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

Akihiro Matsukawa,*[,1] Shinji Kudoh,* Gen-ichiro Sano,[‡] Takako Maeda,* Takaaki Ito,* Nicholas W. Lukacs,[†] Cory M. Hogaboam,[†] Steven L. Kunkel,[†] and Sergio A. Lira[‡]

*Department of Pathology and Experimental Medicine, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan; [†]Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan, USA; and [‡]Immunobiology Center, Mount Sinai School of Medicine, New York, New York, USA

To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.04-1728fje; doi: 10.1096/fj.04-1728fje

SPECIFIC AIMS

Studies to date with regard to CC chemokine receptor 8 (CCR8) have focused on its potential role in adaptive immunity. In the present study, we explored the role of CCR8 in innate immune response during septic peritonitis induced by cecal ligation and puncture (CLP).

PRINCIPAL FINDINGS

1. Expression of CCR8 in peritoneal macrophages during CLP

Freshly isolated peripheral blood mononuclear cells showed negligible levels of CCR8 expression as estimated by RT-PCR and Western blot. However, RT-PCR and immunocytochemistry detected mRNA and protein expression of CCR8 was in resident peritoneal macrophages from $CCR8^{+/+}$ mice. CCR8 was also detected in elicited leukocytes obtained from $CCR8^{+/+}$ mice that underwent CLP. Both infiltrating macrophages and neutrophils were stained with anti-CCR8 IgG, and the staining intensity was stronger than resident macrophages. In contrast, resident peritoneal macrophages and elicited leukocytes during CLP from $CCR8^{-/-}$ mice did not express CCR8.

2. $CCR8^{-/-}$ 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. As a result, all $CCR8^{-/-}$ mice (23/23) survived for 2 days after CLP whereas 12 of 21 $CCR8^{+/+}$ mice were dead on day 2. Twenty-one of 23 $CCR8^{-/-}$ mice survived for 7 days whereas only 5 $CCR8^{+/+}$ mice (5 of 21) survived for 7 days. These data clearly demonstrated that $CCR8^{-/-}$ mice were resistant to the lethality induced by CLP.

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

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. The mean peritoneal CFU count in $CCR8^{-/-}$ mice was 10^3-fold lower than that in $CCR8^{+/+}$ mice (**Fig. 1*A***). At this time point, bacteremia was observed in 4 of 16 $CCR8^{+/+}$ mice but not in $CCR8^{-/-}$ mice (0 of 13) (Fig. 1*B*). Enhanced bacterial clearance in $CCR8^{-/-}$ mice was also observed when mice intraperitoneally received live bacteria (1×10^8 CFU/mouse) recovered from $CCR8^{+/+}$ mice that had undergone CLP (Fig. 1*C*). There were no statistical differences 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.

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

We examined the phagocytic and killing activities of peritoneal macrophages and neutrophils from $CCR8^{+/+}$ and $CCR8^{-/-}$ mice. In vitro bactericidal activities of macrophages, but not neutrophils, were augmented in $CCR8^{-/-}$ macrophages relative to $CCR8^{+/+}$ cells. Effector molecules for bacterial killing

[1] Correspondence: Department of Pathology and Experimental Medicine, Graduate School of Medical Sciences, Kumamoto University, 1-1-1, Honjo, Kumamoto 860-8556, Japan. E-mail: matsu@kaiju.medic.kumamoto-u.ac.jp

0892-6638/06/0020-0302 © FASEB

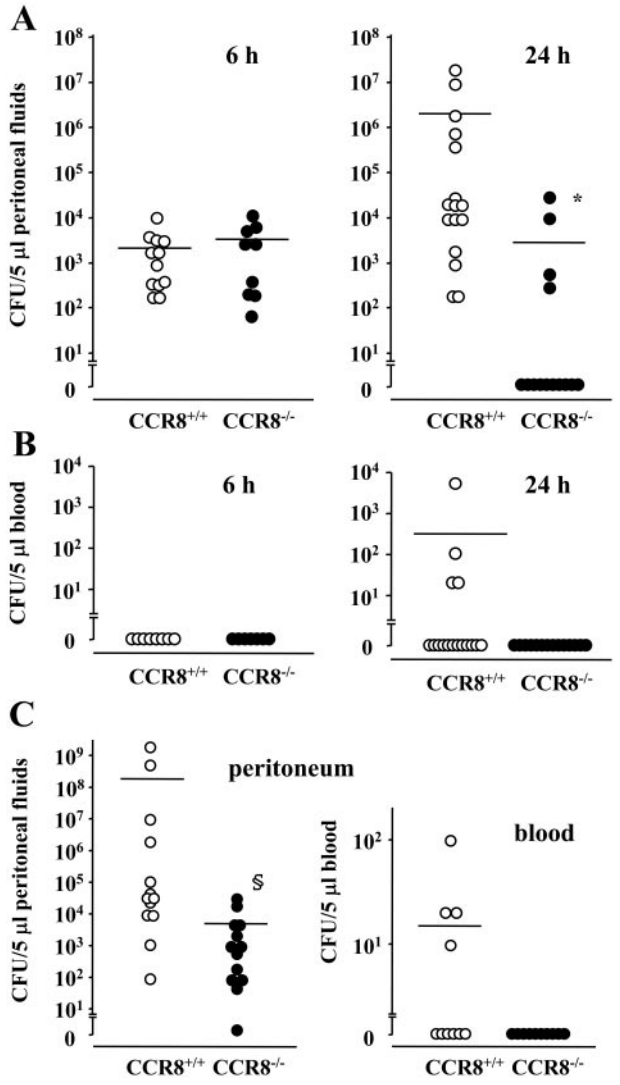

Figure 1. Enhanced bacterial clearance in $CCR8^{-/-}$ mice. *A, B)* At 6 and 24 h after CLP, the peritoneal lavage fluids (*A*) and peripheral blood (*B*) were harvested from each mouse, serially diluted with sterile saline, and 5 μL of each dilution was plated on TSA blood agar plates and incubated overnight at 37°C, after which the number of aerobic bacteria colonies was counted. 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 intraperitoneally received live bacteria (1×10^8 CFU/mouse) recovered from $CCR8^{+/+}$ mice undergone CLP. At 24 h after the inoculation, the peritoneal fluids were harvested. Ten μL of peritoneal fluids (13 mice each) and peripheral blood (10 mice each) was 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.

were subsequently examined, which demonstrated that LPS stimulated $CCR8^{-/-}$ macrophages to produce higher levels of superoxide anion generation, lysosomal enzyme (β-glucronidase) release, and nitric oxide production than $CCR8^{+/+}$ macrophages (**Fig. 2*A***). Superoxide generation in $CCR8^{-/-}$ neutrophils was not augmented upon stimulation with LPS. $CCR8^{-/-}$ 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. 2*B*). Thus, $CCR8^{-/-}$ macrophages, but not neutrophils, exhibited enhanced bactericidal activities compared with $CCR8^{+/+}$ cells. Higher production of cytokines and chemokines by $CCR8^{-/-}$ macrophages could represent an important mechanism favoring bacterial removal from the $CCR8^{-/-}$ hosts.

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

Bacteria killing in $CCR8^{-/-}$ mice may lead to decreased systemic response, followed by CLP. $CCR8^{-/-}$ mice displayed 60% and 41% decreases in plasma level of TNFα and IL-12, respectively, although they were not statistically significant. On the other hand, anti-inflammatory cytokine IL-10 and IL-13 were significantly augmented in $CCR8^{-/-}$ mice compared with $CCR8^{+/+}$ mice. Kidney is a major target organ during sepsis and the dysfunction was noted in $CCR8^{+/+}$ mice that underwent CLP, as determined by plasma level of blood urea nitrogen (BUN) and creatinine. In $CCR8^{-/-}$ mice, BUN, and creatinine level after CLP were comparable to those from untreated mice, demonstrating

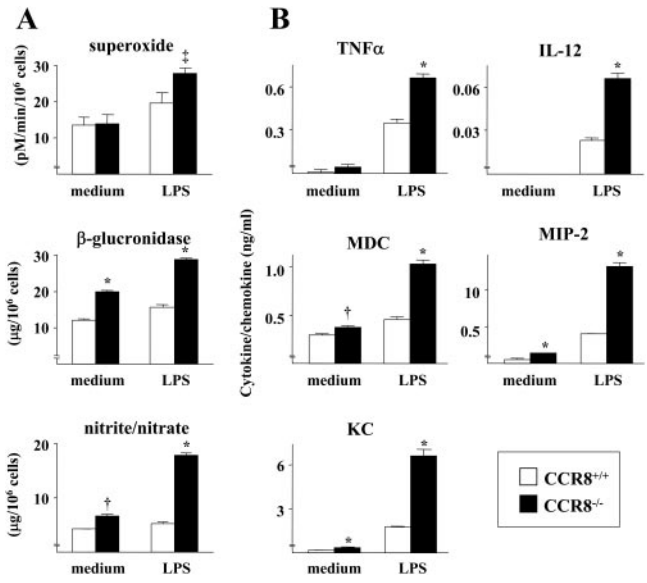

Figure 2. Augmented macrophage function in $CCR8^{-/-}$ mice. Peritoneal cells were harvested from $CCR8^{+/+}$ and $CCR8^{-/-}$ mice and the cells (1.5×10^6cells) were incubated for 1 h at 37°C, and nonadherent cells were removed. *A)* For superoxide generation, the adherent macrophages were stimulated 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. $^{\ddagger}P < 0.05$, $^{\dagger}P < 0.001$, $^{*}P < 0.0001$, vs. $CCR8^{+/+}$ macrophages.

that CCR8$^{-/-}$ mice evaded renal injury induced by CLP. The systemic response is the outcome of the local response. Alleviation of renal injury in CCR8$^{-/-}$ mice appears to be ascribed to the decreased systemic inflammatory responses via an effective clearance of bacteria during CLP.

CONCLUSIONS AND SIGNIFICANCE

CCR8$^{-/-}$ mice were resistant to CLP-induced lethality as compared with CCR8$^{+/+}$ mice, and this resistance was associated with an augmented bacterial clearance in CCR8$^{-/-}$ mice. Peritoneal macrophages, but not neutrophils, appear to be responsible for the enhanced innate immune responses in CCR8$^{-/-}$ mice. Thus, ablation of CCR8 function appeared to have an unexpected positive impact on innate immune response during septic peritonitis. These findings raise several questions, including: Are the systemic or 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 during CLP. TCA3 did not reduce cytokine production from LPS-stimulated macrophages or in vitro bactericidal activities of peritoneal macrophages. TCA3 injection 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. CCR8 may be constitutively active, as this has been reported for some viral chemokine receptors 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. Identification of CCR8 ligands or molecules with antagonistic activity against CCR8 remains to be established.

Perhaps more important, 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. 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. A recent investigation suggests 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. These are intriguing observations because it has been shown that monocytes/macrophages that differentiate into DC-like cells mount poor innate immune response. 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 (**Fig. 3**).

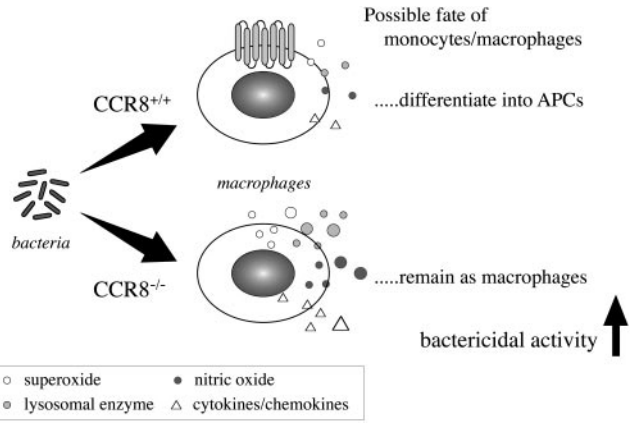

Figure 3. Summary of macrophage function in the presence or absence of CC chemokine receptor 8 (CCR8). In CCR8$^{+/+}$ mice, bacteria infection stimulates macrophages to induce superoxide generation, lysosomal enzyme release, and nitric oxide generation, effector molecules for bacteria killing. Cytokine and chemokines known to activate leukocytes are also produced by the stimulation. In case of CCR8$^{-/-}$ mice, these innate immune abilities of macrophages are augmented, resulting in an enhanced bacterial clearance that is beneficial to the host defense during infection. CCR8 may interfere with innate immune responses by regulating differentiation of macrophages to DC.

Results in the present study provide a new paradigm for the role of CCR8 in an innate immunity. Ablation of CCR8 function enhances innate immunity in a model of sepsis, resulting in an alleviation of systemic impact during septic peritonitis. Sepsis and septic shock are associated with high morbidity and mortality; 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. FJ