not shown). Thus, $CCR8^{-/-}$ mice cleared bacteria much more effectively than $CCR8^{+/+}$ mice without augmenting the leukocyte infiltration, suggesting that bactericidal activities of leukocytes may be augmented in $CCR8^{-/-}$ mice.

Augmented innate immune response in $CCR8^{-/-}$ macrophages

We, therefore, examined the phagocytic and killing activities of peritoneal macrophages and neutrophis from $CCR8^{+/+}$ and $CCR8^{-/-}$ mice. As shown in Table 1, the CFU counts inside macrophages after 1 h culture were similar between $CCR8^{+/+}$ and $CCR8^{-/-}$ mice, suggesting an equal phagocytic activity of the cells. After an additional 2 h culture, the CFU counts inside $CCR8^{-/-}$ macrophages were significantly lower than $CCR8^{+/+}$ macrophages (Table 1). In contrast, in vitro bactericidal activities of neutrophils were not augmented in $CCR8^{-/-}$ neutrophils. Effector molecules for bacterial killing were subsequently examined in the culture supernatants of LPS-stimulated peritoneal macrophages, which included superoxide anion (O_2^-) generation, lysosomal enzyme (β-glucronidase) release, and NO production (21). As shown in Fig. 4*A*, $CCR8^{-/-}$ macrophages produced higher levels of these molecules than $CCR8^{+/+}$ macrophages. Superoxide generation in $CCR8^{-/-}$ neutrophils was not augmented upon stimulation with LPS (data not shown). Thus, $CCR8^{-/-}$ macrophages, but not neutrophils, exhibited enhanced bactericidal activities when compared with $CCR8^{+/+}$ cells.

Next we investigated the ability of peritoneal macrophages to secrete cytokines known to augment the bactericidal activities of leukocytes. For this, $CCR8^{+/+}$ and $CCR8^{-/-}$ macrophages were cultured in the presence or absence of LPS, and cytokine levels in the supernatants were measured. The data in Fig. 4*B* demonstrated that $CCR8^{-/-}$ macrophages produced significantly higher levels of TNFα, IL-12, and macrophage-derived chemokine (MDC)/CCL22 than $CCR8^{+/+}$ macrophages. Elevated levels of the neutrophil activating chemokines macrophage inflammatory protein (MIP)-2 and KC were also detected in the supernatants of $CCR8^{-/-}$ macrophages relative to $CCR8^{+/+}$ macrophages (Fig. 4*B*). Higher production of cytokines and chemokines by $CCR8^{-/-}$ macrophages could represent an important mechanism favoring bacterial removal from the $CCR8^{-/-}$ hosts.

Altered cytokine response and prevention of renal injury in $CCR8^{-/-}$ mice

Effective bacteria killing in $CCR8^{-/-}$ mice may lead to decreased systemic response followed by CLP. Subsequently, plasma levels of several cytokines and organ damage after CLP were investigated. The data in Fig. 6 demonstrated that $CCR8^{-/-}$ mice displayed 60 and 41% decreases

in plasma levels of TNFα and IL-12, respectively, although they were not statistically significant. However, anti-inflammatory cytokine IL-10 and IL-13 were significantly augmented in $CCR8^{-/-}$ mice, compared with $CCR8^{+/+}$ mice (Fig. 5). Kidney is a major target organ during sepsis, and the dysfunction can be fatal to the host (23). CLP caused an elevation in the level of blood urea nitrogen (BUN) and creatinine in $CCR8^{+/+}$ mice, representing renal injury in this model (Fig. 6). In $CCR8^{-/-}$ mice, levels of BUN and creatinine after CLP were comparable with those from untreated mice (Fig. 6). Thus, $CCR8^{-/-}$ mice evaded renal injury induced by CLP, an event that was associated with an altered cytokine balance in favor of anti-inflammatory effects.

DISCUSSION

Effective host defense against bacterial infection is dependent on the recruitment and activation of phagocytic cells (21), which is governed by chemokine and chemokine receptors (2, 3). In the present study, we provide evidence suggesting that the chemokine receptor CCR8 may have a novel and unexpected role that is important in innate immunity. Most of the work on this receptor thus far has focused on its potential role in adaptive immunity. The findings presented here now implicate CCR8 in innate immunity and constitute the first example of negative regulation of innate immunity by a chemokine receptor.

The increased survival of the $CCR8^{-/-}$ mice in the CLP model of sepsis was associated with an enhanced bactericidal activity of the $CCR8^{-/-}$ macrophages. In this context, altered neutrophil function may account for the different degree of bacterial clearance during CLP, as approximately two-thirds of infiltrating leukocytes were neutrophils. Despite the presence of CCR8 on infiltrating neutrophils, in vitro superoxide generation as well as bacteria killing of neutrophils were not augmented in $CCR8^{-/-}$ mice relative to $CCR8^{+/+}$ mice. Thus, neutrophils did not appear to directly contribute to the altered bacterial clearance in $CCR8^{-/-}$ mice. Although macrophages may influence bactericidal activity of neutrophils, allowing $CCR8^{-/-}$ mice to achieve effective innate immunity, our present data suggest that ablation of CCR8 function on macrophages appears to have an unexpected positive impact on innate immune response during septic peritonitis.

The results in the present study raise several questions, such as: Are the local levels of the murine CCR8 ligand TCA3 altered during peritonitis, and if so, does TCA3 favor development of septic peritonitis? Our results suggest that direct ligation of CCR8 by TCA3 does not seem to favor the development of septic peritonitis in wild-type $CCR8^{+/+}$ mice. Appreciable levels of TCA3 were not detected in the peritoneum during CLP (0.05±0.02 and 0.20±0.07 ng/ml, 6 and 24 h post-CLP, respectively, 6 mice each). TCA3 (10–100 ng/ml) did not reduce cytokine production from LPS-stimulated macrophages or in vitro bactericidal activities of peritoneal macrophages. Furthermore, intraperitoneal injection of TCA3 (1 μg/mouse) at the time of CLP-surgery did not induce increased bacterial load in the peritoneum or bacteremia during CLP (our own unpublished observations). Other CCR8 ligand(s) may provide bacteria with environmental niche. In this context, thymus and activation-regulated cytokine (TARC/CCL17) and macrophage inflammatory protein-1 β (MIP-1β/CCL4) can be the possible ligand (24), but others demonstrate that these chemokines do not bind CCR8 at physiologically relevant concentrations (25). CCR8 may be constitutively active, as this has been reported for some viral chemokine receptors (26, 27) and could be relevant in this particular model. Endogenous

antagonist(s) may cause the negative regulation, however, no such molecules have been identified to date in humans or mice. MC148, a virally encoded CCR8 antagonist, has been identified in the genome of the human poxvirus molluscum contagiosum (28). Viral chemokines such as vMIP-II and vMCC-1 act as potent antagonists without inducing signaling (29), which suggests the existence of molecules with similar properties in humans and mice. Identification of CCR8 ligands or molecules with antagonistic activity against CCR8 remains to be established.

Finally, and perhaps more importantly, why are the $CCR8^{-/-}$ macrophages more efficient at mounting bactericidal responses? Could CCR8 interfere with innate immune responses? In the present study, we showed that no appreciable level of CCR8 was found in peripheral blood mononuclear cells, but the expression was evident in peritoneal resident macrophages. CCR8 expression was augmented in the elicited leukocytes during CLP. Consistent with our observations, Trebst et al. have recently demonstrated that CCR8 expression on monocytes is up-regulated in response to monocyte/macrophage-activating stimuli, despite no appreciable CCR8 expression in peripheral blood monocytes (30). Thus, CCR8 expression appears to be associated with macrophage differentiation and/or activation status. An attractive hypothesis is that CCR8 may interfere with innate immune responses by regulating differentiation of macrophages. Studies done by Qu et al. suggest that this may be occurring in the $CCR8^{-/-}$ mice. Their results indicate that different sets of monocytes are recruited into the peritoneum of $CCR8^{+/+}$ and $CCR8^{-/-}$ mice after intraperitoneal challenge of thioglycollate. Nearly half of the monocytes in $CCR8^{+/+}$ mice differentiated into DC after 2 days culture in the presence of GM-CSF; whereas only a small number of monocytes from $CCR8^{-/-}$ did so (31). These are intriguing observations because it has been shown that monocytes/macrophages that differentiate into DC-like cells mount poor innate immune responses (22, 32). Thus, signaling through CCR8 may actually control the differentiation of macrophages and favor the accumulation in the peritoneum of $CCR8^{-/-}$ macrophages with higher bactericidal activities. Up-regulated LPS-induced cytokine response in $CCR8^{-/-}$ macrophages could be associated with the developmental fate of macrophages.

Failure in restricting the invading pathogens to a localized area of tissue results in an overwhelming systemic response via an enhanced cytokine/chemokine production, leading to systemic inflammatory response syndrome (SIRS) (33). This is likely the mechanism whereby $CCR8^{-/-}$ mice are resistant to septic peritonitis induced by CLP. In this model, the $CCR8^{-/-}$ mice evaded renal injury during CLP as evidenced by clinical chemistry. Of interest was the observation that plasma cytokine response during CLP in $CCR8^{-/-}$ mice was balanced in favor of anti-inflammatory effects. Although effective bacteria clearance in $CCR8^{-/-}$ mice appears to be primarily responsible for the reduced production of TNFα and IL-12, augmented production of anti-inflammatory cytokines is expected to play a role in decreasing the level of TNFα and IL-12, possibly contributing to an alleviation of renal injury. Studies are required to elucidate how CCR8 regulates cytokine balance during CLP.

We have thus far elucidated important roles of chemokine–chemokine receptor system in an innate immunity during CLP. For instance, CC chemokines CCL2/MCP-1, CCL3/MIP-1α, CCL6/C10, and CCL22/MDC are beneficial to the septic peritonitis by augmenting an innate immune response (13, 14, 20, 34, 35). Thus, inflammatory responses governed by chemokines are primarily beneficial to the host defense during CLP. However, an excessive inflammation

appears to be pathologic, as neutralization of macrophage inflammatory protein (MIP)-2 and its receptor CXCR2, which plays a crucial role in neutrophil infiltration/activation, is deleterious to the host (36, 37). The host response in the innate immunity is controlled by IL-10, IL-12, and IL-13 (20, 38, 39), in which Stat proteins are important in balancing the cytokine responses (19, 40). The results in the present study provide a new paradigm for the role of chemokine-chemokine receptor system in an innate immunity. As shown here, ablation of CCR8 function enhanced innate immunity in a model of sepsis, resulting in an alleviation of systemic impact during septic peritonitis. Sepsis and septic shock are conditions associated with high morbidity and mortality, and few effective therapies exist to treat these diseases. The results reported here suggest that therapeutic intervention targeting CCR8 may provide a novel strategy for the treatment of sepsis and septic shock.

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Table 1

Phagocytic and killing activities of macrophages in vitro

Incubation (h)[a]	$CCR8^{+/+}$ Macrophages	$CCR8^{-/-}$ Macrophages
1	23.0 ± 3.2	24.3 ± 2.3
3	13.8 ± 2.9	5.9 ± 0.9‡
Killing rate (%)[b]	40	76

[a] Resident peritoneal macrophages were infected with bacteria recovered from CLP mice. After 1 h incubation, the wells were extensively washed and the cells were lysed with 0.5% Triton X-100 for phagocytic activity. Wells were replaced with fresh medium and cultured for an additional 2 h, after which the cells were lysed with 0.5% Triton X-100 for killing activity. The lysates were serially diluted and placed on TSA blood agar plates, and CFU within the cells were determined. Data were expressed as CFU ($\times 10^3$)/ml (mean±SE). [b]Killing rate = [1–(CFU at 3 h/CFU at 1 h)] × 100. The data are representative of the two individual experiments (n=6). ‡$P < 0.05$, when compared with $CCR8^{+/+}$ macrophages (two-tailed Mann-Whitney test).

Fig. 1

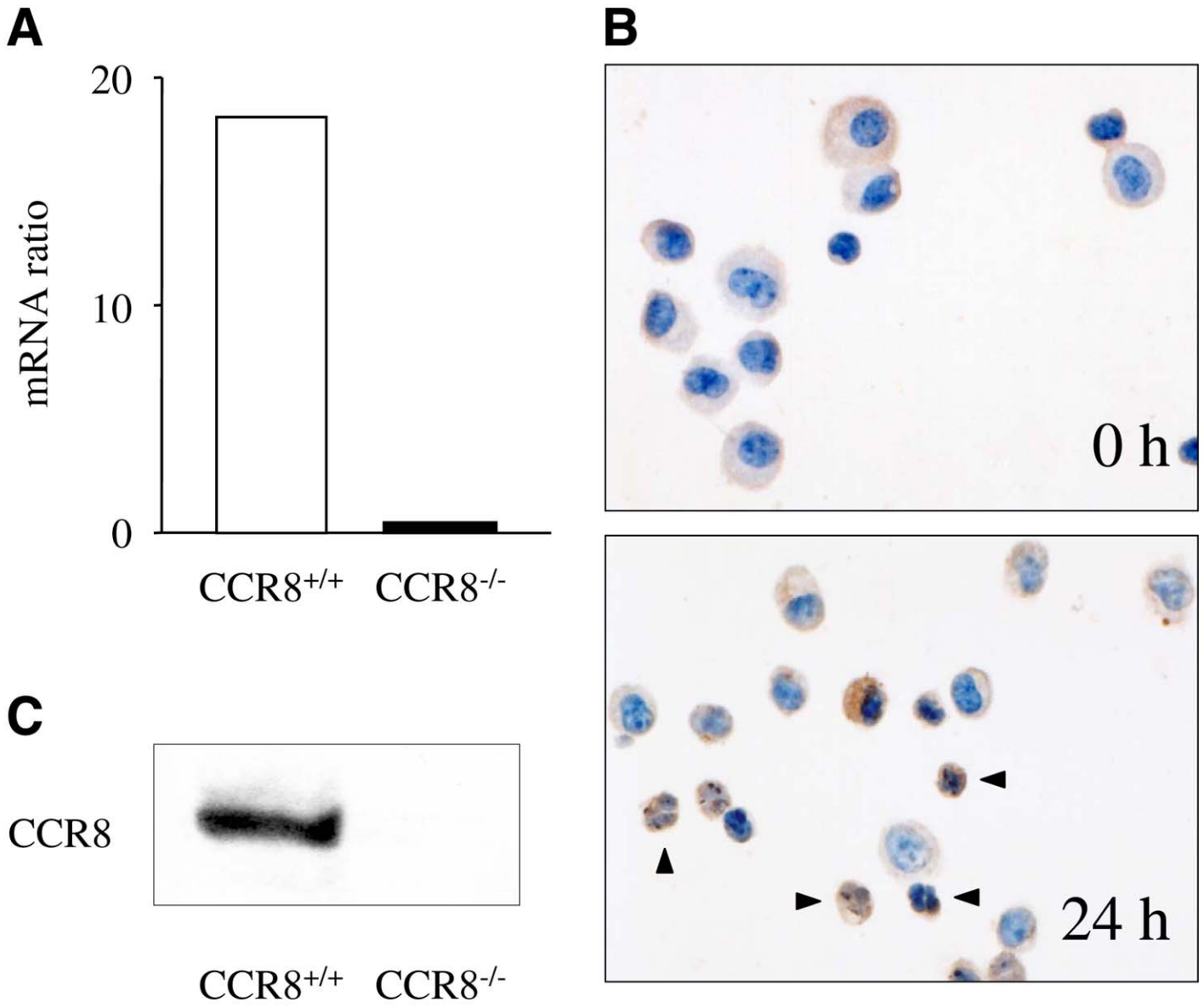

Figure 1. CCR8 is expressed in resident and elicited leukocytes during CLP. ***A***) mRNA was isolated from resident peritoneal macrophages from $CCR8^{+/+}$ and $CCR8^{-/-}$ mice and subjected to quantitative RT-PCR. Data were mean from five mice. ***B***) Resident peritoneal macrophages (0 h) and exudate cells at 24 h post-CLP were harvested from $CCR8^{+/+}$ mice, and the cells were stained with anti-CCR8 IgG. A representative photograph. Arrowheads indicate infiltrating neutrophils. Original magnification ×400. ***C***) Peritoneal exudate cells from $CCR8^{+/+}$ and $CCR8^{-/-}$ mice were harvested at 24 h after CLP, and the lysates were immunoblotted with anti-CCR8 IgG. Representative data.

Fig. 2

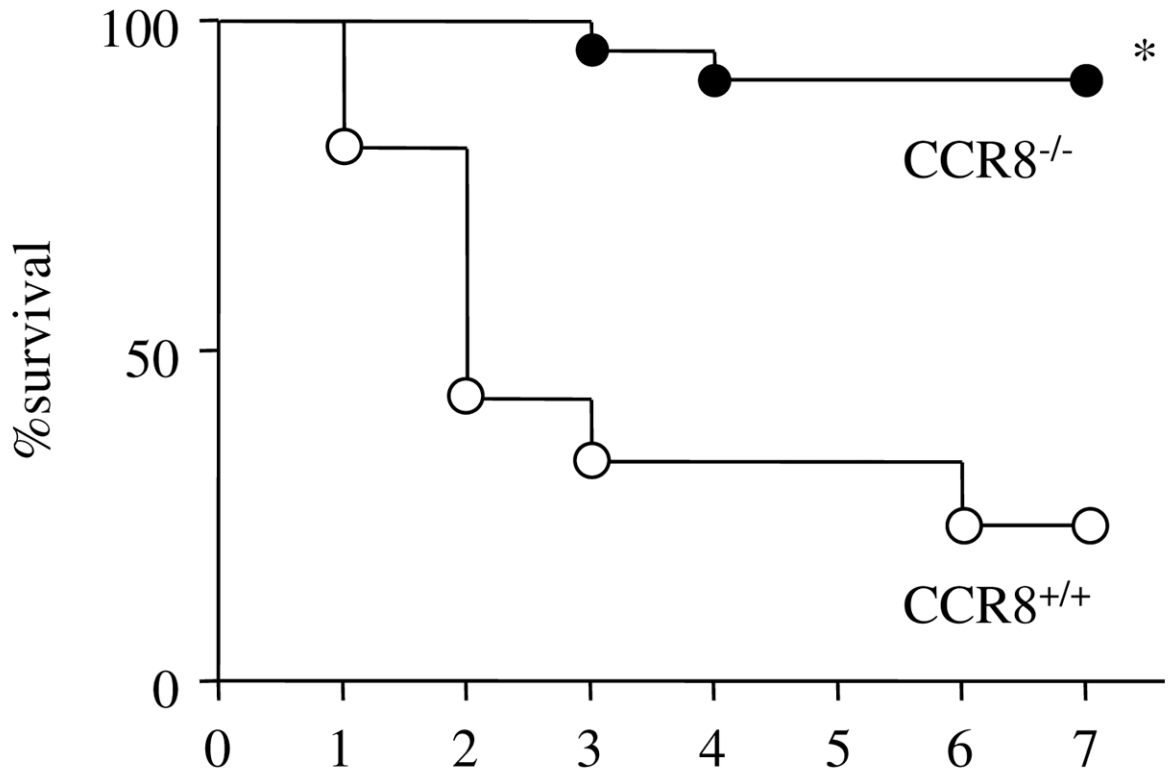

Figure 2. Mice lacking CCR8 are resistant to CLP-induced lethality. The survival rates in $CCR8^{+/+}$ and $CCR8^{-/-}$ mice were monitored for 7 days after CLP (21 and 23 mice, respectively). Three different experiments were performed, and the data were pooled. The results were very similar in individual experiments. $*P < 0.0001$ vs. $CCR8^{+/+}$ mice.

Fig. 3

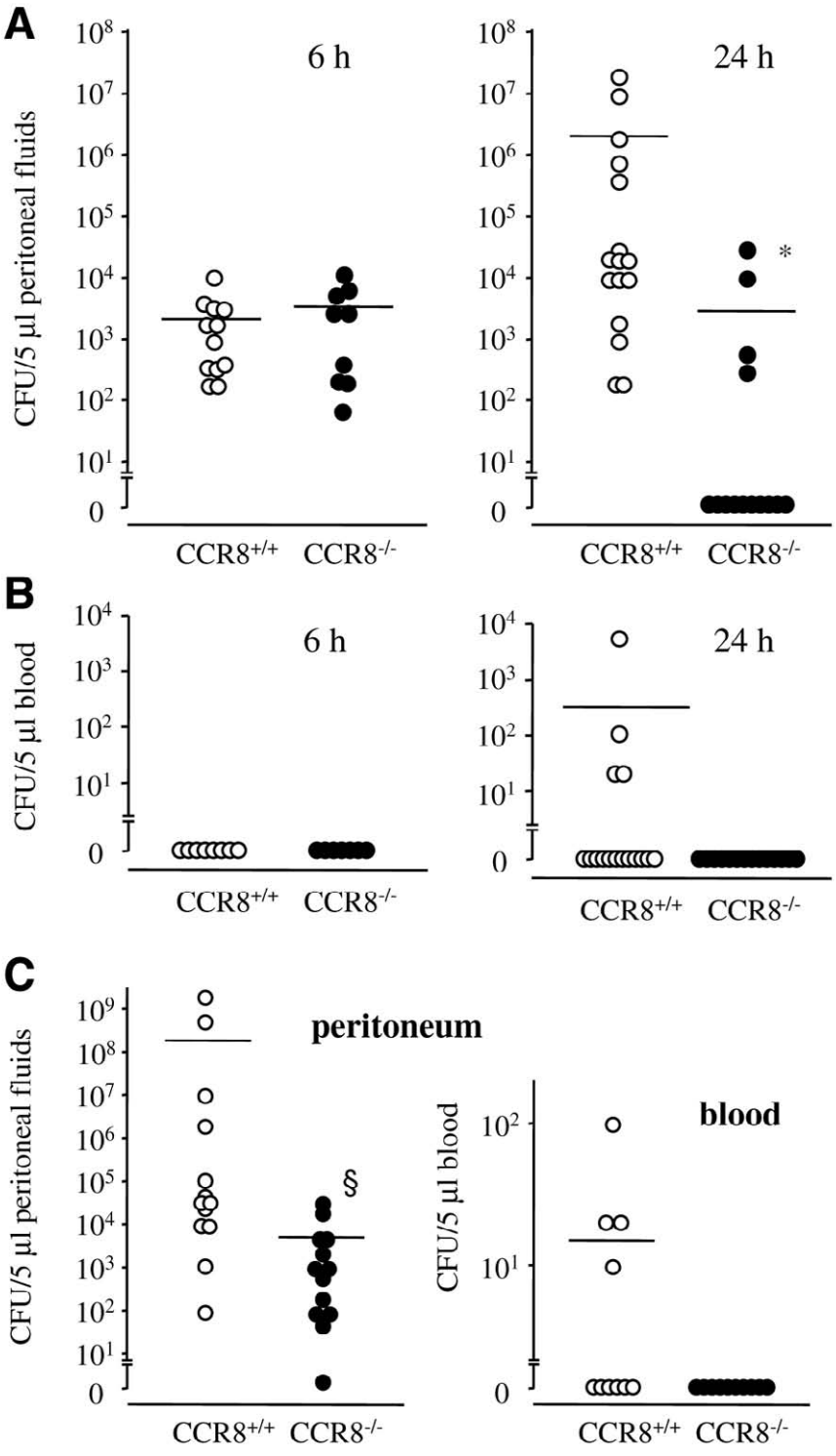

Figure 3. Enhanced bacterial clearance in CCR8$^{-/-}$ mice. ***A***, ***B***) At 6 and 24 h after CLP, mice were anesthetized, bled, and euthanized, and the peritoneal fluids were harvested. Peritoneal fluids (10 µl) (***A***) and peripheral blood (***B***) were serially diluted, and 5 µl of each dilution was plated on TSA blood agar plates. Twenty-eight CCR8$^{+/+}$ mice (6 h: 12 mice; 24 h: 16 mice) and 23 CCR8$^{-/-}$ mice (6 h: 9 mice; 24 h: 14 mice) were used. ***C***) Mice were intraperitoneally received live bacteria (1×10^8 CFU/mouse) recovered from CCR8$^{+/+}$ mice undergone CLP. At 24 h after the inoculation, mice were anesthetized, bled, and euthanized, and the peritoneal fluids were harvested. Peritoneal fluids (10 µl) (13 mice each) and peripheral blood (10 mice each) were serially diluted, and 5 µl of each dilution was plated on TSA blood agar plates. Line represents mean CFU count. $^{\S}P < 0.01$. $^{*}P < 0.0001$ vs. CCR8$^{+/+}$ mice.

Fig. 4

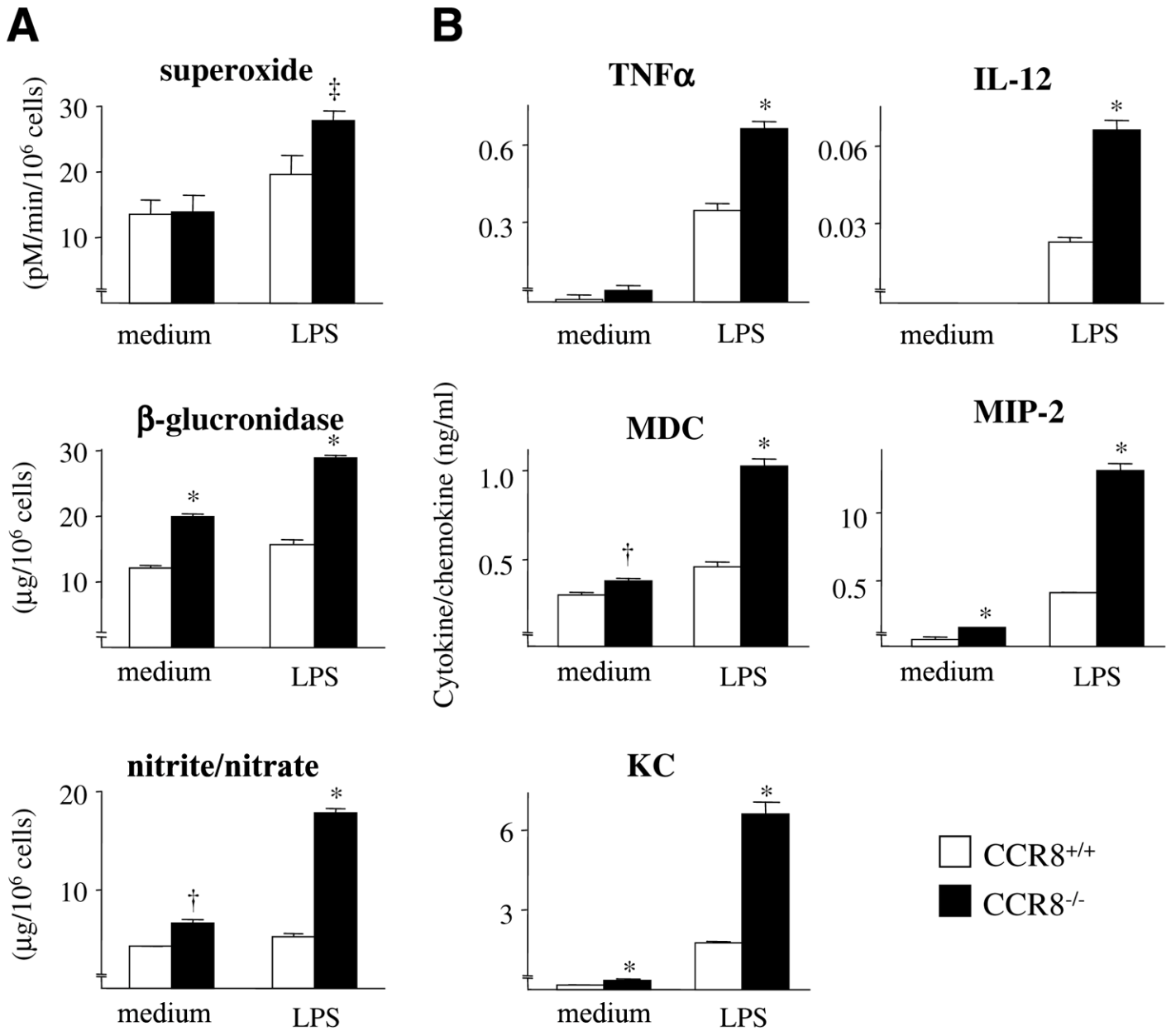

Figure 4. Augmented macrophage function in CCR8$^{-/-}$ mice. Peritoneal cells were harvested from CCR8$^{+/+}$ and CCR8$^{-/-}$ mice; the cells (1.5×10^6cells) were incubated for 1 h at 37°C and non-adherent cells were removed. ***A***) For superoxide generation, the adherent macrophages were stimulated with LPS (1 μg/ml) for 30 min, and the cultures were assayed. For β-glucuronidase release and nitric oxide production, the adherent macrophages were cultured with LPS (1 μg/ml) for 24 h. ***B***) The adherent macrophages were stimulated with LPS (1 μg/ml) for 24 h at 37°C, and cytokines and chemokines in the supernatants were measured. $^{‡}P < 0.05$. $^{†}P < 0.001$. $^{*}P < 0.0001$ vs. CCR8$^{+/+}$ macrophages.

Fig. 5

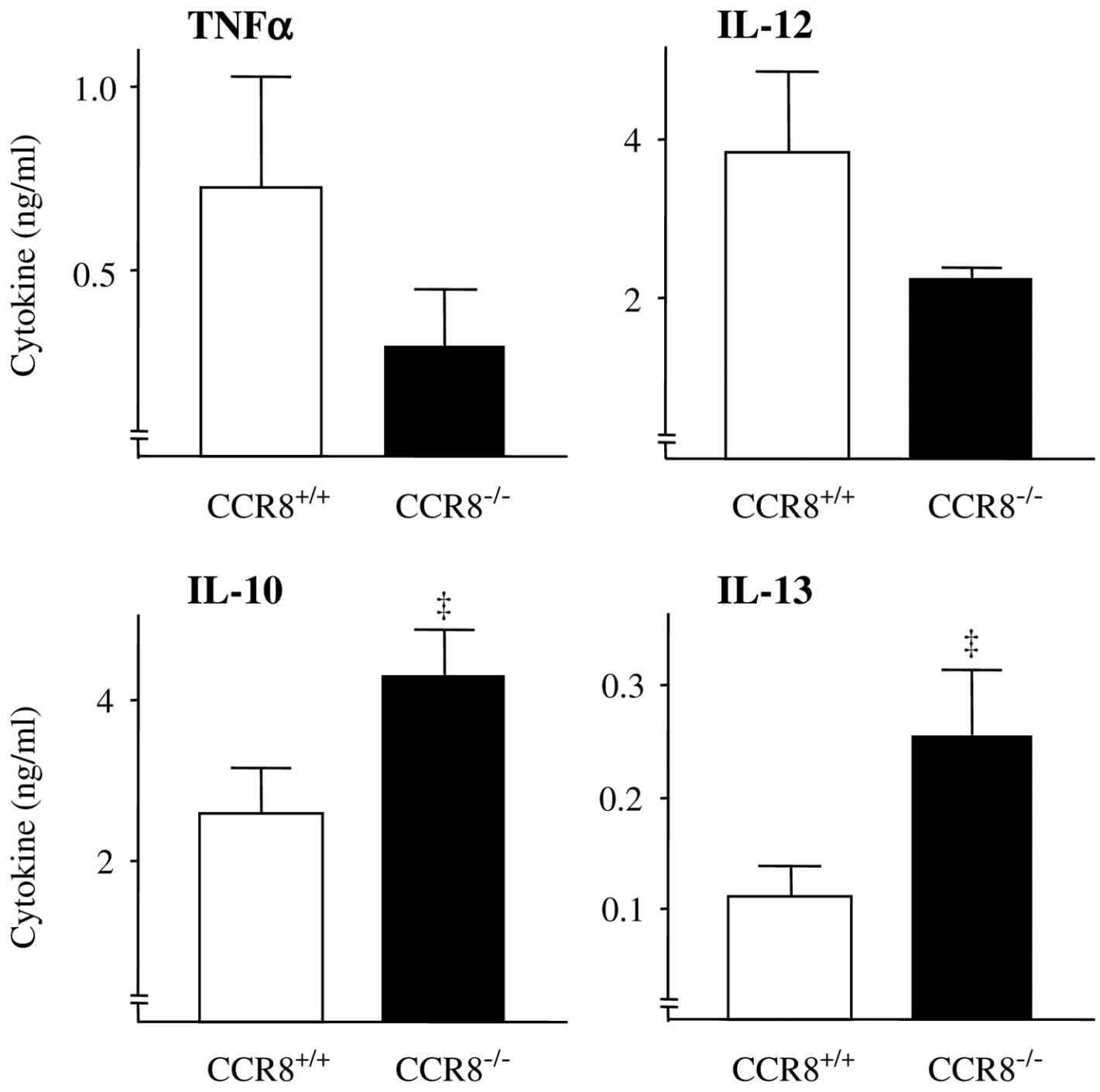

Figure 5. Altered cytokine response during CLP. At 24 h after CLP, $CCR8^{+/+}$ and $CCR8^{-/-}$ mice (8 mice each) were euthanized and bled, and the plasma were harvested. Plasma levels of TNF-α, IL-10, IL-12, and IL-13 were measured. $^{‡}P < 0.05$ vs. $CCR8^{+/+}$ mice.

Fig. 6

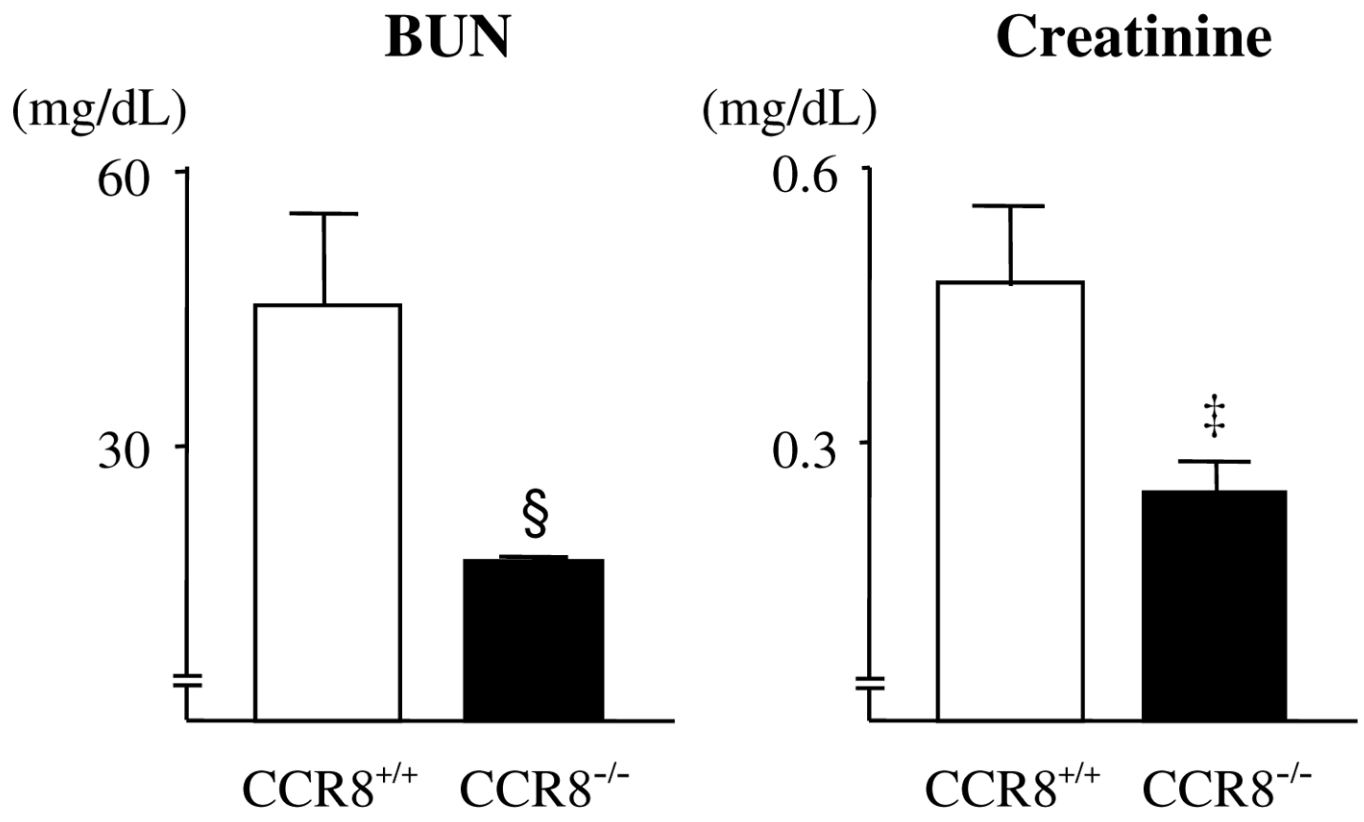

Figure 6. CCR8$^{-/-}$ mice ameliorate renal injury induced by CLP. At 24 h after CLP, CCR8$^{+/+}$ and CCR8$^{-/-}$ mice (8 mice each) were euthanized and bled, and the plasma were harvested. The amounts of BUN and creatinine in the plasma were measured. $^{‡}P < 0.05$, $^{§}P < 0.01$ vs. CCR8$^{+/+}$ mice. Dotted line represents the mean data obtained from untreated mice (5 mice each).