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Critical Protective Role for MCP-1 in Pneumonic *Burkholderia mallei* Infection

Andrew Goodyear,* Abby Jones,* Ryan Troyer,* Helle Bielefeldt-Ohmann,*† and Steven Dow*†

*Burkholderia mallei* is a Gram-negative bacterial pathogen of domestic equidae and humans that can cause severe, rapidly life-threatening pneumonic infections. Little is known regarding the role of chemokines and early cellular immune responses in protective immunity to pulmonary infection with *B. mallei*. Although the role of MCP-1 in Gram-positive bacterial infections has been previously investigated, the role of MCP-1 in immunity to acute pneumonia caused by Gram-negative bacteria, such as *B. mallei*, has not been assessed. In a mouse model of pneumonic *B. mallei* infection, we found that both MCP-1−/− mice and CCR2−/− mice were extremely susceptible to pulmonary infection with *B. mallei*, compared with wild-type (WT) C57Bl/6 mice. Bacterial burden and organ lesions were significantly increased in CCR2−/− mice, compared with WT animals, following *B. mallei* challenge. Monocyte and dendritic cell recruitment into the lungs of CCR2−/− mice was significantly reduced in comparison with that in WT mice following *B. mallei* infection, whereas neutrophil recruitment was actually increased. Depletion of monocytes and macrophages prior to infection also greatly raised the susceptibility of WT mice to infection. Production of IL-12 and IFN-γ in the lungs after *B. mallei* infection was significantly impaired in both MCP-1−/− and CCR2−/− mice, whereas treatment of CCR2−/− mice with rIFN-γ restored protection against lethal challenge with *B. mallei*. Thus, we conclude that MCP-1 plays a key role in regulating cellular immunity and IFN-γ production following pneumonic infection with *B. mallei* and therefore may also figure importantly in other Gram-negative pneumonias. *The Journal of Immunology*, 2010, 184: 1445–1454.

*Burkholderia mallei* is an important Gram-negative bacterial pathogen that readily infects humans, generally following exposure to infected animals (1–3). Infection in humans can be contracted by inhalation or via infection of wounds or mucous membranes. Horses and donkeys are the primary animal hosts for *B. mallei* and can develop acute infection involving the lungs and lymph nodes (glanders) or chronic cutaneous infection (farcy) (1). Notably, *B. mallei* is highly infectious to humans and other animals when contracted by inhalation (2).

Renewed attention has recently been focused on *B. mallei* and the closely related bacterium *Burkholderia pseudomallei*. *B. pseudomallei* is endemic in parts of southeast Asia and northern Australia, where many patients are infected annually (3–5). Although *B. mallei* has been eliminated from most developed countries, it, too, remains endemic in some parts of the world, including the Middle East, Asia, and South America. In addition, both organisms have high potential for use as bioweapons, owing to their ease of aerosol transmission, their resistance to many common antibiotics, and their ability to establish both acute and chronic infections (2). Indeed, *B. mallei* has been previously weaponized and deployed in several wars (6). Thus, there are compelling reasons to better understand the pathogenesis of *B. mallei* infection, including early pulmonary immune responses to respiratory infection.

Both IFN-γ and TNF-α are critical for protection from *B. pseudomallei* after systemic infection (4, 7, 8). In addition, neutrophils were discovered to play an important early protective role in the local control of pulmonary infection with *B. pseudomallei* (9). Macrophages were also found to be important for control of disseminated infection with *B. pseudomallei* following parental challenge (10). Much less is known regarding immunological mechanisms responsible for protection from pneumonic *B. mallei* infection, although an aerosol infection model has been developed in BALB/c mice (11). In that model, pneumonic infection elicited rapidly fatal disease characterized by acute, focal necrotizing alveolitis and pneumonia.

Because little is known about innate immune responses and control of *B. mallei* infection, we conducted studies to identify critical protective components of the innate immune response to pneumonic *B. mallei* infection. In particular, we focused on the role of the chemokine MCP-1 in controlling acute pulmonary infection with *B. mallei*. Previously, it was found that MCP-1 and inflammatory macrophages played a major part in controlling infection with the Gram-positive bacterium *Listeria monocytogenes* (12). For example, mice lacking the MCP-1 receptor (CCR2−/− mice) were much more susceptible to infection with *L. monocytogenes* than were wild-type (WT) mice (13, 14). However, the role of MCP-1 in controlling bacterial infections, in general, has not been systematically examined and may not be readily predictable. For example, MCP-1 was found to be not necessary for control of low-dose pulmonary infection with *Mycobacterium tuberculosis* (15, 16). Thus, the function of MCP-1 in controlling bacterial infections may vary depending on the pathogen, the route of exposure, and the challenge dose.
To address the role of MCP-1 and monocytes in controlling B. mallei infection, we used a model of pneumonic B. mallei infection in C57Bl/6 mice. The kinetics of pulmonary infection, bacterial dissemination, and cytokine responses were investigated in WT mice and in MCP-1−/− and CCR2−/− mice. In addition, liposomal clodronate depletion studies were used to assess the role of monocytes and macrophages in controlling pneumonia induced by B. mallei infection. The link between MCP-1 and production of IFN-γ was also explored. Our findings indicated that both MCP-1 and its receptor CCR2 were critical in generating protective immunity to pneumonic B. mallei infection and that their effectiveness was mediated, at least in part, through regulation of IFN-γ production.

Materials and Methods

Mice

C57Bl/6 mice and MCP-1−/− and CCR2−/− mice on the C57Bl/6 background used in these studies were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in-house. All mice used in experiments were 6–8 wk of age at the time of infection, and the mice were housed under pathogen-free conditions.

Bacterial strains and infections

B. mallei strain ATCC23344 was used in these studies. This strain was kindly provided by Dr. Herbert Schweitzer, Colorado State University. Prior to use for in vivo infections, B. mallei ATCC23344 was serially passaged three times in BALB/c mice; then stocks were prepared and frozen at −80°C. Animal passage of B. mallei has previously been reported to increase virulence, which we also noted in our studies (17, 18). Before each challenge study, fresh broth cultures of B. mallei were grown in Brucella broth with 4% glycerol (BB4G) (Remel, Lenexa, KS) until bacteria reached the log phase of growth; then titers were determined based on optical density values, and appropriate bacterial dilutions were prepared in sterile PBS. Inoculum titers for each experiment were confirmed by plating the inoculum on BB4G agar plates (Remel). Challenge studies were also done with B. pseudomallei (strain 1026b), which was provided by Dr. Herbert Schweitzer. Stocks of B. pseudomallei were thawed immediately prior to animal inoculation, and the challenge dose was confirmed by plating postinfection.

For intranasal (i.n.) infection, mice were anesthetized by i.p. injection of ketamine and xylazine. Mice were then infected i.n. with a total volume of 20 μl bacterial inoculum (10 μl per nostril). Preliminary experiments were performed to identify the optimal i.n. infection conditions. Three hours after i.n. infection, mice were euthanized, and the lungs from each mouse were plated. On the basis of the number of bacteria reaching the lungs, we determined that there was no difference in efficacy between infection volumes of 20 and 40 μl (p = 0.79). Efficiency of infection experiments in mice infected i.n. with a total volume of 20 μl (10 μl per nostril) demonstrated that ~40% of the original inoculum was deposited in the lungs (data not shown; n = 3 mice per group; data pooled from 3 independent experiments).

All procedures involving Burkholderia were performed in a Biosafety Level 3 facility, in accordance with approved BSL3 and Select Agent protocols. All animal studies were approved by the Colorado State University Institutional Animal Care and Use Committee.

Determination of bacterial burden

Lung, liver, and spleen tissues from infected mice were collected separately and placed in 5 ml sterile PBS on ice. Organs were homogenized using a Stomacher 80 Biomaster (Seward, Boemia, NY), and supernatants were serially diluted in saline and plated on BB4G agar plates (Remel). Agar plates were incubated at 37°C for 48 h, and colonies were counted. The limit of detection for determination of bacterial burden in organ homogenates was 100 CFU/organ. For quantification of bacteremia, blood was collected in Microtainer tubes with lithium heparin (BD Biosciences, San Jose, CA), and serial dilutions were prepared in saline and plated on BB4G agar plates. The limit of detection for determination of bacterial burden in the blood was 10 CFU/ml.

Collection and preparation of bronchoalveolar lavage and lung cells for flow cytometry

Airway cells were obtained by bronchoalveolar lavage (BAL), as previously described (19). Immediately postextubation, an 18-gauge catheter was inserted into the trachea of each mouse, and the lungs were insufflated with ~1 ml of PBS containing 1 mM EDTA, which was then immediately withdrawn from the lungs by suction. This procedure was repeated six times for each mouse, and the fluid collected was pooled. Cells were recovered from the BAL by centrifugation. Postlavage, lung digestion was performed, as previously described (20). Briefly, lungs were minced and digested in HBSS containing 2.5 mg/ml collagenase, 10 U/ml DNase, and 10 μg/ml soybean trypsin inhibitor (all reagents from Sigma-Aldrich, St. Louis, MO). After digestion, cells were triturated through an 18-gauge needle and passed through a 70-μm cell strainer (BD Biosciences). RBCs were lysed using ammonium chloride, washed twice in HBSS, immunostained, and then stored on ice until analyzed. All cells were resuspended in FACS buffer (PBS with 2% FBS and 0.05% sodium azide) before immunolabeling and flow cytometric analysis.

Flow cytometry

Directly conjugated Abs for flow cytometry were purchased from eBioscience (San Diego, CA) or BD Biosciences. The following eBioscience Abs were used for flow cytometry staining: anti-CD11b (allophycocyanin conjugated; clone M1/70), anti-CD11c (PE conjugated; clone N418), anti-CD45 (Pacific Blue conjugated; clone 30-F11), anti-NK1.1 (biotin conjugated; clone PK136), anti-CD4 (PE conjugated; clone GK1.5), and anti-CD8 (allophycocyanin conjugated; clone 53-6.7). The following BD Biosciences Abs were used: anti-Ly6C (biotin conjugated; clone AL-21) and anti-Ly6G (FITC conjugated; clone 1A8). Pacific Orange and Alexa 488 streptavidin conjugates were purchased from Invitrogen (Carlsbad, CA). Before staining, nonspecific Ab binding was blocked by addition of FACS block consisting of normal mouse serum (Jackson ImmunoResearch, West Grove, PA), human IgG (Jackson ImmunoResearch), and unlabeled anti-mouse CD16/162 (clone 93, eBioscience) for 5 min at room temperature. Staining was performed in FACS buffer for 30 min on ice, followed by washing with FACS buffer. In the case of biotinylated Abs, the streptavidin-fluorochrome conjugate was added next for 20 min on ice. After a final wash, the cells were fixed in 1% paraformaldehyde in PBS for 24 h at 4°C, washed once, resuspended in FACS buffer, and stored at 4°C until analyzed. Flow cytometry was performed with a CyAn ADP Analyzer flow cytometer using Summit software (Beckman Coulter, Fullerton, CA). Analysis was done using FlowJo software (Tree Star, Ashland, OR). Samples were gated on forward and side scatter characteristics for viable cells. For investigation of monocytes, neutrophils, dendritic cells (DCs), and alveolar macrophages (AMs), live cells were then gated on lymphoid vs. myeloid leukocytes for analysis. AMVs were defined as CD11c+ and CD11b+ cells. DCs were defined as CD11c+ and CD11b+. Neutrophils were defined as CD11b+ and Ly6G+ cells, and monocytes were defined as CD11b+ and Ly6C+ but Ly6G−. For investigation of NK and T cells, live cells were gated on lymphocytes based on their low forward and side scatter characteristics. NK cells were identified as CD45+ and NK 1.1+ cells.

Assessment of cytokine and NO concentrations in BAL, fluid, and lung homogenate

The BAL supernatant was separated from the cells by centrifugation, sterile filtered, and frozen at −80°C prior to cytokine analysis. Lavaged lungs were dissected and placed into 4 ml PBS. Lungs were homogenized using a Stomacher 80 Biomaster (Seward). Homogenate was centrifuged at 3000 × g for 15 min at 4°C; supernatants were sterile filtered and frozen at −80°C until analyzed.

BAL fluid and lung homogenates from CCR2−/− and concurrently infected WT mice were assayed for cytokine production of IFN-γ, TNF-α, and IL-12p40 using the cytometric bead array (CBA) (BD Biosciences), and KC was measured using a commercially available ELISA DuoSet Kit (R&D Systems, Minneapolis, MN), according to the manufacturer’s directions. BAL fluid from MCP-1−/− and concurrently infected WT mice was assayed for the presence of IFN-γ, IL-12p40, KC, and TNF-α by ELISA, using DuoSet Kits (R&D Systems). Samples were assayed for the presence of NO, using a commercially available Griess Reagent System (Cayman Chemicals, Ann Arbor, MI).

Data acquisition from CBA experiments was performed using a CyAn ADP Analyzer flow cytometer, with data analysis done using Summit software (Beckman Coulter) and FlowJo software (Tree Star). For ELISA and Griess reaction assays, optical density readings were determined using a Multiskan Ascent ELISA plate reader and Ascent software (Thermo Scientific, Waltham, MA).

Determination of MCP-1 concentrations

Organs were harvested, and half of each lung and spleen were homogenized in 4 ml saline, using a Stomacher 80 Biomaster (Seward). The organ homogenate was centrifuged at 3000 × g for 15 min at 4°C; supernatants were sterile filtered and then stored frozen at −80°C prior to analysis. Plasma was prepared from blood collected via cardiac puncture and also
frozen at −80°C prior to cytokine analysis. MCP-1 concentrations in organ homogenates and plasma were determined using a CBA kit (BD Biosciences), according to the manufacturer’s directions. Data acquisition was performed with a FACSscan flow cytometer, with data analyzed using CellQuest software (BD Biosciences) and FlowJo software (Tree Star).

**Cytokine and chemokine analysis by quantitative real-time PCR**

Lung tissues were placed in 1 ml Trizol Reagent (Invitrogen), homogenized using a Tissue Tearor (BioSpec Products, Bartlesville, OK), and then frozen at −80°C. Total RNA was extracted following storage in Trizol Reagent, using the manufacturer’s instructions (Invitrogen). Any remaining DNA was then eliminated by treatment with Amplification Grade DNase I (Invitrogen), and RNA was further purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) RNA clean-up protocol.

Reverse transcription to generate cDNA was carried out with 4 μg total lung RNA, using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Cytokine and chemokine cDNA was quantified using specific primers and FAM dye-labeled TaqMan probes designed by Applied Biosystems. Reactions were prepared using Taqman Universal PCR Master Mix (Applied Biosystems) and run in 96-well format on an iCycler (BioRad, Hercules, CA). Cytokine and chemokine gene expression was normalized to abundance of the Hprt1 gene, and the ΔΔCt method was used to calculate fold change in relative gene expression.

**Clodronate depletion**

Clodronate liposomes (CLs) were prepared as described previously and used within a week of preparation (21). Control PBS liposomes (PLs) were prepared according to the same protocol used for CLs, but liposomes were rehydrated in a molar-equivalent concentration of PBS instead of clodronate. For depletion of monocytes and macrophages, mice were injected i.v. with 0.2 ml CLs 24 h prior to infection with *B. mallei*. Control mice received 0.2 ml PLs i.v. 24 h prior to infection.

**Histological analysis**

Liver and spleen tissues were collected immediately after euthanasia and placed in 10 ml 10% neutral buffered formalin (Sigma-Aldrich) for 24 h. For lung histology, the lung lobe was first inflated with formalin via the trachea for 5 min prior to removal, then placed in formalin for 24 h. After 24 h, organs were transferred into a solution of 70% ethanol for 7 d. Tissues were then embedded in paraffin, sectioned, and stained with H&E. Tissues were examined and photographed by a veterinary pathologist experienced in mouse pathology (H. Bielefeldt-Ohmann).

**In vivo treatment with rIFN-γ**

Mice were injected i.p. with 1 × 105 U murine rIFN-γ (Peprotech, Rocky Hill, NJ) or diluent (PBS + 0.1% BSA). IFN-γ treatments were initiated at the time of infection and continued once daily for 5 d postinfection. This treatment protocol was adapted from one used previously in a C57Bl/6 mice, according to the manufacturer’s directions. Data acquisition was performed with a FACSscan flow cytometer, with data analyzed using CellQuest software (BD Biosciences) and FlowJo software (Tree Star).

**Statistical analysis**

Statistical analyses were performed using Prism 5.0 software (GraphPad, San Diego, CA). Survival times were analyzed by Kaplan–Meier analysis, followed by the log-rank test. Additional analyses used either a two-tailed Student *t* test or one-way ANOVA followed by a Tukey multiple means comparison test (comparison of more than two groups). Differences were considered statistically significant for *p* < 0.05.

**Results**

**MCP-1−/− and CCR2−/− mice are highly susceptible to inhaled *B. mallei* infection**

Prior studies in a systemic *L. monocytogenes* infection model demonstrated a critical role for CCR2, but not MCP-1, in controlling infection (13, 14). However, a role for MCP-1 or its receptor has not been previously investigated in animal models of acute pneumonia due to Gram-negative bacterial infection. To address these questions, we established a respiratory infection model of acute pneumonia infection with *B. mallei* in C57Bl/6 mice. In this model, infection of WT C57Bl/6 mice with a high-dose challenge (5000 CFU by i.n. administration) led to lethal infection within 3–4 d of inoculation (data not shown). To investigate factors that might regulate susceptibility to pulmonary challenge with *B. mallei*, we also developed a low-dose challenge model in C57Bl/6 mice, in which mice were challenged i.n. with 500 CFU (~0.5 LD50) *B. mallei*. In this model, infected mice developed early signs of pneumonia, but most recovered 2–3 d postinoculation (Fig. 1). It should also be noted that the majority of mice that recovered from low-dose challenge later succumbed to chronic infection of the spleen and liver, typically 45–60 d postinfection (Fig. 1). For example, in addition to mice euthanized because of progressive and clinically apparent chronic disease, determination of bacterial burdens revealed that 33.3% of asymptomatic mice surviving to day 60 had chronic infection of the spleen (data not shown). Therefore, 55% of mice surviving acute infection developed some form of chronic disease by day 60.

To determine whether MCP-1 played a role in regulating susceptibility to *B. mallei* pneumonia, we subjected WT and MCP-1−/− mice (*n* = 5 per group) to low-dose respiratory challenge with 500 CFU *B. mallei*. We found that MCP-1−/− mice all developed rapid and overwhelming infection within 3–4 d of challenge, whereas 90% of WT mice survived the same challenge dose (Fig. 1). These results indicated clearly that MCP-1 played a critical role in generating protective immunity to *B. mallei* pulmonary infection.

We wished next to determine whether the chemokine receptor CCR2 also figured importantly in regulating immunity to *B. mallei* challenge. Although CCR2 is considered the primary biologically relevant receptor for MCP-1, it is known that MCP-1 can also bind to other receptors in addition to CCR2, including CCR3 and CCR4 (23–26). Furthermore, CCR2 can bind ligands other than MCP-1, including MCP-2, MCP-3, and MCP-5 (27, 28). The relevance of this chemokine and receptor complexity was demonstrated clearly in the *Listeria* infection model, in which it was found that CCR2−/− mice were significantly more susceptible to infection than were MCP-1−/− mice (14). Moreover, increased numbers of monocytes were retained in the bone marrow of CCR2−/− mice when compared with MCP-1−/− mice (13).

Thus, it was important to determine in the *B. mallei* infection model whether, in fact, CCR2 was the key MCP-1 receptor regulating resistance to infection. We compared the susceptibility of MCP-1−/− and CCR2−/− mice to infection with a low-dose...
challenge with *B. mallei*. Compared with WT mice, CCR2<sup>−/−</sup> mice were significantly (*p* < 0.01) more susceptible to infection (Fig. 1). Importantly, however, and unlike the case with *Listeria* infection, MCP-1<sup>−/−</sup> mice were as susceptible as CCR2<sup>−/−</sup> mice to low-dose *B. mallei* infection (Fig. 1). Thus, in the *B. mallei* pneumonia model, MCP-1 expression and CCR2 expression were equivalent in terms of regulating resistance to infection.

*B. mallei* and *B. pseudomallei* are genetically similar, and the pathogenesis of disease caused by infection with these two organisms is thought to be very similar. Therefore, we also assessed the susceptibility of CCR2<sup>−/−</sup> mice to low-dose respiratory infection with *B. pseudomallei*. However, when CCR2<sup>−/−</sup> mice (*n* = 5 per group) were challenged i.n. with 1500 CFU (∼0.5 LD<sub>50</sub>) of *B. pseudomallei*, we found that all of the challenged mice survived (data not shown), whereas an equivalent infectious dose (∼0.5 LD<sub>50</sub>) was rapidly lethal in 100% of *B. mallei*-challenged CCR2<sup>−/−</sup> mice (Fig. 1). In addition, we noted that C57Bl/6 WT mice were equally susceptible to infection with *B. pseudomallei* and *B. mallei* (data not shown), indicating that the background strain of mouse could not explain the differences observed in the CCR2<sup>−/−</sup> mice. Therefore, important differences appeared to exist between these two strains of *Burkholderia* with respect to the role of MCP-1 in controlling pneumonic infections.

MCP-1<sup>−/−</sup> and CCR2<sup>−/−</sup> mice are unable to control bacterial replication in the lungs or systemic tissues

To investigate the mechanisms underlying the extreme susceptibility of MCP-1<sup>−/−</sup> and CCR2<sup>−/−</sup> mice to infection with *B. mallei*, bacterial burdens in lung, liver, and spleen were assessed in mice 72 h after low-dose i.n. infection. Bacterial burdens were significantly increased in both MCP-1<sup>−/−</sup> and CCR2<sup>−/−</sup> mice compared with WT mice (Fig. 2). Notably, even within 3 d of infection, bacterial burdens were extremely high in the spleens and livers of MCP-1<sup>−/−</sup> and CCR2<sup>−/−</sup> mice. However, bacterial burdens in MCP-1<sup>−/−</sup> mice were not significantly different from those in CCR2<sup>−/−</sup> mice (*p* = 0.39 for lung; *p* = 0.78 for liver; *p* = 0.20 for spleen). Thus, MCP-1 production and signaling via the CCR2 receptor markedly increased the resistance of mice both to *B. mallei* infection in the lungs and to dissemination and replication in extrapulmonary sites.

MCP-1<sup>−/−</sup> and CCR2<sup>−/−</sup> mice develop marked organ disease following low-dose challenge with *B. mallei*

*B. mallei* has been shown previously to elicit marked pulmonary pathologic conditions in mice, when inhaled at high doses (11). However, in this case we wished to compare the pathologic conditions of WT mice with those of MCP-1<sup>−/−</sup> and CCR2<sup>−/−</sup> mice following a low-dose respiratory challenge. Therefore, mice (*n* = 5 per group) were infected i.n. with 500 CFU *B. mallei*, and tissues were collected for histological examination 72 h postinfection.

In *B. mallei*-infected WT mice, multifocal lesions were observed in the lungs, consisting of moderate to severe alveolar infiltration of neutrophils and macrophages, along with mild fibrin transudation and mild parenchymal and leukocytoclastic necrosis with variable occlusion of alveolar spaces (Fig. 3). Occasionally, bronchioles with luminal accumulation of leukocytes and cellular debris were observed, but the respiratory epithelium remained intact. A mild increase in neutrophils in the splenic red pulp was noted, and in the liver there were mild multifocal sinusoidal infiltrates of neutrophils and macrophages, with accompanying piecemeal hepatocyte necrosis.

Much more severe lesions were noted in the lungs of MCP-1<sup>−/−</sup> mice. There were multifocally coalescing and extensive areas of severe bronchopneumonia, with pronounced neutrophil and macrophage infiltration, along with severe fibrin transudation and parenchymal and leukocytoclastic necrosis (Fig. 3). Also noted were severe accumulations of cellular debris and degenerate leukocytes within the lumina of bronchi and bronchioles, with scattered areas of epithelial degeneration, necrosis, and exfoliation. Marked perivascular edema, severe endothelial hypertrophy and transendothelial leukocyte migration, and frank vasculitis in multiple regions of the lung were present as well. In the spleen, the white pulp was mildly to moderately lymphocyte depleted, and there was moderate accumulation of neutrophils in the red pulp. In the liver, there was multifocal moderate hepatocyte necrosis accompanied by mild to moderate neutrophil and macrophage infiltration.

Lung lesions in CCR2<sup>−/−</sup> mice were similar in severity and extent to those noted in MCP-1<sup>−/−</sup> mice (Fig. 3). The spleens of CCR2<sup>−/−</sup> mice contained a marked accumulation of neutrophils in the red
pulp, causing expansion of the organ. Lesions in the liver of CCR2\(^{-/-}\) mice were similar to those noted for MCP-1\(^{-/-}\) mice.

Thus, the overall histological picture in MCP-1\(^{-/-}\) and CCR2\(^{-/-}\) mice infected with B. mallei, compared with that in WT mice, showed much more extensive formation of inflammatory lesions characterized by infiltration of large numbers of neutrophils and in some cases macrophages, with tissue destruction and necrosis. By immunohistochemical analysis, using a polyclonal Ab against the B. pseudomallei capsule (a kind gift from Dr. David Waag, U.S. Army Medical Research Institute of Infectious Diseases), organisms were observed within AVMs and in foci of inflammation in the lungs of WT mice, whereas in the lungs of CCR2\(^{-/-}\) mice, many more organisms were observed, both within infected cells and extracellularly (data not shown).

**Effects of Burkholderia infection on production of MCP-1**

The preceding results indicated that MCP-1 production was critical for generating protection from B. mallei infection. We conducted experiments to investigate the kinetics of MCP-1 production in the bloodstream, lungs, and spleen of infected mice. WT mice (n = 5 per group) were infected with B. mallei (500 CFU B. mallei delivered i.n.), then sacrificed at 24 h, 48 h, and 72 h postinfection, to assess MCP-1 concentrations. Concentrations of MCP-1 in plasma increased rapidly postinfection, with significant increases noted by 24 h postinoculation, then decreased as the infection was cleared and the mice recovered (Fig. 4). Production of MCP-1 in the lungs and spleen increased more slowly postinfection, with high concentrations first detected at 48 h and returning to baseline concentrations by 72 h (Fig. 4). Thus, pneumonic infection with B. mallei rapidly triggered circulating concentrations of MCP-1 in the bloodstream, followed 24 h later by MCP-1 production in the lung and spleen.

The amount of MCP-1 produced in response to B. mallei infection was also related to the challenge dose. For example, in the low-dose challenge model, transient production of low amounts of MCP-1 was observed, followed by a drop in MCP-1 concentrations as the infection was controlled. However, MCP-1 concentrations in mice subjected to high-dose B. mallei challenge (10^5 CFU i.n.) were significantly higher than in low-dose infected mice and remained elevated until the mice were euthanized because of progressive infection (data not shown).

Experiments were also done to assess the kinetics of replication of B. mallei after respiratory infection. Bacterial burdens in the blood, lungs, and spleen were determined following i.n. infection with 500 CFU B. mallei. As expected, B. mallei replicated rapidly in the lungs after respiratory challenge, but by 48 h the infection was controlled and bacterial counts began to decline (Fig. 4). Bacteria were undetectable in the bloodstream at this time point, and only low numbers of bacteria were detectable in the spleen.

Notably, however, in animals subjected to high-dose challenge, B. mallei replicated much more rapidly in the liver and spleen, with bacterial titers increasing by a factor of almost 7-fold from the time of challenge until euthanasia 3 d later (data not shown). Interestingly, the magnitude of B. mallei bacteremia was less than expected, given the rapid dissemination to liver and spleen. In fact, B. mallei was virtually undetectable in the bloodstream until 48 h postinfection, despite the fact that the liver and spleen already contained large numbers of bacteria by 24 h postinoculation (data not shown). These results indicated that high-level bacteremia was not necessary for efficient dissemination of B. mallei to extrapulmonary organs. Moreover, once the organism reached organs, such as the liver and spleen, replication was essentially unchecked in the first few days postinfection, even in immunologically intact animals.

**Airway inflammatory cell responses to B. mallei infection in WT and CCR2\(^{-/-}\) mice**

Given that MCP-1 is a key regulator of monocyte mobilization and recruitment, cellular inflammatory responses to B. mallei infection in the lungs were assessed next. WT and CCR2\(^{-/-}\) mice (n = 5–6 per group) were subjected to low-dose challenge with B. mallei, and 48 h postinfection, airway cells were collected by BAL and lung cells were obtained by enzymatic digestion of lung tissue. BAL and lung cells were immunostained and analyzed by multicolor flow cytometry, to evaluate monocytes, neutrophils, DCs, AVMs, T cells, and NK cells.

Infection with B. mallei led to a marked inflammatory response in the lungs of infected mice, both WT and CCR2\(^{-/-}\). Infection induced a large influx of neutrophils, myeloid DCs, and monocytes into the lungs, whereas little effect was seen on the numbers of NK cells, T cells, and AVMs (Fig. 5, Supplemental Fig. 1). In addition, total cell counts in the airways were not significantly different between WT and CCR2\(^{-/-}\) mice (data not shown). We observed a striking decrease in monocytes in the airways and lung parenchyma of CCR2\(^{-/-}\) mice compared with WT mice (Fig. 5). Moreover, there were significantly fewer DCs (CD11b\(^{+/+}\)/CD11c\(^{+/+}\)) in the BAL and lungs of B. mallei-infected CCR2\(^{-/-}\) mice compared with infected WT mice (Fig. 5).
Although no differences were observed in AVMs in the BAL or lungs of WT and CCR2−/− mice, the CCR2−/− mice had significantly more neutrophils in the lung parenchyma than did the WT mice (Fig. 5). There were no differences in NK cells in the BAL or the lung, and although similar levels of T cells were seen in the BAL, significant increases in both CD4 and CD8 T cells were noted in the lungs of WT mice (Supplemental Fig. 1). Thus, lack of CCR2 expression appeared to primarily affect monocyte, inflammatory DC, and neutrophil recruitment and accumulation in the lungs and airways following B. mallei infection. Interestingly, neutrophil recruitment was increased in the lungs of CCR2−/− mice compared with WT mice (Fig 5). This increase in neutrophils in the lungs of CCR2−/− mice is most consistent with an intact neutrophil response to the increased bacterial burden present in the lungs of CCR2−/− mice (Fig 2). Although both CD4 and CD8 T cells were reduced in the lungs of CCR2−/− mice, no differences were seen in NK cells, which are the main producers of protective cytokines during the early innate immune response to acute Burkholderia infection (29, 30).

**Effects of monocyte depletion on protection from B. mallei infection**

MCP-1 plays a key role in regulating release of monocytes from the bone marrow and directing their recruitment to sites of inflammation (12). The preceding experiments revealed decreased DC and monocyte recruitment to the lungs of B. mallei-infected mice. However, the reduction in monocyte and DC recruitment to the airways may not have been directly responsible for the inability of CCR2−/− mice to control B. mallei infection, because these mice may have other defects in innate immune responses. To address this question in a different manner, we directly depleted monocytes from the bone marrow of WT mice, using i.v. injection of CLs (31–33). Treatment with CLs has been shown previously to transiently deplete inflammatory monocytes from the bone marrow and blood of mice (32). Thus, the CL depletion technique allowed us to assess the effects of transient monocytepenia on the ability of mice to control pulmonary infection with *B. mallei*.

WT mice (*n* = 4 or 5 per group) were pretreated by i.v. administration of either CLs or control PLs. Injection of CLs resulted in a transient 60–70% reduction in numbers of circulating monocytes (data not shown). Twenty-four hours postinjection, mice were subjected to low-dose *B. mallei* i.n. challenge. We found that mice pretreated with CLs had significantly decreased survival times, compared with mice pretreated with PLs (Fig. 6). In addition, pre-treatment with CLs also resulted in significantly increased bacterial counts in lungs, spleens, and livers of CL-treated mice compared to mice receiving PLs (Fig. 6). These data provided additional evidence for a critical role for monocytes in generating early protective immunity against pneumonic *B. mallei* infection and in controlling dissemination from the lungs to extrapulmonary sites.

**CCR2−/− mice are impaired in their ability to produce critical antibacterial cytokines**

The preceding experiments indicated that MCP-1−/− and CCR2−/− mice were both markedly impaired in their ability to mount effective innate immune responses against *B. mallei* infection. To determine whether the extreme susceptibility of these mice to infection could also have resulted from dysregulation of key antibacterial effector mechanisms, we compared proinflammatory cytokine and NO responses in the lungs of WT and of MCP-1−/− and CCR2−/− mice following low-dose infection with *B. mallei*. WT, MCP-1−/−, and CCR2−/− mice were subjected to low-dose i.n challenge, and BAL and lung tissues were collected 48 h later. BAL fluid and lung homogenates were analyzed by CBA, ELISA, quantitative real-time-PCR (QRT-PCR), and biochemical assays.

Cytokine ([IFN-γ, TNF-α, and IL-12]) and NO responses to *B. mallei* infection were compared in the BAL and lung homogenates of infected WT and CCR2−/− mice 48 h postinfection (Fig. 7). Interestingly, CCR2−/− mice actually mounted significantly stronger TNF-α and KC responses in the airways and lung parenchyma than did WT mice following *B. mallei* infection (Fig. 7). In contrast to the findings in CCR2−/− mice, TNF-α and KC levels in the BAL fluid...
of MCP-1<sup>−/−</sup> mice were not significantly different from those in WT mice (Supplemental Fig. 2).

The most dramatic cytokine differences were observed when IFN-γ and IL-12 responses in the airways and lungs were compared. Both MCP-1<sup>−/−</sup> and CCR2<sup>−/−</sup> mice, compared with WT mice, were markedly impaired in their ability to produce IFN-γ in response to infection (Fig. 7, Supplemental Fig. 2). Moreover, the IL-12 re-
sponses in the airways of both MCP-1<sup>−/−</sup> and CCR2<sup>−/−</sup> mice were also significantly impaired. IL-12 responses in the lungs of CCR2<sup>−/−</sup> mice were reduced; however, the difference did not reach the level of statistical significance (<i>p</i> = 0.1) (Fig. 7, Supplemental Fig. 2). Thus, the inability to produce or respond to MCP-1 resulted in markedly attenuated IFN-γ responses to <i>B. mallei</i> infection, whereas TNF-α and KC responses were either increased (CCR2<sup>−/−</sup> mice) or unchanged (MCP-1<sup>−/−</sup> mice).

The ability of MCP-1<sup>−/−</sup> and CCR2<sup>−/−</sup> mice to mount an NO response to <i>B. mallei</i> infection was also assessed. Samples of BAL and lung homogenate collected 48 h after <i>B. mallei</i> infection were analyzed to assess total NO production. Despite the differences in IFN-γ production, we did not observe differences in NO production in either the BAL or the lung homogenates in CCR2<sup>−/−</sup> or MCP-1<sup>−/−</sup> mice compared with WT mice (data not shown).

The effects of pneumonic <i>B. mallei</i> infection on expression of other relevant cytokines and chemokines in the lungs were also examined 48 h postinfection, using QRT-PCR. Levels of gene expression in lung tissues of the following cytokines and chemokines were compared in WT and in MCP-1<sup>−/−</sup> and CCR2<sup>−/−</sup> mice (<i>n</i> = 4 mice per group): IFN-β, IFN-α, MIP-2α, CXCL1, and MIP-1. A significant decrease in the expression of MIP-2α (RANTES) mRNA in the lungs of CCR2<sup>−/−</sup> mice was noted, whereas no significant differences were observed in other cytokines measured (data not shown). Thus, lack of MCP-1 expression or inability to respond to MCP-1 affected certain components of the innate immune response to <i>B. mallei</i> infection, most notably IFN-γ, while leaving other innate immune responses intact.

Discussion

Despite the importance of <i>B. mallei</i> as a zoonotic pathogen and potential bioweapon agent, little is known regarding key early protective immune responses to infection with this organism. The studies presented in this study provide vital new information about the pathogenesis of pneumonic <i>B. mallei</i> infection and identify a critical protective role for MCP-1 in the early innate immune response to this pathogen. It is also noteworthy that the antibacterial effects of MCP-1 appeared to be mediated through regulation of IFN-γ production, as well as by stimulating monocyte and DC recruitment to the airways. In addition, MCP-1 and CCR2 were found to be important for controlling the dissemination of <i>B. mallei</i> from the lungs to extrapulmonary sites following initial pneumonic infection.
The role of MCP-1 and CCR2 in immunity to bacterial infection has been investigated previously in three different infection models. In the *L. monocytogenes* parenteral infection model, CCR2 expression was a critical regulator of early protective innate immune responses to infection, whereas MCP-1 expression had a less important role (13, 14). In the *L. monocytogenes* model, the infection route was i.p., and the primary target organ for bacterial infection was the spleen (34, 35). In mice infected with *Listeria*, CCR2 expression was found to be necessary for recruitment of a population of DCs to the spleen that produced high levels of TNF-α and inducible NO synthase (iNOS) and generated anti-bacterial immunity (34). In contrast to the *Listeria* infection model, in a low-dose inhalational *M. tuberculosis* challenge model, CCR2 expression was found to play no role in regulating resistance to low-dose challenge (36). However, in a high-dose inhalational *M. tuberculosis* challenge model and in an i.v. challenge model, CCR2 expression was found to be important for protective immunity (16, 37). Finally, respiratory infection with *Streptococcus pneumoniae* in MCP-1−/− mice also resulted in increased bacterial burden and lung abnormalities. Similar to the findings in our study, MCP-1−/− mice were found to have decreases in DCs and increases in neutrophils. In addition, TNF-α and KC production was equivalent or increased in MCP-1−/− mice compared with WT mice, although IFN-γ production and cytokine levels in CCR2−/− mice were not investigated (38).

To our knowledge, the studies presented in this study are the first to assess the role of MCP-1 in a model of acute pulmonary infection with a Gram-negative bacterium. In contrast to results obtained in *Listeria* studies, in which TNF-α responses were significantly diminished in the spleens of *L. monocytogenes*-infected CCR2−/− mice, TNF-α responses in the lungs of CCR2−/− mice infected with *B. mallei* were preserved. Although increased TNF-α concentrations in *B. mallei*-infected CCR2−/− mice could be due to the increased bacterial burdens seen in these mice, TNF-α levels were still significantly increased in CCR2−/− mice compared with WT mice when cytokine levels were normalized to bacterial burden (data not shown). Despite the increased bacterial burden in CCR2−/− mice, we found that IFN-γ responses were markedly reduced in the lungs of CCR2−/− mice infected with *B. mallei*. In contrast, IFN-γ responses were maintained in the spleens of CCR2−/− mice infected with *L. monocytogenes* (34). Thus, we postulated that the loss of IFN-γ production in the lungs of *B. mallei*-infected CCR2−/− mice was one of the key immunological mechanisms underlying the extreme susceptibility of these mice to infection. In support of this idea, we found that treatment with exogenous IFN-γ could significantly restore resistance to *B. mallei* infection in CCR2−/− mice.

The differing roles of MCP-1 and CCR2 in regulating resistance to infection have also been observed previously in a *Leishmania* infection model (39, 40). In addition, MCP-1 has been shown to serve an important protective function in protozoal and fungal infections. For example, CCR2−/− mice were found to be more susceptible to infection with *Toxoplasma gondii* (41). Increased susceptibility to *Toxoplasma* infection correlated with reduced recruitment of Gr-1− monocytes to the peritoneum postinfection (41, 42). The recruited monocytes were found to contribute to killing of *T. gondii* in an NO-dependent manner. Interestingly, however, *Toxoplasma*-infected CCR2−/− mice did not manifest a defect in production of IFN-γ or TNF-α. In a model of pulmonary infection with the fungal organism *Cryptococcus neoformans*, CCR2−/− mice also had increased susceptibility to infection, which was associated with decreased macrophage recruitment to the lungs (43). Thus, CCR2 and MCP-1 appear to be significant in controlling infection with several different pathogens, all of which share the common feature of being intracellular organisms. However, as noted above, it is also apparent that lack of CCR2 expression impairs innate and adaptive immune responses to varying degrees, depending on the infecting organism, the site of infection, and the size of the challenge dose and route of infection (44).

Whereas little is known regarding innate or adaptive immune responses to *B. mallei*, more is known about the closely related pathogen *B. pseudomallei*. Control of *B. pseudomallei* infection previously was shown to depend on production of IFN-γ and TNF (7, 8). Interestingly, however, and in agreement with our results, in vivo control of *B. pseudomallei* infection in C57Bl6 mice was found to be largely independent of iNOS expression (10). Neutrophils were also shown to be instrumental in controlling pulmonary infection with *B. pseudomallei* (9). The role of neutrophils in response to *B. mallei* infection, however, has not been investigated. In this study, we observed that CCR2−/− mice had increased susceptibility to respiratory infection with *B. mallei*, but not *B. pseudomallei*, and that CCR2−/− mice had significant increases in lung neutrophils when compared with WT mice. These results suggest a differential role for neutrophils in response to infection with these two closely related bacteria. Although neutrophils may be sufficient to protect CCR2−/− mice from *B. pseudomallei* infection, neutrophils recruited in response to *B. mallei* infection of CCR2−/− mice were not capable of controlling infection. Other studies, however, have shown that depletion of monocytes and macrophages, using the CL injection technique, resulted in increased susceptibility of mice to *B. pseudomallei* infection following i.p. challenge (10). The results of our studies in CCR2−/− mice are therefore consistent with the idea that recruited monocytes and inflammatory DCs served a key protective function in the lungs against *B. mallei* infection, possibly by acting as a source of IFN-γ (9).

Monocytes recruited to sites of infection may play several roles in controlling bacterial infections. In the *Listeria* infection model, a subset of DCs thought to be derived from inflammatory monocytes were largely responsible for controlling bacterial infection by producing TNF-α and iNOS (34). In fact, inflammatory monocytes are believed to be the principal precursor for certain types of DCs in several sites, including the skin, draining lymph nodes, spleen, and lung (12, 31, 32, 45). In the present study, we observed that CCR2−/− mice were significantly impaired in their ability to recruit both monocytes and inflammatory myeloid DCs, but not neutrophils, into their lungs in response to *B. mallei* infection (Fig. 5). Within the lungs and airways, inflammatory monocytes and DCs may suppress bacterial replication in infected target cells by producing antibacterial cytokines, such as TNF-α and IFN-γ. For
example, we showed recently that in vitro treatment of infected AVMs (a primary target for early B. mallei infection in vivo) with IFN-γ significantly suppressed intracellular replication of B. mallei (46). Inflammatory monocytes may produce IFN-γ directly, as noted previously, or they may indirectly induce production of IFN-γ by other cells in the lung, especially NK cells (9).

The lack of IFN-γ production in the lungs of CCR2−/− mice in response to B. mallei infection could reflect failure of recruitment of IFN-γ-producing cells to the lungs or may instead reflect a more general impairment in the ability of CCR2−/− mice to mount IFN-γ responses. The latter explanation is less likely, however, inasmuch as we found that CCR2−/− mice produced strong IFN-γ responses (equivalent to those in WT mice) when challenged in vivo with cationic liposome-DNA complexes, which are potent inducers of IFN-γ production (data not shown and [47]).

The importance of IFN-γ was also demonstrated in experiments in which IFN-γ treatment was sufficient to provide CCR2−/− mice with long-term protection against B. mallei infection (Fig. 8). In contrast to WT mice, of which 55% developed chronic disease by day 60, however, 100% of surviving IFN-γ-treated CCR2−/− mice developed chronic disease by day 60. These results suggest that although IFN-γ is sufficient to provide protection against acute disease, monocytes may have additional important functions in the development of adaptive immune responses necessary for control of chronic B. mallei infection.

We conclude, therefore, that MCP-1 and CCR2 are key components of pulmonary innate immune responses to infection with a highly virulent Gram-negative pathogen, such as B. mallei. The effects of MCP-1 and CCR2 appear to be mediated through recruitment of key antibacterial effector cells, principally monocytes and DCs, to sites of bacterial infection. These findings suggest that therapeutic recruitment of monocytes and DCs into the airways may serve as an effective means of generating rapid protection against respiratory exposure to pathogenic bacteria. Indeed, treatment with MCP-1 or therapeutics that induce monocyte recruitment has proved to be effective against bacterial pathogens (48–50). In addition, we have recently found that respiratory delivery of a potent immunotherapeutic, cationic liposome-DNA complex, can protect mice from lethal infection with both B. mallei and B. pseudomallei (46). Finally, it will also be important to determine more generally the role of MCP-1 and inflammatory monocytes in controlling infections, not only with intracellular bacterial pathogens but also with extracellular pulmonary pathogens, such as Staphylococcus and Pseudomonas.

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Disclosures

The authors have no financial conflicts of interest.

References

**Supplemental Figure 1. Lymphocyte responses in the airways and lung parenchyma of wild type and CCR2<sup>±</sup> mice following B. mallei infection.** Wild type and CCR2<sup>±</sup> mice (n = 5 per group) were subjected to low-dose respiratory challenge with B. mallei, as described in Methods. After 48 hours, the mice were sacrificed and BAL and lung cells were collected, counted, and immunostained as described in methods. Cell populations were identified as follows: NK Cells CD45<sup>+</sup>/ NK-1.1<sup>+</sup>; CD8<sup>+</sup> T cells: Low FSC and SSC/CD8<sup>+</sup>; CD4 T Cells: Low FSC and SSC/CD4<sup>+</sup>. (A) and (B). Graphical representation of cell populations in the BAL (A) and lung parenchyma (B). Data are plotted as the mean ± SEM of each cell population’s percentage of all live cells. Data are representative of two independent experiments. Statistical differences between wild type and CCR2<sup>±</sup> mice were determined using a two-tailed Students t-test (** = p < 0.01)

**Supplemental Figure 2. Cytokine responses in the BAL of B. mallei infected wild type and MCP-1<sup>-</sup> mice.** Mice (n = 3-4 per group) were subjected to low-dose respiratory challenge with B. mallei, and then sacrificed at 48 hours after infection. Airway lavage samples were collected for analysis of TNF-α, IFN-γ, IL-12p40 and KC concentrations, as described in Methods. The mean cytokine concentrations were compared statistically between groups using a two-tailed Students t-test. (* = p < 0.05 between WT and CCR2<sup>-</sup> values). Data were pooled from two independent experiments.