Infection Improves Survival during Influenza Infection

Markedly Pulmonary Immune Pathology and CCR2-Antagonist Prophylaxis Reduces

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Infection with influenza virus induces severe pulmonary immune pathology that leads to substantial human mortality. Although antiviral therapy is effective in preventing infection, no current therapy can prevent or treat influenza-induced lung injury. Previously, we reported that influenza-induced pulmonary immune pathology is mediated by inflammatory monocytes trafficking to virus-infected lungs via CCR2 and that influenza-induced morbidity and mortality are reduced in CCR2-deficient mice. In this study, we evaluated the effect of pharmacologically blocking CCR2 with a small molecule inhibitor (PF-04178903) on the entry of monocytes into lungs and subsequent morbidity and mortality in influenza-infected mice. Subcutaneous injection of mice with PF-04178903 was initiated 1 d prior to infection with influenza strain H1N1A/Puerto Rico/8/34. Compared with vehicle controls, PF-04178903–treated mice demonstrated a marked reduction in mortality (75 versus 0%) and had significant reductions in weight loss and hypothermia during subsequent influenza infection. Drug-treated mice also displayed significant reductions in bronchoalveolar lavage fluid total protein, albumin, and lactose dehydrogenase activity. Administration of PF-04178903 did not alter viral titers, severity of secondary bacteria infections (Streptococcus pneumoniae), or levels of anti-influenza–neutralizing Abs. Drug-treated mice displayed an increase in influenza nucleoprotein-specific cytotoxic T cell activity. Our results suggest that CCR2 antagonists may represent an effective prophylaxis against influenza-induced pulmonary immune pathology.
pharmacological versus genetic manipulation of CCR2 activity remained to be evaluated directly in vivo.

Monocyte-derived cells play important roles in innate responses to pathogens, as well as in the polarization and expansion of lymphocytes (21). This raises concerns that CCR2 inhibition may result in decreased immune responses to influenza infection. Published studies disagree as to whether CCR2-deficient mice have increased viral titers after influenza infection (11, 20). These mice are also more susceptible to some bacterial infections (9, 10). Therefore, influenza viral titers and bacterial load in the lungs after secondary bacteria infections were examined in this study to assess possible side effects caused by CCR2 inhibitor treatment. Viral-neutralizing Abs and CTL responses were also assessed, because they make up two of the most effective arms of immunity against influenza virus (2).

In this study, we administered a small-molecule CCR2 inhibitor (PF-04178903) in an attempt to modulate lung injury during influenza infection in a murine model. We used the H1N1 A/Porto Rico/8/34 (PR8) strain to mimic the lung injury seen during highly pathogenic flu infection. This strain causes high mortality in mice, induces severe immune pathology, and localizes to the lungs. We found that PF-04178903-treated mice had reduced pathology, morbidity, and mortality during influenza infection when the drug was administered prior to infection; this prophylaxis did not impact immune responses against influenza virus or secondary bacterial infections.

Materials and Methods

Mice, drug treatment, and influenza infection

C57BL/6 mice and CD45.1 mice were purchased from Charles River Laboratories (Wilmington, MA) or The Jackson Laboratory (Bar Harbor, ME). All mice used in these experiments were 10–14 wk-old females. The CCR2 antagonist used for these studies (1,5-anhydro-2,3-dideoxy-3-[(1R,3S)-3-isopropyl-3-({4-[4-(trifluoromethyl)pyridin-2-yl]piperazin-1-yl}carbonyl)cyclopentyl]laminoo)-4-O-methylpentitol) was provided by Pfizer (Groton, CT) and is designated PF-04178903. This compound exhibits nanomolar potency in ligand-competition binding or in vitro chemotaxis assays against murine CCR2, with ~10-fold less potency observed for murine CCR5 in comparable assays. Prior to use, compound was dissolved in PBS at 8.3 mg/ml and injected s.c. twice daily at a dose of 10 mg/kg. For flow-cytometric initiation of compound dosing with ketamine (100 mg/kg)/xylazine (10 mg/kg), mice were anesthetized with 1% isoflurane in 90% O2/10% CO2 for 30 min. Mice were injected into recipient C57BL/6 mice that had been infected with influenza virus (2). Cells were analyzed using a BD LSRII flow cytometer.

Total BAL protein, albumin concentration, and lactate dehydrogenase activity

Influenza-infected mice were treated with PF-04178903 starting at day −1 and were sacrificed on day 5, 7, or 9 along with PBS-injected control mice. Three microliters of BAL fluid was obtained, as described above, and cells were removed by centrifugation. Protein concentrations in the supernatant fluid were determined via Bradford assay (Pierce, Rockford, IL), according to the manufacturer’s instructions. Albumin concentrations in BAL fluid were measured using a mouse albumin ELISA kit (Invitrogen, Carlsbad, CA). Lactate dehydrogenase (LDH) activities in BAL fluid were measured using an LDH-based toxicity assay kit (Sigma-Aldrich, St. Louis, MO).

Viral-titer measurements

At selected times postinfection, lungs from control or influenza-infected mice were perfused with PBS and homogenized by rubbing lung tissues between frosted microscope slides (Fisher Scientific, Pittsburgh, PA). Influenza viral titers in lung homogenates were quantified by viral-plaque assay (16). Briefly, lung homogenates were serially diluted in PBS containing Ca2+ and Mg2+ and 0.1% BSA, plated on confluent monolayers of MDCK cells, and allowed to adsorb for 1 h at 37˚C in a tissue-culture incubator. Inocula were then removed, and the monolayer was overlaid with 1× MEM-containing agar and TPCK trypsin (Sigma-Aldrich) at a final concentration of 0.1 μg/ml. Plates were incubated for 2 d in a tissue-culture incubator (37˚C, 5% CO2) to allow plaques to form. When plaques were clearly visible, agar was removed, and the plates were stained with 1% crystal violet in methanol to aid in enumeration of PFU.

Secondary bacterial pneumonia infection assays

Type 3 Streptococcus pneumoniae (ATCC 6303) was rehydrated and grown in Bacto Todd-Hewitt broth (BD Biosciences, San Jose, CA) overnight at 37˚C. One milliliter of overnight culture was diluted 1:10 in fresh media and incubated for 6 h at 37˚C to attain log phase. On day 5 following infection (day 6 after initiation of PF-04178903 treatment), mice were anesthetized by i.p. injection of ketamine and xylazine and inoculated with 105 CFU S. pneumoniae intranasally. Two days after S. pneumoniae inoculation (day 7), mice were euthanized, and their lungs were harvested and homogenized as described above for viral PFU assays. Lung homogenates were serially diluted 1:10 and plated on Columbia agar with 5% sheep blood (BD Biosciences) to determine lung bacteria CFU.

Serum viral-neutralizing Ab titers

Mice were treated with PF-04178903 starting on day −1 and were infected with influenza on day 0. Serum samples were collected on days 21 and 28, heat-inactivated, serially diluted 10-fold in MEM containing 5% BSA into 96-well plates, and incubated with influenza virus (10 TCID50/ml) for 1 h at 37˚C. MDCK cells (105) were added to each well, and the plates were incubated at 37˚C. Culture media were changed 48 h later to MEM with 2% BSA and were incubated for 72 h longer. Next, 0.5% chicken RBCs were added to all wells. Plates were incubated at 4˚C for 1 h and observed for agglutination. The serum viral-neutralizing titer is defined as the reciprocal of the highest dilution of serum at which wells show no agglutination of chicken RBCs.

In vivo CTL assays

CTL assays were performed as previously described (23), with some modifications. Briefly, target cells were prepared by lysing RBCs from naive CD45.1 splenocytes. The remaining cells were washed and split into two populations. One population was pulsed with 2 × 105 M influenza membrane peptide (NP: 366–374) (Anspec, San Jose, CA) incubated at 37˚C for 45 min, and labeled with 4 μM CFSE (CFSElow cells). The second control target population was pulsed with control OVA peptide and labeled with 0.4 μM CFSE (CFSEhigh cells). For i.v. injection, 4 × 107 cells from each population were mixed together in 200 μl PBS and injected into recipient C57BL/6 mice that had been infected with influenza PR8 virus 9 d earlier and treated with PF-04178903 or vehicle control. Six hours later, recipient mice were sacrificed, and mediastinal lymph nodes (LNs) and spleens were harvested. Cell suspensions were analyzed by flow cytometry, and each target population was identified by its specific CFSE fluorescence intensity. Up to 4000 CD45.1+CFSE+ cells were collected for analysis.
analysis. To calculate specific lysis, the following formula was used:
percentage specific lysis = \[\frac{1}{2} \left( \frac{\text{ratio influenza nucleoprotein peptide primed/ratio OVA primed}}{100} \right)\].

Statistics
All numerical data are presented as mean ± SD. The comparison between survival curves was performed with the log-rank test using GraphPad Prism software (GraphPad, San Diego, CA). This test is equivalent to the Mantel–Haenszel test. All other data were analyzed by ANOVA or the unpaired Student t test using Prism software, as indicated in the figure legends.

Results
Inhibition of influenza-induced cell accumulation in the lungs of mice by PF-04178903
The decrease in lung inflammation and mortality seen in CCR2-deficient mice during influenza infection (16, 20) motivated us to investigate whether the murine-reactive, small-molecule CCR2-antagonist PF-04178903 could reduce monocytic cell infiltration into influenza-infected lungs and, ultimately, decrease the mortality in infected mice. To test the effect of the drug on reducing monocytic cell infiltration, mice were infected with PR8 (H1N1) and then injected with PF-04178903 starting on day 0. On day 5, lungs of infected mice were harvested and subjected to flow-cytometric analysis. The cell populations in BAL and lung digests were gated as in our previous publication (16) and are shown in Fig. 1A–H. The forward- and side-scatter characteristics of the various cell populations are shown in Supplemental Fig. 1.

As anticipated, PF-04178903–treated mice exhibited a marked reduction in the percentage of inflammatory (Gr-1+) monocytes present in BAL and lung parenchyma (Fig. 1C,1D). This was mirrored by a similar reduction in CD11b+Gr-1+CD11cintMHCIIint double-intermediate (DI) cells (Fig. 1E,1F), CD11cMHCIIhi DCs

FIGURE 1. Monocytes, DCs, and CD11b+Gr-1+ DI cells are decreased in the lungs of PF-04178903–treated mice during influenza infection. Mice were treated with PF-04178903 or PBS starting immediately after virus inoculation. On day 5 of influenza infection, mice were sacrificed. Cells from BAL (A, C, E, G) and lung digests (B, D, F, H) were harvested from PF-04178903–treated and PBS-treated mice and analyzed by flow cytometry. Results are from individual mice representative of two separate experiments. Numbers represent the percentage of cells within the gates. Total cell numbers (per mouse) of individual cell types obtained from BAL (I) or lung digests (J) of PF-04178903–treated and PBS-treated mice were calculated. Bars represent the mean ± SD for three mice per group. *p < 0.05; **p < 0.005; ***p < 0.0005, Student t test. AM, alveolar macrophage; DI, CD11cintMHCIIint; DN, double-negative (CD11c−MHCII−) cells; MAC, total macrophages; mono, Gr-1− monocytes.
(Fig. 1A, 1B), and CD11c<sup>H</sup>MHCII<sup>lo</sup>CD11b<sup>+</sup> exMACs (Fig. 1A, 1B, 1G, 1H). The CD11b<sup>Gri-1<sup>-</sup></sup> cells in the DI gate are transitional cells that arise as inflammatory monocytes develop into DCs or macrophages and increase the expression of CD11c and MHC class II (16). In BAL fluid, PF-04178903 reduced the numbers of Gr1<sup>+</sup> monocytes, DCs, and CD11b<sup>Gri-1<sup>-</sup></sup> DI cells by 75% (Fig. 1I). In lungs, DC numbers were reduced 70%, and the numbers of Gr1<sup>+</sup> monocytes and CD11b<sup>Gri-1<sup>-</sup></sup> DI cells were reduced 80% in compound-treated mice (Fig. 1I). As expected, the numbers of neutrophils [PMNs, CD11b<sup>+</sup>CD11c<sup>−</sup>Ly6G<sup>+</sup>] and alveolar macrophages [CD11c<sup>+</sup>MHCII<sup>lo</sup>CD11b<sup>+</sup>] (Fig. 1A, 1G) were not significantly different between control and PF-04178903–treated mice (Fig. 1I, 1J). There was an observed trend toward a reduction in the number of exMACs in the lungs after PF-04178903 treatment (70%), but it did not reach statistical significance. The extent of reduction in the numbers of monocytes and monocyte-derived cells in PF-04178903–treated lungs (70–80%) was slightly lower than was seen in CCR2-deficient lungs (80–85%) (16). We also examined inflammatory cell populations in the lungs of mice treated with PF-04178903 beginning 1 d prior to influenza infection (day –1). We found no significant differences on day 3 between lung cell populations in mice treated with PF-04178903 on day –1 or on day 0 (data not shown).

CCR2 is required for the migration of inflammatory monocytes from the bone marrow into the blood and for the extravasation of these cells from the blood into tissues (24). In mice treated with PF-04178903 starting on day –1, the number of blood monocytes did not display the increase that typically occurs during influenza infection (Fig. 2). This finding suggests that the reduced accumulation of monocytes and monocyte-derived cells seen in the lungs of PF-04178903–treated mice is due, at least in part, to a reduction in inflammatory monocyte mobilization from the bone marrow.

**Inhibition of influenza-induced lung injury by PF-04178903**

The finding that CCR2-antagonist treatment reduced the number of inflammatory mononuclear cells in influenza-infected lungs suggested that it might also reduce lung injury after influenza infection. Initial studies examined prophylactic use of the CCR2 antagonist. Mice were injected with the drug 1 d before influenza infection (two injections before intranasal viral infection). On days 5 and 7 after influenza infection, BAL fluid was collected from control and PF-04178903–treated mice and assayed for markers of lung injury. On day 5, LDH activity and albumin concentrations in BAL fluid were significantly decreased in PF-04178903–treated mice (Fig. 3A, 3B), whereas on day 7, albumin and total protein concentration in BAL fluid were significantly decreased (Fig. 3B, 3C). These data indicated that PF-04178903 prophylaxis could effectively reduce pulmonary pathology in influenza-infected lungs. However, these parameters were not reduced in PF-04178903–treated mice to the full extent seen earlier in CCR2-deficient mice (16). CCR2-deficient mice averaged 60% reductions in total BAL protein and LDH activity on day 5 of influenza infection versus 40% (total BAL protein) and 50% (LDH activity) for PF-04178903–treated mice.

**Inhibition of influenza-induced morbidity and mortality by CCR2 antagonist**

To determine whether the prophylactic use of PF-04178903 could reduce morbidity and mortality in influenza-infected mice, they were injected with drug 1 d before influenza infection, twice per day until day 10 after viral inoculation. Mice were weighed, and rectal temperatures were measured daily until day 18. This dosing regimen resulted in no influenza-induced mortality, compared with 75% mortality seen in the vehicle-treated mice (Fig. 4A). Drug-treated mice also displayed a significant reduction in weight loss and hypothermia compared with control mice throughout the course of infection (Fig. 4A).

To determine whether PF-04178903 is effective in reducing mortality when given after influenza infection, mice were treated with PF-04178903 (PF) or PBS (Ctrl) starting 1 d before influenza inoculation. On days 3, 5, and 7 of influenza infection, mice were sacrificed, and their blood was collected and subjected to flow-cytometric analysis. A. The profile of CD115 versus Gr-1 staining of blood CD11b<sup>+</sup>Ly6G<sup>+</sup> cells is shown. B. Total cell number of blood inflammatory monocytes in PF-04178903–treated and Ctrl mice. Data are representative of two independent experiments. Bars represent mean ± SD for four mice per group. *p < 0.05, Student t test.

**FIGURE 2.** The number of inflammatory monocytes in the blood of control or PF-treated mice after influenza infection. Mice were treated with PF-04178903 (PF) or PBS (Ctrl) starting 1 d before influenza virus inoculation. On days 3, 5, and 7 of influenza infection, mice were sacrificed, and their blood was collected and subjected to flow-cytometric analysis. A. The profile of CD115 versus Gr-1 staining of blood CD11b<sup>+</sup>Ly6G<sup>+</sup> cells is shown. B. Total cell number of blood inflammatory monocytes in PF-04178903–treated and Ctrl mice. Data are representative of two independent experiments. Bars represent mean ± SD for four mice per group. *p < 0.05, Student t test.

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infected with influenza virus on day 0, and whole lungs from infected mice were collected and homogenized on days 3, 5, 7, and 9. As shown in Fig. 5A, lung viral titers peaked on day 5 and then rapidly decreased to almost undetectable levels on day 9 in PF-04178903–treated and control (PBS-injected) mice. Lung viral titers did not differ significantly between PF-04178903–treated and control mice at any time (Fig. 5A), demonstrating that prophylactic use of a CCR2 inhibitor does not impair viral clearance.

Although influenza infection can be lethal itself, many post-influenza deaths are caused by secondary bacterial pneumonias (25). To examine whether CCR2 inhibition aggravates secondary bacterial infection with S. pneumoniae, PF-04178903 treatment was started on day 21, mice were infected with a sublethal dose of H1N1 PR8 strain on day 0, and inoculated with S. pneumoniae intranasally on day 5. Two days later, lung homogenates of infected mice were assayed for bacterial burden. As previously reported (25, 26), influenza infection results in a marked increase in lung bacterial titers (Fig. 5B). However, there was no difference in bacterial burden between PF-04178903–treated and control mice (Fig. 5A), demonstrating that prophylactic use of a CCR2 inhibitor does not impair viral clearance.

Although influenza infection can be lethal itself, many post-influenza deaths are caused by secondary bacterial pneumonias (25). To examine whether CCR2 inhibition aggravates secondary bacterial infection with S. pneumoniae, PF-04178903 treatment was started on day −1, mice were infected with a sublethal dose of H1N1 PR8 strain on day 0, and inoculated with S. pneumoniae intranasally on day 5. Two days later, lung homogenates of infected mice were assayed for bacterial burden. As previously reported (25, 26), influenza infection results in a marked increase in lung bacterial titers (Fig. 5B). However, there was no difference in bacterial burden between PF-04178903–treated and control mice (Fig. 5B), showing that the use of a CCR2 antagonist in mice infected with a highly pathogenic influenza does not affect the severity of secondary bacterial infection.

**FIGURE 3.** Influenza-induced lung injury is reduced in PF-04178903–treated mice. Mice were treated with PF-04178903 (PF) or PBS (Ctrl) starting 1 d before influenza virus inoculation. On days 5 and 7 of influenza infection, mice were sacrificed, and their BAL fluid was collected and assayed for LDH activity (A), albumin concentration (B), and total protein concentration (C). The value of albumin in BAL fluid from naive mice is 150 μg/ml. The values of LDH activity and total protein concentration for naive BAL fluid are both near zero. Data are representative of two independent experiments. Bars represent the mean ± SD for four or five mice per group. The p values were calculated using the Student t test.

**FIGURE 4.** Influenza-induced morbidity and mortality are reduced by PF-04178903 prophylaxis. Mice were treated s.c. with PF-04178903 or PBS starting 1 d before (A) or immediately following (B) influenza inoculation and were treated twice daily until day 10. Mortality, weight loss, and rectal temperature were monitored daily until day 22. Data are representative of two independent experiments (n = 6–10 mice). The p value for the survival curve was calculated using the log-rank test. The p values for overall weight loss and temperature curves were calculated by repeated-measures ANOVA. *p < 0.05, Student t test comparing individual time points.

**CCR2-antagonist treatment does not decrease viral-neutralizing Ab titers or CTL responses**

CCR2-deficient mice, which have decreased numbers of LN monocyte-derived DCs, display decreased Th1 responses to infections (27–30). To determine whether CCR2 inhibition results in reduced adaptive immune responses to influenza, we examined anti-influenza–neutralizing Ab titers and CTL responses in PF-04178903–treated mice. Mice were infected with influenza on day 0 and treated with PF-04178903 from days −1 to 10. Sera were collected for Ab titers on days 21 and 28. As shown in Fig. 6A, levels of serum anti-influenza–neutralizing Abs were comparable in PF-04178903–treated and control mice, suggesting that CCR2 antagonism does not impact the production of viral-neutralizing Abs.

For in vivo CTL assays, recipient mice were treated with PF-04178903 and infected with influenza as above. On day 9, donor splenocytes were pulsed with viral nucleoprotein peptide or an unrelated peptide, the two cell populations were differentially labeled with CFSE, and the labeled cells were transferred to recipient mice. Six hours later, the mice were sacrificed, and the presence of donor cells in mediastinal LNs and spleens was assessed. A greater proportion of NP-pulsed (CSFEhi) cells was eliminated from the spleens (Fig. 6B) and mediastinal LNs (data not shown) in PF-04178903–treated mice than in control mice, leading to a significantly increased CTL activity in PF-04178903–treated mice (Fig. 6C). Interestingly, NP-specific CTL activity was higher in spleens than in mediastinal LNs (Fig. 6C), demonstrating that Ag-specific CD8+ T cells distribute systematically after generation, and this is not inhibited by CCR2 antagonism. We concluded that CCR2 inhibition with small-molecule inhibitors does
not reduce CTL activity against influenza virus but actually enhances it.

Discussion
Infection with highly pathogenic influenza virus causes significant pulmonary immune pathology. No drug has been reported to be effective in preventing or treating this pathology. In this report, we demonstrated that CCR2 inhibition in adult mice decreased influenza-induced pulmonary immune pathology and mortality. The small-molecule CCR2 inhibitor PF-04178903 used prophylactically to treat mice with severe influenza infection resulted in decreased recruitment/accumulation of monocyte-derived cells in the lungs and reduced lung injury as measured by LDH activity, albumin concentration, and total protein concentration in BAL fluid. PF-01478903 treatment also resulted in decreased weight loss, decreased hypothermia, and a 100% survival rate, a marked improvement compared with control mice. These results are consistent with earlier influenza infection studies conducted in CCR2-deficient mice (16, 20). The correlation of fewer inflammatory monocytederived cells in the lungs and reduced pulmonary damage suggests that CCR2 inhibition diminishes morbidity and mortality by blocking the accumulation of inflammatory monocytes in influenza-infected lungs.

Prophylactic use of the CCR2 inhibitor PF-04178903 does not result in any apparent side effects. We found no decrease in immunity against influenza virus or increase in severity of secondary bacterial infection. Drug-treated mice exhibited comparable levels of viral titers, influenza-specific neutralizing Ab titers, and bacterial loads after secondary bacteria infections compared with control mice. Unexpectedly, the prophylactic dosing of PF-04178903 resulted in increased CTL activity against influenza virus protein-loaded cells. Multiple DC subsets were shown to present viral Ag to CD4 T cells (31). CD8^{+} DCs and CD103^{+} migrating lung DCs are specifically implicated in the induction of CD8^{+} virus-specific T cells (32, 33). Thus, although monocyte-derived DCs are likely to play a role in T cell activation during influenza infection, it seems that other DC subsets that are not CCR2 dependent are sufficient to perform this function. We speculate that the high levels of cytokines produced by monocyte-derived DCs and exMACs during influenza infection may interfere with normal T cell activation. It was shown that overproduction of NO during viral infections can suppress Th1 responses, leading to Th2-biased immune responses (18, 34–36). This may result in fewer cytotoxic T cells being primed (37, 38). Decreased inducible NO synthase expression was seen in CCR2-deficient mice (16), and it is likely to occur in PF-04178903-treated mice. This may explain the increased CTL activity in drug-treated mice.

We observed that drug-treated mice do not have increased bacterial burdens after secondary *S. pneumoniae* infection. This finding was unexpected because CCR2-deficient mice were shown to have decreased resistance to some bacterial infections—an effect that is probably due to fewer macrophages in infected tissues (9, 10). However, macrophages in influenza-infected lungs seem to be dysfunctional and, therefore, of little benefit in the clearance of bacteria. This would explain the markedly increased susceptibility of influenza-infected mice to bacterial infection (25). It seems that reducing the numbers of such dysfunctional cells has little effect on bacterial clearance. Macrophage dysfunction may
be caused by the abnormal elevation of proinflammatory and anti-inflammatory cytokines elicited by influenza infections (25). IL-10 was proposed to be the key mediator for this dysfunctional process: inhibiting IL-10 in influenza-infected mice improved the survival rate from secondary bacteria infections (39, 40). Moreover, other investigators reported that the decreased migration of neutrophils, along with the reduced phagocytosis and reactive oxygen species generation in neutrophils during influenza infections, contributes to the failure to clear secondary bacteria infections (26, 41, 42). It seems that, with limited neutrophil functions, the bacteria infection is difficult to control with or without the help of macrophages.

Interestingly, administration of PF-04178903 after influenza infection of mice did not reduce overall mortality or morbidity. It is unclear why PF-04178903 failed as a therapeutic. The most obvious cause of decreased efficacy in mice treated postinfection would be a decrease in the inhibition of inflammatory monocyte accumulation in lungs. However, we found no significant differences in inflammatory cell accumulation in the lungs of mice treated at day −1 versus those treated at day 0 (data not shown). Still, we cannot rule out the possibility that a small number of monocytes, capable of inducing injury, entered the lungs very early in the course of infection in day 0-treated mice but fell below the limits of detection. It is also possible that treatment with PF-04178903 inhibits the function or activation of inflammatory monocytes at baseline but that this effect is lost once these cells are activated by influenza virus. Such “timing” effects would be due to fundamental aspects of CCR2 biology and would be expected with any CCR2 antagonist. Alternatively, it is possible that the lack of efficacy of PF-04178903 treatment postinfection is due to some unrecognized characteristic of this specific compound and that some other CCR2 antagonist may perform better when used as a treatment. We are examining these possibilities.

Small-molecule CCR2 antagonists have been tested in several murine models of inflammatory diseases, including experimental autoimmune encephalomyelitis, adjuvant arthritis, dry eye disease, and hepatic steatosis and lipotoxicity (43–45). In these models, CCR2 antagonist treatment reduces the accumulation of monocyte-derived cells in target organs, decreases inflammation, and maintains organ functions. In this study, we showed that prophylactic CCR2 inhibition reduced the excessive immune responses that occur during influenza infection. To our knowledge, this is the first instance in which CCR2 antagonists have been used to reduce infection-induced immune pathology. Our results suggest that CCR2 inhibition may be useful in other infection-induced inflammatory diseases.

Although current antiviral drugs can be effective in treating highly pathogenic influenza infection, they must be used early in the course of infection (7). The CCR2 inhibitor used in our study also has to be administered early to prevent subsequent mortality. In our model, mortality was not caused by high viral load. Viral titers actually decreased before infection peaked. In contrast, human H5N1 avian flu infections are often associated with continued high viral loads in alveolar epithelial cells and alveolar macrophages, and blocking viral replication seems to be critical for patient survival (7, 8). It is possible that the combined use of antiviral drugs and CCR2 inhibitors would be more successful in blocking immune pathology later in the course of influenza infection, because combined therapy would block viral replication and inflammation at the same time. Therefore, although CCR2 inhibition has the potential to be used as prophylaxis, it should also be evaluated in the later course of infection along with antiviral drugs.

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Disclosures

The authors have no financial conflicts of interest.

References


Suppl. Figure 1. FSC/SSC of Cell populations in the lungs of influenza-infected mice.
Mice were infected by inoculating influenza virus PR8 intranasally. After 5 days, the lungs were digested and processed for flow analysis. FSC/SSC of each cell populations is shown.