The chemokine receptor CCR6 is an important component of the innate immune response

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In our initial studies we found that naïve CCR6-deficient (CCR6−/−) C57BL/6 mice possessed significantly lower number of both F4/80+ macrophages and dendritic cells (DC), but higher number of B cells in the peritoneal cavity, as compared to naïve wild type (WT) controls. Furthermore, peritoneal macrophages isolated from CCR6−/− mice expressed significantly lower levels of inflammatory cytokines and nitric oxide following lipopolysaccharide (LPS) stimulation, as compared to WT macrophages. In a severe experimental peritonitis model induced by cecal ligation and puncture (CLP), CCR6−/− mice were protected when compared with WT controls. At 24 h following the induction of peritonitis, CCR6−/− mice exhibited significantly lower levels of inflammatory cytokines/chemokines in both the peritoneal cavity and blood. Interestingly, DC recruitment into the peritoneal cavity was impaired in CCR6−/− mice during the evolution of CLP-induced peritonitis. Peritoneal macrophages isolated from surviving CCR6−/− mice 3 days after CLP-induced peritonitis exhibited an enhanced LPS response compared with similarly treated WT peritoneal macrophages. These data illustrate that CCR6 deficiency alters the innate response via attenuating the hyperactive local and systemic inflammatory response during CLP-induced peritonitis.

Introduction

Despite advances in intensive care unit interventions and the use of specific antibiotics, sepsis with concomitant multiple organ failure is the most common cause of death in many acute care units. In this setting, approximately one-third of 750,000 people annually affected by sepsis die [1–4]. Sepsis is characterized by an initial overwhelming systemic inflammatory response syndrome (SIRS) (also known as a cytokine storm), which is followed by a compensatory anti-inflammatory response syndromes (CARS) [5]. Innate immune cells, including macrophage, PMN and DC are the main inflammatory cell types responsible for excessive cytokine production during the evolution of this response. These cells both produce and respond to proinflammatory cytokines and chemokines, suggesting that they might be appropriate targets for the treatment of severe sepsis. A number of novel therapeutics, specifically developed to prevent sepsis-induced mortality have failed, with the exception of recombinant activated protein C [6], supporting the concept that severe sepsis is dynamic and complex disease.

Chemokines are a family of structurally related chemotactic proteins, whose functions have been well studied on many fundamental aspects of immunology, including the development, homeostasis and host inflammatory response. The effects of specific chemo-
The latter information may provide the mechanism for CCR6/CCL20 has also been reported to be involved in T cell activation and host immunity against invasive pathogen sites. Furthermore, CCR6 –/– peritoneal macrophages show an attenuated response to TLR agonist stimulation compared with similarly treated WT controls. In contrast, there were significantly more CD19+ B cells in the peritoneal cavities of CCR6–/– mice compared with controls (1.5 ± 0.25 × 10⁶ vs. 1.2 ± 0.3 × 10⁶/µl/mouse, n = 5) (Table 1). No difference was found in the number of peritoneal CD4+ and CD8+ T cells, as well as NK cells between the CCR6–/– and WT groups. Therefore, CCR6 depletion alters the normal composition of peritoneal cells and results in a decrease in the cell types of myeloid origin, but an increase in B cell number.

Naive CCR6–/– peritoneal macrophages display an attenuated LPS-response

Macrophages are major participants in innate immunity, because of their ability to phagocytose pathogens, generate cytotoxic oxygen and nitrogen intermediates, and express chemokines and cytokines that either attract or activate other immune cells. However, the over-production of inflammatory factors during host defense leads to serious deleterious effects in normal tissues if left uncontrolled. We next sought to determine whether the presence of CCR6 affected the production of peritoneal macrophage-derived inflammatory mediators. Peritoneal macrophages from WT and CCR6–/– mice were harvested by peritoneal lavage and stimulated with 1 µg/mL LPS. After 4 h, CCR6–/– peritoneal macrophages contained significantly lower mRNA levels of IL-12p35, IL-12p40, IL-10, TNF-α, CCL2, CCL3, and CXCL10 (Fig. 1A). Furthermore, after 24 h of stimula-

### Results

#### Depletion of CCR6 alters inflammatory cell populations in naïve mice

Given that CCR6 have been reported to regulate the normal DC localization in Peyer’s patches [24, 25], we tested whether the depletion of CCR6 altered the localization of resident cell populations in the peritoneal cavity of naïve mice. Flow cytometric analysis was used to compare the numbers of several resident peritoneal cell types between WT C57BL/6 (CCR6+/+) and C57BL/6 CCR6–/– mice. Whereas there was no difference in the number of total peritoneal cells between two groups, we found CCR6–/– mice had significant fewer CD11b+/F4/80+ macrophages (50.4 ± 7.7 × 10⁴ vs. 72.4 ± 8.4 × 10⁴/µl/mouse, n = 5) and CD11c+ MHCII+ DC (0.8 ± 0.1 × 10⁴ vs. 1.5 ± 0.2 × 10⁴/µl/mouse, n = 5) compared with WT controls (Table 1). In contrast, there were significantly more CD19+ B cells in the peritoneal cavities of CCR6–/– mice compared with controls (1.5 ± 0.25 × 10⁶ vs. 1.2 ± 0.3 × 10⁶/µl/mouse, n = 5) (Table 1). No difference was found in the number of peritoneal CD4+ and CD8+ T cells, as well as NK cells between the CCR6–/– and WT groups. Therefore, CCR6 depletion alters the normal composition of peritoneal cells and results in a decrease in the cell types of myeloid origin, but an increase in B cell number.

| Table 1. Peritoneal leukocyte subsets in naïve micea) |
|-----------------|-----------------|-----------------|
| Group           | CCR6+/+         | CCR6–/–         |
| Total cells     | 293.5 ± 46      | 305.5 ± 48.8    |
| CD11b+F4/80+    | 72.4 ± 8.4      | 50.4 ± 7.7      |
| CD19+b)         | 115.5 ± 27.6    | 151.6 ± 25.4    |
| CD4+            | 15.3 ± 0.4      | 18.7 ± 0.4      |
| CD8+            | 4.8 ± 0.3       | 5.9 ± 0.3       |
| NK1.1+          | 3.1 ± 0.4       | 2.7 ± 0.4       |
| CD11c+ MHCII+b) | 1.5 ± 0.2       | 0.8 ± 0.1       |

a) All values are (×10⁶)/µl/mouse.

b) Significant difference between the two groups.
tion with LPS, CCR6−/− peritoneal macrophages produced significantly lower protein levels of IL-12p70, IL-10, TNF-α, CCL2, CCL3 and CXCL10 (Fig. 1B), which is consistent with their mRNA levels after 4 h of LPS stimulation.

Naive CCR6−/− peritoneal macrophages were also assessed for the levels of nitrite, which reflect the expression of NO, one of the major macrophage-derived effector molecules that is cytotoxic [26] and can induce host tissue injury [27]. Peritoneal macrophages isolated from CCR6−/− mice generated significantly lower level of nitrite in response to either a 100 ng/mL LPS challenge (3.7 ± 1.0 vs. 8.3 ± 1.1 μM, n = 5), or challenged with LPS plus 20 U/mL IFN-γ (4.7 ± 0.98 vs. 15.3 ± 2.2 μM, n = 5), respectively, as compared to WT peritoneal macrophages (Fig. 1C). Collectively, our data indicated that the absence of CCR6 resulted in an attenuated LPS-response by naïve peritoneal macrophages.

Previously, our group has shown that CCR4−/− peritoneal macrophages exhibited a constitutive phenotype of alternative activation. Since we observed that CCR6−/− peritoneal macrophages showed an attenuated LPS response, we further tested if CCR6−/− macrophages were also skewed to alternative activation status. Alternative activated macrophages has been tightly linked to the up-regulation of several molecules, including mannose receptor (MR), arginase 1 and the found in inflammatory zone 1 (FIZZ1) [28]. There was no difference in mRNA levels of MR, arginase 1 and FIZZ1 between CCR6−/− and WT peritoneal macrophages in response to either medium alone or LPS (Fig. 1D). Both WT and CCR6−/− peritoneal macrophages generated similar amount of FIZZ1 protein in in vitro culture system (Fig. 1E). These data suggest that CCR6−/− peritoneal macrophages are not skewed to alternative activation.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Cytokine production by peritoneal macrophages from naïve WT and CCR6−/− mice. Peritoneal macrophages were collected by peritoneal lavage and stimulated with 1 μg/mL LPS. Messenger RNA levels (A) and protein production (B) of inflammatory cytokines were measured by Taqman and ELISA, respectively. (C) Nitrite production was measured following the activation of peritoneal macrophages with 20 U/mL IFN-γ, 100 ng/mL LPS, or both IFN-γ and LPS. (D) Messenger RNA levels of mannose receptor, FIZZ1 and arginase 1 were measured by Taqman. (E) Protein level of FIZZ1 was measured by ELISA. The results shown are representative of five experiments and are expressed as mean ± SEM. *p ≤ 0.05 compared with cytokine levels measured in WT peritoneal macrophages, n = 5.
CCR6–/– mice are resistant to CLP-induced peritonitis

The difference in LPS response between WT and CCR6–/– peritoneal macrophages prompted us to test the role of CCR6 in an experimental model of severe sepsis induced by CLP. In initial studies, 100% mortality was observed in WT mice without antibiotic treatment as early as 3 days post-CLP (Fig. 2A). However, CCR6–/– mice were significantly protected from CLP-induced lethality ($p = 0.011$), and showed a 25% mortality rate at day 2 and 38% mortality rate at day 6; these latter mice were long-term survivors. Clearly, the absence of CCR6 provided a survival benefit compared with CCR6 competent mice. Antibiotic treatment for 3 days following CLP attenuated the severity of CLP and increased survival rates both in the WT and CCR6–/– groups (Fig. 2B). However, CCR6–/– mice were more resistant to CLP-induced mortality compared to WT controls ($p = 0.001$). Specifically, none of CCR6–/– mice died at day 3 after CLP. At day 6 after CLP, 88% of the CCR6–/– mice were alive compared with 25% of the WT mice at this time.

CCR6–/– mice express an attenuated cytokine profile after CLP-induced peritonitis

The polymicrobial peritonitis induced by CLP is associated with an amplified inflammatory response, in which the local and systemic expression of many cytokines and chemokines are augmented [29]. We compared the cytokine profiles in peritoneal lavage fluid and blood between WT and CCR6–/– mice before and after CLP-induced peritonitis. Compared to WT mice, CCR6–/– mice produced a similar amount of inflammatory cytokines, including IL-12p70, IL-10, TNF-α, KC, CCL2, CCL3 and CXCL10 in peritoneal cavity and blood at 4 h after surgery. However, CCR6–/– mice showed significantly lower levels of inflammatory cytokines both

![Figure 2](image)

**Figure 2.** Survival study during CLP-induced severe sepsis. WT and CCR6–/– mice were subjected to CLP surgery, either in the absence (A) or in presence (B) of antibiotics treatment. Survival was monitored for 6 days following surgery. Each group: $n = 10$

![Figure 3](image)

**Figure 3.** Cytokines profile in WT and CCR6–/– mice following CLP operation. To compare the production of inflammatory cytokines in peritoneal cavities (A) and blood (B) between WT and CCR6–/– mice after CLP, ELISA was performed to measured protein levels of IL-12p70, IL-10, TNF-α, KC, CCL2, CCL3 and CXCL10 in peritoneal lavage fluid and serum. * $p \leq 0.05$ compared with cytokine protein levels measured in WT mice 24 h after CLP, $n = 5$. The results shown are representative of three individual experiments.
in the peritoneal cavity (Fig. 3A) and in blood (Fig. 3B) at 24 h after CLP-induced peritonitis. We also observed that CCR6−/− mice had significantly lower levels of inflammatory cytokines in both the lungs and livers, as compared to WT controls (data not shown).

**Leukocyte recruitment into the peritoneal cavity post CLP-induced peritonitis**

Based on the observation that CCR6−/− displayed a decreased cytokine production in mice, we designed a set of investigations to determine if the alterations were due to changes in leukocyte recruitment into the peritoneal cavity following CLP-induced peritonitis. Two methods were used to assess leukocyte peritoneal infiltration in WT and CCR6−/− mice at various times after CLP (Fig. 4). Cytospins were prepared and the leukocyte composition, including PMN and mononuclear cells, was determined by microscopic differential counting. No difference in total numbers of leukocytes (Fig. 4A), mononuclear cells (Fig. 4B) and PMN (Fig. 4C) in the peritoneal cavity was observed between the two groups of mice after CLP-induced peritonitis.

Our earlier observations demonstrated a difference in cellular composition in the peritoneal cavity between naïve WT and CCR6−/− mice and other investigations showed alterations in the recruitment of immature DC during inflammatory responses [20, 21]. Thus, we investigated changes in DC population in CCR6−/− mice and found significantly less MHCII⁺CD11c⁺ DC in the peritoneal cavity before and 4 and 24 h after CLP-induced peritonitis, as compared to WT mice (Fig. 4D). However, no difference was observed in the number of CD11b⁺F4/80⁺ macrophage (data not shown).

**CCL20 shows no effect on peritoneal macrophages and CLP-induced mortality**

CCL20, the only chemokine ligand for CCR6, is up-regulated during inflammation [30, 31] and by specific proinflammatory cytokines [32]. An increase in CCL20 level in the peritoneal cavity was observed in WT mice with CLP-induced peritonitis with a peak at 24 h (Fig. 5A). CCR6−/− mice showed a significantly lower level of CCL20 in the peritoneal cavity 24 h after CLP-induced peritonitis, as compared to WT mice, which was consistent with other inflammatory cytokines. This might be due to an attenuated inflammatory response in CCR6−/− mice. We next sought to determine whether the presence of CCL20 exacerbated the dysregulated immune response and promoted CLP-induced mortality in WT mice. When endogenous CCL20 was passively neutralized with anti-CCL20 2 h prior to CLP-induced peritonitis and each following day, survival was not altered, as compared to mice treated with IgG control (Fig. 5B). These data suggested that the interaction between CCL20 and CCR6 was not involved in the survival of CCR6−/− mice after CLP-induced peritonitis. We also tested whether CCL20 altered the LPS response of WT peritoneal macrophages. LPS induced inflammatory cytokine production by WT peritoneal macrophages, which was not altered by the presence of CCL20 in either lower (1 ng/mL) or high (100 ng/mL) concentrations (Fig. 5C).

**CCR6−/− macrophages show enhanced LPS-responsiveness after CLP-induced peritonitis**

It has been well established that macrophages develop an unresponsiveness to LPS challenge after LPS pretreatment, a process referred to as endotoxin tolerance [33]. The mechanism for this tolerance response is not fully clear. Since we observed that CCR6−/− mice had an attenuated inflammatory immune response and higher survival rate during CLP-induced severe sepsis, we investigated whether the septic response induced LPS tolerance in CCR6−/− peritoneal macrophages. We initially focused on cytokine produc-
tion in response to LPS stimulation. Three days after CLP, the levels of inflammatory cytokines generally decreased to the base line, indicating the end of the acute phase of severe sepsis. In contrast to naïve macrophages, CCR6−/− peritoneal macrophages, at day 3 after CLP-induced peritonitis, had significantly higher LPS-induced mRNA levels of IL-12p35, IL-12p40, IL-10, TNF-α, CCL3 and CXCL10, but lower CCL2 mRNA level compared with WT peritoneal macrophages isolated at the same time after CLP (Fig. 6A). Consistently, after 24 h of stimulation with LPS, CCR6−/− peritoneal macrophages produced significantly higher protein levels of IL-12p70, IL-10, TNF-α, CCL3 and CXCL10, but less CCL2 (Fig. 6B). These data suggest that CCR6−/− peritoneal macrophages do not exhibit severe sepsis-induced tolerance.

To test if the greater LPS responsiveness of CCR6−/− macrophages day 3 after CLP-induced peritonitis is an intrinsic property of CCR6−/− macrophages, or it is because of an attenuated cytokine storm affecting CCR6−/− peritoneal macrophages during CLP-induced peritonitis (Fig. 3A), we next performed a classical LPS rechallenge experiment using ex vivo peritoneal macrophage isolated from naïve WT and CCR6−/− mice. Naïve CCR6−/− peritoneal macrophages produced significantly lower protein levels of IL-12p70, IL-10, TNF-α, CCL2, CCL3 and CXCL10 in response to one-time LPS challenge compared to similarly treated WT control, which we have previously observed (Fig. 1). Both WT and CCR6−/− peritoneal macrophages, which have been pretreated with 10 ng/mL LPS, showed unresponsiveness to a second challenge with 1 μg/mL LPS, indicating that CCR6−/− peritoneal macrophages possessed a similar unresponsiveness to LPS challenge after LPS pretreatment as WT controls (Fig. 7).

Discussion

In the present study, the role of CCR6 during experimental severe sepsis was investigated. We found that mice lacking CCR6 exhibited a constitutive decrease in resident peritoneal macrophage populations, as well as DC numbers, as compared to WT mice. Although cytokine production by pulmonary DC and bone marrow-derived DC in response to TLR agonist stimulation was comparable between WT and CCR6−/− mice (data not shown), we observed that naïve CCR6−/− peritoneal macrophage exhibited a decreased production of inflammatory cytokines and NO in response to LPS stimulation compared with similarly treated WT controls. CCR6−/− mice were significantly protected from CLP-induced lethality and showed an attenuated localized and systemic inflammatory cytokine production compared with WT mice. Surprisingly, CCL20, the only chemokine ligand of CCR6, did not appear to play a role in the pathology of experimental sepsis, since localized neutralization of endogenous CCL20 had no effect on survival in septic WT mice, and CCL20 failed to influence the LPS responsiveness of WT peritoneal macrophages.
Figure 6. Cytokine production by peritoneal macrophages from WT and CCR6−/− mice day 3 post-CLP. WT and CCR6−/− mice (n = 5) were sacrificed at day 3 after CLP surgery. Peritoneal macrophages were collected by peritoneal lavage and exposed to 1 μg/mL LPS. (A) Four hours after LPS stimulation, mRNA levels of inflammatory cytokines in peritoneal macrophages were measured by Taqman. *, t = p ≤ 0.05 compared with mRNA levels of inflammatory cytokines in WT peritoneal macrophages. (B) Twenty-four hours after LPS stimulation, protein levels of inflammatory cytokines in culture supernatants were measured by ELISA. α, β p ≤ 0.05 compared with protein levels of inflammatory cytokines produced by WT peritoneal macrophages.

Figure 7. LPS rechallenge of peritoneal macrophages from naïve WT and CCR6−/− mice. Peritoneal macrophages were collected by peritoneal lavage and pretreated with 10 ng/mL LPS for 24 h. After two washes, these cells were stimulated with 1 μg/mL LPS for 24 h. Supernatants were collected to measure cytokine protein levels by ELISA. * p ≤ 0.05 compared with cytokine levels measured in WT peritoneal macrophages, n = 5.
Chemokines and chemokine receptors were originally described to be essential mediators directing homeostatic cell trafficking and cellular recruitment during inflammatory responses. However, the biological activities of chemokines are much more than only chemotactic factors. They are now known to play important roles in a variety of activities including maintenance of homeostasis, angiogenesis/angiostasis, wound healing, tumor growth and metastasis, cellular differentiation and activation, lymphocyte development and trafficking, lymphoid organ development, and influencing the type 1/type 2 balance of immune responses [34, 35]. Monocyte/macrophage trafficking has been shown to depend on the interactions between chemokines and chemokine receptors. For example, CCR2 mediated homeostatic monocyte emigration from bone marrow into circulation, and inflammation-induced monocyte recruitment from bone marrow to peripheral infected tissue also required CCR2 signaling [36]. Although it has been reported that CCR2 was not involved in tissue entry of monocyte from the circulation [36], new data has shown that CCR2 was necessary for efficient monocyte recruitment from the blood into inflamed tissue [37]. CCR2–/– mice also showed an impaired recruitment of macrophage into the peritoneal cavity in response to thioglycollate stimulation [38]. Thus, CCR2–/– exhibited deficient monocyte recruitment into infected sites in response to a broad range of stimuli [39, 40].

While we were not surprised to observe a decrease in peritoneal DC population in naïve status and impaired DC recruitment in inflammatory responses in CCR6–/– mice, which was consistent with other studies [17, 22, 24, 25], it was not predicted that the population of peritoneal macrophages also was decreased. In contrast to numerous reports showing monocyte/macrophage chemotactic property of chemokines and chemokine receptors, little is known about the influence of chemokine signaling on monocyte/macrophage lineage development and normal function. The chemokine receptor CXCR4 has been reported to be expressed on hematopoietic stem cells (HSC) and the interaction of CXCR4 with its cognate ligand, CXCL12, was essential for the colonization of bone marrow by HSC during development [41]. CXCR4 was also involved in HSC homing to bone marrow [42], as well as their survival and proliferation [43]. Thus, it is highly possible that chemokine signaling has a role in directing normal hematopoiesis, including the development of monocyte/macrophage lineage. Our data showed that CCR6 controlled the localization of macrophages and DC in the peritoneal cavity and altered the phenotype of naïve peritoneal macrophages, suggesting that CCR6 signaling may be involved in the development of monocyte/macrophage lineage. However, the molecular mechanism whereby CCR6 influences the development and function of peritoneal macrophages is not clear. The possibilities are that CCR6 functions either during the development of monocyte/macrophage lineage from progenitor cells in bone marrow, or influences migration of monocyte/macrophage to peripheral tissues. Thus, the role of CCR6 in the development of monocyte/macrophage lineage is worthy of further investigation.

A number of in vitro and in vivo studies have underscored the importance of chemokines/chemokine receptors in the initiation of the immune response to sepsis, as well as a direct correlation with tissue pathology, organ dysfunction and failure, and lethality. Immunoneutralization of CXCR2 with specific antibody decreased PMN recruitment and CLP-induced lethality [13, 44]. CCR1–/– mice had accelerated cytokine production and were protected against the deleterious effects of sepsis, due in part to the fact that CCR1 in peritoneal macrophages displayed both an early and enhanced cytokine/chemokine expression profile and anti-bacterial response. CCR4 depletion has been shown to protect mice from systemic challenge with either LPS [10] or other TLR agonists, as well as protect mice against bacterial peritonitis [9]. CCR4–/– peritoneal macrophages were demonstrated to possess a constitutive phenotype of an alternatively activated macrophage and exhibit features associated with alternative activation, including enhanced production of type-2 cytokines, CCL2, CCL17 and FIZZ1 protein [9]. CCR8–/– mice were also resistant to CLP-induced lethality, which mechanistically may be related to the phenotype of CCR8–/– peritoneal macrophages. These inflammatory cells were highly activated, exhibited enhanced bacterial activities, and generated high levels of superoxide, lysosomal enzymes and NO in response to LPS stimulation [8, 12]. Collectively, our data in the present study and the above reports suggests that chemokines/chemokine receptors have a role in the development and maintenance of normal function of peritoneal macrophage, since chemokine receptor deficiencies alter the functions of peritoneal macrophages, including cytokine production in response to TLR agonists.

Although CCL20 is the main ligand for CCR6, β-defensin has also been shown to bind CCR6 with a lower affinity [14]. Defensins, a family of arginine-rich cationic peptide, have direct antimicrobial effect and are considered an important effector molecule in innate immune response [45, 46]. Furthermore, defensins were also shown to chemottract immune cells, including monocytes/macrophages [47, 48], mast cells [49] and DC [14]. This suggests that defensins contribute either direct or indirectly to the mobilization of host immune defense in response to pathological infections. In the present study, we observed an attenuated inflammatory cytokine production and
survival advantage in CCR6−/− mice during severe sepsis, which was not related to CCL20 function. Thus, this finding raised the question whether β-defensin was involved in exacerbating dysregulated immune response and cytokine production in WT mice. Interestingly, it has been shown that human α-defensin promoted the expression and production of CXCL5 and CXCL8 by bronchial epithelial cells [50] and increased the production of TNF-α and IL-1 by monocytes [51]. Thus, it is possible that, during CLP-induced bacterial peritonitis, β-defensin promoted the inflammatory cytokine production by innate immune cells, including peritoneal macrophages and DC in a CCR6-dependent mechanism.

In summary, we have shown that genetic depletion of CCR6 decreased cells of myeloid origin including macrophage and DC in peritoneal cavity. Peritoneal macrophages from CCR6−/− generated significantly lower amounts of inflammatory cytokines and NO compared with similarly treated WT controls. Furthermore, CCR6 depletion increased survival rate during CLP-induced severe sepsis, which was associated with an attenuated cytokine storm, an indicator of severity of sepsis. Thus, these data suggested that CCR6 is a key mediator in modulating the innate inflammatory response during a septic insult and is potential target for immunotherapy in septic patients.

Materials and methods

Mice

Specific pathogen-free female C57BL/6 WT mice were purchased from Taconic Farms (Germantown, NY). Female CCR6-deficient (CCR6−/−) mice were originally generated in Dr. Lira’s laboratory and were subsequently backcrossed eight generations onto a C57BL/6 background [17, 24]. Mice were housed in the animal care facility at the University of Michigan. The University Committee on Use and Care of Animal (UCUCA) at the University of Michigan approved all experimental procedures.

Experimental sepsis induced by CLP

CLP surgery was performed on mice as previously described [52]. In brief, 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 surgical conditions, a 1-cm midline incision was made to the ventral surface of the abdomen, and the cecum was exposed. The cecum was partially ligated at its base with a 3.0 silk suture and punctured nine times with 21-gauge needle. The cecum was returned to the peritoneal cavity, and the abdominal incision was closed using surgical staples. Mice were rehydrated with 1 mL saline s.c. and placed on a heating pad until they recovered from anesthetic. At 4, 24 and 72 h after surgery, CLP mice were anesthetized and bled. Peritoneal lavage was performed with 2 mL of cold sterile saline. Serum and cell-free peritoneal fluid were collected for chemokine/cytokine protein analyses. RBC were lysed in ammonium chloride buffer (150 mM NH₄Cl, 10 mM NaHCO₃, 1 mM EDTA- tetrasodium), and the total cell numbers were determined using a hemocytometer. Cytospins (Shandon, Waltham, MA) were prepared and stained with Diff-Quik solutions (Dade Behring, Dudingen, Switzerland). The leukocyte composition was analyzed, and the percentage of PMN and mononuclear cells was multiplied by the total cell count to determine their absolute number.

Survival studies following CLP

The first set of survival studies was performed to determine the effect of the presence of CCR6 on survival in CLP-induced sepsis animal model. WT and CCR6−/− mice were subjected to CLP surgery in the presence or absence of the treatment with antibiotic INVANZ (Ertapenem) i.p. injected at 75 mg/kg (Merck & Co, Whitehouse station, NJ) 6 h after surgery and every 24 h until day 3 post-CLP. Survival was monitored for 6 days following surgery. The second set of survival studies focused on the role of endogenous CCL20 following induction of acute septic peritonitis. WT mice were i.p. injected with 20 μg per mouse of anti-mouse CCL20 mAb (R&D systems, Rochester, MN) 2 h before CLP and every day following surgery to block endogenous CCL20. Rat IgG1 was used as a control. Survival was monitored for 6 days following surgery.

Flow cytometry analysis

Total peritoneal cells were harvested by peritoneal lavage with 10 mL cold sterile saline twice from naïve WT or CCR6−/− mice or at indicated time points following CLP. After lysing RBC with ammonium chloride buffer, total cell numbers were determined using a hemocytometer. Fc binding was blocked via a 10 min incubation with purified rat anti-mouse CD16/CD32 (FcγIII/II receptor). Then, the cells were stained with the following mAb to identify series of mouse cell types: PE-conjugated anti-CD11b in combination with FITC-conjugated anti-F4/80 (macrophage), FITC-anti-CD19 (B cell), PE-anti-I-Α in combination with FITC-anti-CD11c (DC), PE-anti-NK1.1 (NK cell), PE-anti-CD4 in combination with FITC-anti-CD8 (CD4+ and CD8+ T cell). The appropriate IgG isotypes were used as controls. All antibodies and IgG isotypes were purchased from BD PharMingen (San Diego, CA), except for anti-F4/80 (Serotec, Raleigh, NC). The cells were fixed in 1% paraformaldehyde and kept in the dark at 4°C until analysis with a FACSCaliber (CELLQuest™ software; Becton Dickinson, Mountain View, CA).

Peritoneal macrophage collection and in vitro stimulation

Total peritoneal cells were harvested by peritoneal lavage with 10 mL cold sterile saline twice from WT or CCR6−/− mice in naïve status or at day 3 post-CLP. Lavage was pooled for mice in the same group. RBC were lysed in ammonium chloride buffer. The remaining cells were thoroughly washed with
saline and counted in a hemocytometer. Cytospins were prepared and stained with Diff-Quik solutions, and the number of peritoneal macrophages was determined. Cells were resuspended at a concentration of $10^6$ macrophages/mL in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 5% FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg streptomycin. Macrophages were plated in plastic 48-well cell culture plate (4 x $10^5$/well) (Corning, Acton, MA) and incubated 2 h at 37°C in 5% CO2. Nonadherent cells were removed, and adherent cells were washed with complete RPMI 1640 medium, followed by the stimulation with 1 µg/mL LPS (Sigma-Aldrich, St. Louis, MO). Four hours later, total RNA was isolated from cultured peritoneal macrophages using Trizol reagent and quantitative real-time PCR (TaqMan) was performed to measure cytokine/chemokine gene expression. Twenty-four hours later, cell-free supernatant from each sample was collected and stored at ~80°C until cytokine/chemokine protein assay by ELISA. For the LPS rechallenge experiments, isolated peritoneal macrophages were pretreated with 10 ng/mL LPS for 24 h, washed twice and then exposed to a second challenge with 1 µg/mL LPS. Twenty-four hours later, the supernatants were collected to measure cytokine protein levels by ELISA.

RNA isolation and real-time PCR (TaqMan)

Total RNA was isolated from cultured peritoneal macrophages using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. A total of 2.0 µg of RNA was reverse transcribed to yield cDNA in a 25 µL reaction mixture containing 1X first strand (Life Technologies, Gaithersburg, MD), 250 ng of oligo (dT)12–18 primer, 1.6 mM dNTP (Invitrogen), 5 U of RNase inhibitor (Invitrogen), and 100 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). Complementary DNA was then analyzed by quantitative, real-time PCR using a TaqMan 7500 sequence detection system (Applied Biosystems). GAPDH was analyzed as an internal control. The fold differences in mRNA expression between treatment groups were determined by the Sequence Detection Systems software (Applied Biosystems).

ELISA

Concentrations of IL-10, IL-12p70, TNF-α, CCL2, CCL3, CXCL10 were measured in cell-free peritoneal lavage fluid, serum, and cell culture supernatants using a standardized sandwich ELISA technique as previously described in detail [17, 53]. Briefly, C96 Maxisorp Nunc-Immuno plates (Fisher Scientific) were coated with 1–5 µg/mL capture Ab in coating buffer overnight at 4°C and washed with PBS containing 0.05% Tween 20. All Ab used for ELISA were purchased from R&D Systems. Nonspecific binding sites were blocked with 2% BSA in PBS for 90 min at 37°C. Plates were rinsed four times with wash buffer and cell-free supernatants were loaded and incubated for 1 h at 37°C. After four washings, a biotinylated detection polyclonal Ab was added for 45 min at 37°C. The plates were washed again and peroxidase-conjugated streptavidin (Bio-Rad) was added to the well for 30 min at 37°C. Plates were washed, and after the addition of chromogen substrate (Bio-Rad), OD readings were measured at 490 nm using an ELISA plate reader. Recombinant murine cytokines were used to generate the standard curves from which the concentrations present in the samples were calculated. The limit of detection of the assays was 50 pg/mL.

Nitrite production

Nitrite ($\text{NO}_2^-$) levels were determined using the Griess method as previously described [54]. The measurement of this parameter is widely accepted as indicative of NO production. Briefly, naive peritoneal macrophages were plated at $4 \times 10^5$/well of a 96-well cell culture plate (Corning) and rested overnight. Cells were treated with fresh complete RPMI 1640 medium alone, 20 U/mL IFN-γ (PeproTech), 100 ng/mL LPS, or both IFN-γ and LPS. After 48 h at 37°C in 5% CO2, 50 µL of cell-free supernatants was transferred to a flat-bottom 96-well plate and treated with 100 µL of 0.5% sulfanilamide (Sigma-Aldrich) and 0.05% naphthylethylenediamine dihydrochloride (Sigma-Aldrich) in 2.5% phosphoric acid (H3PO4). The absorbance was read at 550 nm in a microplate reader. A standard curve was generated using known concentrations of NaNO2 (Sigma-Aldrich). Similar results were shown in three independent experiments.

Statistics

Statistical analysis was carried out with Prism 4 for Macintosh. 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. Values of $p < 0.05$ were considered statistically significant.

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References


