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*J Immunol* 2006; 176:2562-2567; doi: 10.4049/jimmunol.176.4.2562
http://www.jimmunol.org/content/176/4/2562

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Deletion of CCR1 Attenuates Pathophysiologic Responses during Respiratory Syncytial Virus Infection

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The role of chemokines in chronic inflammatory responses are central to the recruitment of particular subsets of leukocytes. In the present studies, we have examined the role of CCR1 in the developing pathogenesis of respiratory syncytial virus (RSV) in the lungs of infected BALB/c mice. Although we did not observe significant differences in clearance of RSV, we were able to identify decreased pathophysiologic responses in CCR1−/− mice. CCR1−/− mice displayed a significant reduction in both airway hyperresponsiveness and mucus production that corresponded to significant increases in IFN-γ and CXCL10. The goblet cell hypermetaplasia and the expression of mucus-associated gene, gob5, were correspondingly reduced in the CCR1−/− mice. In addition, the Western blot analysis of gob5 protein indicated that CCR1−/− mice have virtually no up-regulation of the protein at day 6 of infection compared with wild-type-infected mice. Results from bone marrow chimeric mice indicated that partial reconstitution of the response could be achieved in the CCR1−/− mice with wild-type bone marrow cells, suggesting that these cells have a role in the response. However, transplanting of CCR1−/− bone marrow into wild-type mice did demonstrate an incomplete deficit in RSV-induced responses, indicating that CCR1+ parenchymal cells may also play a significant role in the process. Thus, the presence of CCR1 appears to have a significant role in the development of detrimental airway physiologic responses during RSV infection. These data suggest that CCR1 may be a potential target during detrimental pulmonary responses during infection. The Journal of Immunology, 2006, 176: 2562–2567.

Infection of young children and infants with respiratory syncytial virus (RSV) can often result in the induction of severe airway responses leading to compromised lung function (1–3). Of particular interest is the up-regulation of mucus production and induction of airway hyperresponsiveness (AHR) that corresponds to the severity of the disease. These two parameters are likely due to the problems associated with the intensity of the viral infection itself and with the inflammatory and immune response that accompanies the process. In particular, the accumulation of leukocytes in the lung and the ensuing damage to the airway-associated cells, epithelial cells in particular, may be the primary step leading to increased lung dysfunction.

A number of publications have described the up-regulation of chemokines both from clinical samples and from in vitro analyses of RSV-infected airway cell populations (4–8). The overproduction of these chemokines from airway epithelial cells have identified a number of interacting pathways, including NF-κB as well as TLR3-dependent and -independent pathways that lead to high levels of both CXCL8 (IL-8) and CCL5 (RANTES) production (9, 10). Results have previously identified that receptors for these chemokines can have significant effects in general on the immune and inflammatory responses (11–13). During RSV infection in mice, the putative receptor for IL-8 homologs, CXCR2, was shown to have a significant effect on RSV-induced pathophysiology, including airway hyperreactivity and mucus overproduction (14). The neutralization of CCL5, which binds to CCR1 and CCR5, during RSV infection in mice can also have significant effects on the airway pathophysiologic responses (15, 16). Likewise, a number of groups have identified altered RSV-induced disease by neutralization of another chemokine, CCL3, with similar receptor-binding patterns that can also significantly reduce detrimental airway responses (17–20). In a previous study that examined the role of CCR1 on RSV infection in C57BL/6 mice, little difference was observed in the viral clearance and inflammation (19). We have previously found that this strain of mice, C57BL/6, displays only mild disease in response to RSV (21). Thus, in the present study, we have examined RSV-induced responses in CCR1−/− mice on a BALB/c background and have identified a role for CCR1 in the development of several disease parameters with clinical relevance.

Materials and Methods

Animals

CCR1−/− mice backcrossed onto a BALB/c background were obtained from Prof. C. Gerard (22). Breeding pairs of mice with a targeted disruption of the CCR1 gene (CCR1−/−; BALB/c backcrossed 10 generations) were maintained under specific pathogen-free conditions at the University Laboratory Animal Medicine facility at the University of Michigan Medical School. CCR1−/− mice showed no overt evidence of abnormal breeding or growth patterns while maintained in the University Laboratory Animal Medicine facility. Age- and sex-matched, wild-type control BALB/c mice (CCR1+/+) were purchased from The Jackson Laboratory.

RSV propagation and titer determination

Human RSV A strain originally isolated at the University of Michigan Hospitals was propagated in Hep2 cells as previously described (23). To determine viral titers, a plaque assay was performed in Vero cells grown until they were semiconfluent. The viral stock was serially diluted and added to each well in duplicate. The plates were incubated for 4–5 days at 37°C while syncytia formed. The methylcellulose solution was removed from each of the wells and the cells were fixed with cold 80% methanol for...
60 min at −80°C. The methanol was removed and the plates were frozen at −80°C for 1 h to lyse the cells before the plates were stained for RSV proteins. Blocking was done using 5% dry milk/PBS. The primary Ab used was goat anti-RSV polyclonal Ab purchased from Chemicon International. This Ab was used at a dilution of 1/500. The secondary Ab used was rabbit anti-goat Ab conjugated to HRP. It was diluted 1/100 for use. The Ab incubations were conducted for 1 h at 37°C. One-Step chloronaphthol purchased from Pierce was added to each well and the cells were incubated for 10 min at room temperature. Cells were washed with PBS and plaques were counted.

**RSV infection**

BALB/c mice were infected intratracheally with 1 × 10^9 PFU of human RSV A strain virus as previously described (21). Animals were then maintained in under biohazard containment in University of Michigan animal facilities.

**Measurement of airway hyperreactivity**

Airway hyperreactivity was measured using a direct ventilation method on anesthetized trachea cannulated and ventilated mice with a mouse plethysmograph (Buxco) as previously described (21). Briefly, mice were anesthetized with sodium pentobarbital, intubated via cannulation of the trachea with a 18-gauge metal tube, and ventilated with a Harvard pump ventilator (0.3-ml tidal volume; 120 breaths/min; 2.5–3.0 positive end-expiratory pressure). A dose-response curve to i.v. methacholine was performed to determine the optimal dose required to induce between 1.5- and 2-fold increase maximal in airway resistance in untreated mice. RSV-infected mice were given an i.v. tail vein injection of methacholine at a dose of 125 mg/kg in 0.1 ml of saline. Data are mean ± SE change in airway resistance (cm H2O/ml/s) for five animals and are representative of data obtained in two separate experiments.

**ELISA analysis**

Lung samples were prepared for ELISA analysis by homogenization in buffer containing Complete protease inhibitor mixture (Roche Diagnostics) and 0.05% Triton X-100 (Sigma-Aldrich). The cytokine concentrations were determined using a standardized sandwich ELISA technique (24). Abs used were the appropriate suggested pairs sold by R&D Systems. Recombinant murine cytokine and chemokines (R&D Systems) were used to generate standard curves, and the limit of detection for the assays was consistently below 50 pg/ml.

**Assessment of gene expression by real-time RT-PCR**

RNA was isolated from lung tissue following homogenization in TRIzol (Invitrogen Life Technologies) according to the manufacturer’s protocol. Briefly, 0.5 μg of total RNA was reverse transcribed in a 25-μl volume. mRNA expression was determined in 1 μl of cDNA by TaqMan real-time PCR using a Prism 7700 sequence detection system (PE Biosystems) with (cm H2O/ml/s) for five animals and are representative of data obtained in two separate experiments.

**Statistical analysis**

All results are expressed as mean ± SE. Statistical significance was calculated by ANOVA followed by Student’s Neuman-Keull’s posttest to calculate the p value. Significance was determined as values of p < 0.05.

**Results**

**CCR1−/− mice do not develop RSV-induced AHR**

It has been previously observed that RSV-infected mice produce a number of chemokines and by blocking CCL5, a major CCR1 ligand, disease can be modulated (15). In earlier studies, CCR1 was found to be significantly up-regulated in the lungs of BALB/c mice during the development of detrimental RSV infections corresponding to AHR (25). Upon examining the expression of a number of other chemokine receptors, including CCR2–CCR8 and CXCR3–CXCR4, we found no other receptor that was significantly up-regulated at the later time points (days 6–12) when AHR was most severe (data not shown). When infected, CCR1−/− mice displayed significantly less change in airway resistance compared with the responses in wild-type mice (Fig. 1A). These results correspond well with our previous data demonstrating that when CCL5, a CCR1 ligand, was neutralized a similar reduction on AHR was observed (15). Interestingly, when we examined viral titers in these animals, there was no significant difference at any time point by plaque assay (Fig. 1B). When later time points, days 8 and 12, were examined, no viral titers were found in either wild-type or CCR1−/− (data not shown). Additionally, no difference in either the RSV G protein quantitative mRNA expression or RSV protein (as measured by ELISA) was observed in the two groups at any time point from day 1 until day 12 of infection (data not shown). Thus, the effects observed in the CCR1−/− mice did not appear to be related to viral clearance, but rather were related to an effect on the response to the virus.

**Alteration of cytokines and chemokines in RSV-infected CCR1−/− mice**

Several studies have demonstrated that cytokine responses are closely associated with the clinical outcome of RSV infections (26–30). We therefore examined the production of several cytokines during RSV infection in wild-type vs CCR1−/− mice. In separate studies, we identified that CCR1−/− mice had increased levels of IFN-γ in lungs of infected mice (Fig. 2). Corresponding with the increased IFN reported above, the lungs from CCR1−/− mice had significantly higher levels of CXCL10 (IFN-γ-inducible protein 10) (Fig. 2B). Interestingly, when we examined other disease-related chemokines, data indicated that CCL5 and CCL22 were significantly decreased during the RSV infection in CCR1−/− mice (Fig. 3A). Thus, not only were there differences in IFN-γ, but also an alteration in chemokines produced during the response that corresponds to development of the most severe disease phenotypes.
Because we observed differences in the expression of chemokines in the lungs of the CCR1<sup>−/−</sup> mice after infection, the leukocyte infiltration was also examined by bronchoalveolar lavage analysis. Total leukocyte numbers were not significantly different in the airways after a 1-ml bronchoalveolar sampling in wild-type vs CCR1<sup>−/−</sup> mice (data not shown). However, when we examined leukocyte subset recruitment by differential staining and morphologic assessment, we observed a difference in only the accumulation of mononuclear lymphocytes. Although similar levels of neutrophils were recruited in the two groups (wild type, 13.6 ± 6.7% vs CCR1<sup>−/−</sup>, 14.4 ± 3.9% at day 8), the lymphocyte levels were significantly lower in the CCR1<sup>−/−</sup> mice at day 8 of infection (Fig. 3B). Thus, the cytokine alterations observed above corresponded with the intensity of the lymphocyte accumulation in the airway.

**CCR1<sup>−/−</sup> mice develop substantially less mucus production in the lungs during RSV infection**

One of the most devastating aspects of RSV is the overproduction of mucus that creates the physical obstruction of infant airways and alters lung function. We and others have reported that RSV infection of BALB/c mice induces significant mucus production in the airways (14, 31–34). In the present studies, we examined whether RSV-infected CCR1<sup>−/−</sup> mice had a defect in their ability to produce mucus. The data in Fig. 4A demonstrate that the CCR1<sup>−/−</sup> mice had no significant increase in mRNA expression of a mucus-associated protein, gob5, whereas the wild-type CCR1<sup>+</sup> mice demonstrated significant up-regulation. The latter mRNA data were verified by Western blot analysis of gob5 protein from whole lungs RSV-infected animals after 8 days of infection using a gob5-specific Ab (Fig. 4B). The Western blot analysis indicated that gob5 was not at all up-regulated in the CCR1<sup>−/−</sup> mice, verifying that this pathway was altered in CCR1<sup>−/−</sup> mice.

**Partial reconstitution of responses in bone marrow chimeras**

To further explore the nature of the role of CCR1 in this response and to identify whether this phenotype is dependent on CCR1<sup>+</sup> immune cells, we made bone marrow chimeras. Wild-type and CCR1<sup>−/−</sup> mice were lethally irradiated and injected i.v. into the tail vein with isolated bone marrow cells (5 × 10<sup>6</sup>/mouse). After 42 days of engraftment, the animals were challenged with RSV (1 × 10<sup>6</sup> PFU) by intratracheal administration. Controls included chimeras made from wild-type and CCR1<sup>−/−</sup> naive mice into a similar background challenged with RSV. After 8 days of RSV infection, the mice were assessed for AHR using box plethysmography in anesthetized and ventilated animals (A). Subsequently, separate studies were conducted to assess differences in viral titers using in vitro plaque assays with whole lungs of mice from animals infected for 4 or 6 days (B). The data in each study represent the mean ± SE from five mice per group. *, p < 0.05 significance.
infection animals were examined. The data in Fig. 5 indicate that while the wild-type bone marrow into CCR1−/− chimeras had a partially reconstituted AHR response, the CCR1−/− bone marrow into wild-type chimeras demonstrated a reduced AHR compared with the wild-type mice. When we examined the airway for mucus overproduction, we found that the wild-type and CCR1 chimeric mice maintained their mucus phenotype. The CCR1−/− mice that received wild-type bone marrow and displayed an increase in AHR had some increase in goblet cell staining compared with CCR1−/− mice (Fig. 6). In the wild-type mice that received the CCR1−/− bone marrow, there was a decrease in goblet cell staining compared with wild-type mice (CCR1+/+). These data indicate two important concepts. First, the wild-type bone marrow-derived cells that express CCR1 appear to enhance the detrimental response in the CCR1−/− lung environment. Second, the radioresistant parenchymal cells present in the wild-type mice appear to provide an environment that allows the maintenance of some of the goblet cell responses. This latter mouse chimera suggests that CCR1 ligands may be responsible for a direct activation of the airway epithelium and subsequent mucus overproduction. This will be explored further in future studies.

Discussion

The regulation of pulmonary immune responses to viral infections can dictate the severity of the infection and relates to the intensity of the pathophysiologic outcome. Although clearance of the virus...
is paramount to limiting the immune reaction, the severity of the pathophysiology depends on the mediators involved in the local pulmonary responses. In the present studies, CCR1\(^{-/-}\) mice displayed significant reductions in AHR and mucus overproduction even though there was no detectable alteration in the clearance of virus. In particular, the ability of the CCR1\(^{-/-}\) mice to limit mucus production was characterized by altered gob5 expression and protein production that has been closely associated with mucus and goblet cell hyper/metaplasia (35, 36). Although we have not completely identified the mechanism of the overall response, we can suggest possible mechanisms based on our findings. First, the CCR1\(^{-/-}\) mice may have a direct alteration in epithelial cell activation during RSV-induced disease that prevents goblet cell development (14). Thus, the direct activation of CCR1 on airway cells may influence the overproduction of mucus, an aspect we continue to assess. Second, the absence of CCR1 may lead to the altered recruitment of particular immune cells within the airway that would otherwise lead to cellular damage and/or altered immune activation. This is supported by the reduced lymphocyte accumulation in the airway. Third, CCR1 may play a role in the activation of cell populations in the lung resulting in the alteration of the response as was characterized by the differential cytokine and chemokine production during RSV infection in the CCR1\(^{-/-}\) mice. It is likely a combination of all of these effects that influence the pulmonary responses. This was best illustrated in the studies using bone marrow chimeras. Establishing CCR1\(^{-/-}\) bone marrow in CCR1\(^{-/-}\) mice partially reconstituted the AHR and some mucus, whereas CCR1\(^{-/-}\) bone marrow in CCR1\(^{-/-}\) mice continued to show evidence of goblet cell hyper/metaplasia in those mice, although it was reduced. These data likely indicate that CCR1 expression on non-bone marrow-derived cells, such as the pulmonary epithelial cells, contributes significantly to the responses. Our future studies will specifically examine these issues directly, but have thus far been hampered due to a lack of appropriate mouse reagents.

An interesting aspect of these findings is that even though there is an alteration in the pathophysiologic outcomes, there was no alteration in the clearance of the virus, which is undetectable by day 8 of infection. These are important findings as we know from studies in this model that the most severe clinical signs of disease, mucus and airway responsiveness, occur only after detectable virus plaques and mRNA have been substantially reduced. From a clinical standpoint of treating severely infected patients, targeting CCR1 would not decrease the viral clearance, but perhaps reduce the pathogenesis. Most immune mediators that have been used in animal models to enhance viral clearance, such as IL-12 and IFN-\(\gamma\), are quite toxic to humans and likely would promote more adverse effects when administered to a patient for the objective to increase viral clearance (26, 29, 37, 38). Instead, it would be better to target the mediators associated with the symptoms of disease and potentially alleviate long-term complications that appear to be associated with severe RSV infections, especially asthma-like syndromes. Interestingly, earlier publications have identified that chemokines, especially the CCR1 ligands, are produced during the period of most severe disease, days 6–12 postinfection (15, 16, 23). The results of the present study further identify that by targeting CCR1 the majority of the pathophysiologic complications can be greatly prevented. Because other CCR1 ligands, CCL3 in particular, have been identified to be associated with severe RSV-induced disease in both human samples and mouse models, there would be further advantages to targeting this receptor (6–8, 17, 18, 20, 23, 39). We have also found that other CCR1 ligands, CCL3 in particular, have been identified to be associated with severe RSV-induced disease in both human samples and mouse models, there would be further advantages to targeting this receptor (6–8, 17, 18, 20, 23, 39). We have also found that other CCR1 ligands, CCL3 in particular, have been identified to be associated with severe RSV-induced disease in both human samples and mouse models, there would be further advantages to targeting this receptor (6–8, 17, 18, 20, 23, 39). We have also found that other CCR1 ligands, CCL3 in particular, have been identified to be associated with severe RSV-induced disease in both human samples and mouse models, there would be further advantages to targeting this receptor (6–8, 17, 18, 20, 23, 39). We have also found that other CCR1 ligands, CCL3 in particular, have been identified to be associated with severe RSV-induced disease in both human samples and mouse models, there would be further advantages to targeting this receptor (6–8, 17, 18, 20, 23, 39).
may have further application in children with severe RSV infections since epidemiologic data indicate that some of these children continue to have chronic airway dysfunction, with many developing severe childhood asthma (3, 43–45). Perhaps by altering these early responses during RSV infection one could subsequently decrease chronic episodes. In a previous set of experiments that indicate that severe RSV infections predispose animals to an enhanced allergic airway response, we found that RSV-infected CCR1$$^{−/−}$$ mice fail to develop a more severe allergic response compared with CCR1$$^{+/+}$$ mice (25). Thus, blocking CCR1 during a severe RSV infection may provide for a decrease in acute disease, as in the present study, but also may alter long-term disease and prove efficacious.

Disclosures

The authors have no financial conflict of interest.

References