CXCL10/CXCR3-mediated responses promote immunity to respiratory syncytial virus infection by augmenting dendritic cell and CD8\(^+\) T cell efficacy

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The induction of inflammatory cytokines during respiratory viral infections contributes to both disease pathogenesis and resolution. The present studies investigated the role of the chemokine CXCL10 and its specific receptor, CXCR3, in the host response to pulmonary respiratory syncytial virus (RSV) infection. Antibody-mediated neutralization of CXCL10 resulted in a significant increase in disease pathogenesis, including airway hyperresponsiveness (AHR), mucus gene expression, and impaired viral clearance. When the pulmonary cytokine levels were examined, only type I IFN and IL-12p70 were significantly reduced. These latter observations were reflected in reduced dendritic cell (DC) numbers and DC maturation in the lungs of RSV-infected mice treated with anti-CXCL10.

Neutralization of the only known receptor for CXCL10, CXCR3, resulted in similar increases in pathogenic responses. When bone marrow-derived DC were incubated with CXCL10 and RSV, an up-regulation of type I IFN was observed. In addition, T lymphocytes were also examined and a significant decrease in the number of RSV M2 peptide-specific CD8\(^+\) T cells was identified. These findings highlight a previously unappreciated role for the CXCL10:CXCR3 signaling axis in RSV-infected animals by recruiting virus-specific T cells into the lung and promoting viral clearance.

**Key words:** Chemokines - Cytokines - Dendritic cells - Lung - Rodent

**Introduction**

Respiratory syncytial virus (RSV) infects nearly all infants by age 2 and is the leading cause of bronchiolitis in children worldwide [1]. It is estimated by the CDC that up to 125 000 pediatric hospitalizations in the United States each year are due to RSV, at an annual cost of over US$300 000 000 [2]. Despite the generation of RSV-specific adaptive immune responses, RSV does not confer protective immunity and recurrent infections throughout life are common [3, 4]. While RSV is especially detrimental in very young infants whose airways are small and easily occluded, RSV is also widely becoming recognized as an important pathogen in transplant recipients, patients with chronic obstructive pulmonary disease (COPD), the elderly, as well as likely other patients with chronic lung disease, especially asthma. Recent data suggest that mortality for all ages combined have been approximately 30 000–100 000 from 1990 to 2000, with an annual average of over 17 000 in the US [5, 6]. These numbers are likely grossly underestimated, as RSV infection has not been examined in adults in a consistent manner. Thus, RSV not only causes significant exacerbated lung disease in young and old, but also is associated with significant mortality. Although anti-RSV antibodies are available and appear to alleviate severe disease, they perform best when given prior to infection, and few other options exist for combating the RSV infections in susceptible patient populations [7].

Clinical studies have suggested that the severity of RSV-induced disease correlates with the influx of leukocytes, resulting in damage to the airways [3]. Chemokines (chemotactic cytokines) have been shown to correlate directly to the intensity
of the inflammatory response and are induced by viral infection. In particular, a number of studies have demonstrated that CXCL8 (IL-8), CCL3 (MIP-1α), and CCL5 (RANTES) released during the RSV infection correlate to the most severe cases of RSV infection in infants [8–10]. More recently, chemokine production has been linked directly to recognition of pathogen-associated molecular patterns (PAMP) that are expressed during the viral infection [11, 12]. In particular, the recognition of dsRNA by either TLR3 or RIG-I pathways induces the production of type I IFN and chemokines, especially CCL5 and CXCL10 [12]. Blockade of CCL5-mediated responses in animal models of RSV appears to attenuate disease pathogenesis by reducing airway hyperreactivity (AHR) and mucus overproduction [13]. Little information exists on the potential role of CXCL10 and its receptor CXCR3 in the development of pathogenesis during RSV infection. The results from previous studies, together with the one presented here, indicate that while both CCL5 and CXCL10 are expressed at significantly high levels in vitro and in vivo during RSV infection, CXCL10 appears to have a protective role in the host by reducing viral load and pathogenesis.

Results

Induction/neutralization of CXCL10 in vivo

Our previous studies have demonstrated the temporal production of CXCL10 during RSV infection [14]. Our first objective in the current studies was to determine the kinetics of CXCL10 induction during RSV infection, and evaluate the efficacy of our neutralizing antibody. BALB/c mice were treated with anti-CXCL10 or control antibodies, and infected with 10⁵ PFU RSV. The levels of CXCL10 in the lungs were assessed by ELISA assay of lung homogenates. RSV infection in BALB/c mice resulted in significant induction of CXCL10 in the lungs. As previously demonstrated, in control antibody-treated mice, a dramatic induction of CXCL10 protein was observed beginning at day 3 post infection, and persisting to day 8 post infection (Table 1). Treatment of mice with neutralizing antibodies to CXCL10 significantly decreased CXCL10 levels in the lungs (Table 1). These data demonstrate that RSV infection results in the dramatic induction of CXCL10 in the lungs, which is significantly reduced via treatment with neutralizing antibodies.

<table>
<thead>
<tr>
<th>Days post infection</th>
<th>Ctrl RSV</th>
<th>Anti-CXCL10</th>
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<tbody>
<tr>
<td>0</td>
<td>1.06 ± 0.09</td>
<td>0.98 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>7.44 ± 1.1</td>
<td>3.92 ± 0.38*</td>
</tr>
<tr>
<td>8</td>
<td>3.83 ± 0.59</td>
<td>1.83 ± 0.29*</td>
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RSV-induced pathophysiology

In our model, RSV infection in BALB/c mice results in an increase in AHR, as well as mucus hypersecretion [15]. To determine whether CXCL10 plays a role in mediating RSV-induced pathophysiology, mice were treated with control or anti-CXCL10 neutralizing antibodies, infected with RSV, and AHR and mucus production were assessed. An increase in airway resistance was observed in response to methacholine in RSV-infected mice (Fig. 1A). Increased AHR was accompanied by increased transcription of the mucus associate genes Muc5ac and Gol5 (Fig. 1B). Anti-CXCL10 antibody treatment significantly increased AHR relative to control antibody-treated mice (Fig. 1A). Similarly, CXCL10-neutralized animals exhibited increased periodic acid-Schiff (PAS) staining (Fig. 1B) and increased mucus-associated gene expression (Fig. 1C). Taken together, these data demonstrate that neutralization of CXCL10 enhances RSV-induced pathophysiology.

Viral clearance

To determine whether CXCL10 plays a role in the clearance of RSV, we determined viral load via plaque assay and via quantitative PCR for RSV G protein transcript. The levels of infectious virus (PFU) were similar in the lungs of control Ig and anti-CXCL10-treated mice at day 3 post infection (Fig. 2A). At day 8 post infection, PFU via plaque assay were below the level of detection. Similarly, RSV G protein expression in the lungs was similar in both groups at day 3 post infection (Fig. 2B). At day 8, however, significantly more RSV G protein expression was present in the lungs of CXCL10-neutralized mice (Fig. 2B). These results suggest that neutralization of CXCL10 leads to impaired clearance of RSV from the lungs.

Pulmonary cytokines

The expression of CXCR3 by T cells has generally been associated with Type 1 cell-mediated responses, Th1 and cytotoxic T1 (Tc1). If Tc1 cells utilize CXCL10 in trafficking to the lungs, blockade of CXCL10 would likely result in a shift in the Th1/Th2 balance in favor of Th2. To determine whether CXCL10 neutralization results in an enhanced Th2 response in the lungs, the levels of IL-4, IL-5, and IFN-γ were assessed in control Ig-treated and CXCL10-neutralized RSV-infected mice, as described in the Materials and methods. No alterations in IFN-γ or Type 2 cytokines were observed in CXCL10-neutralized mice, relative to controls (Fig. 3A). However, a significant decrease in the Type 1-promoting cytokine IL-12p70 was observed in CXCL10-neutralized mice (Fig. 3A). Additionally, a decrease in Ifn-α mRNA was observed in CXCL10-neutralized mice (Fig. 3B). Thus, neutralization of CXCL10 decreased IL-12 protein and Ifn-α mRNA in the lungs, but these results were not associated with an increased Type 2 response in the lungs.
CXCR3 expression by pulmonary leukocytes

To determine which immune cells are affected by CXCL10 neutralization, we characterized the expression of CXCR3 on a variety of leukocyte subsets from the lungs of uninfected and RSV-infected mice. Consistent with reports in the literature, CXCR3 expression was detectable on all cell types assayed, including CD8 T cells, CD4 T cells, B cells, NK cells, monocyte/macrophages, as well as both myeloid DC (mDC) and plasmacytoid DC (pDC) (Fig. 4) [16–19]. The frequency of CXCR3 expression was highest on NK cells and pDC (Fig. 4). Following RSV infection, however, only pDC maintained CXCR3 expression at high frequency (Fig. 4). These data indicate that a variety of cell types in the lungs express CXCR3 prior to, and during, RSV infection, but only pDC maintained CXCR3 expression at high frequency.

Lung DC trafficking/maturation

The decreased Ifna4 and IL-12 in the lungs of CXCL10-neutralized mice led to a focus on lung DC. Resident lung DC (and macrophages) are among the first immune cells to encounter respiratory pathogens. Lung DC take up antigen and traffic to lung-draining lymph nodes, where they subsequently prime T cell responses. Pulmonary immunity to viruses and other pathogens also results in the accumulation of large numbers of DC in the lungs. To determine whether CXCL10 plays a role in the trafficking of these “inflammatory” DC to the lungs during pulmonary RSV infection, mice were treated with control or CXCL10 neutralizing antibodies and the numbers of mDC and pDC in the lungs were assessed. In control IgG-treated mice, RSV infection resulted in the dramatic recruitment of mDC to the lungs (Fig. 5A, approximately...
sixfold over uninfected controls – dashed line). Anti-CXCL10-treated mice had significantly fewer mDC in the lungs (Fig. 5A). A smaller, but significant increase in pDC was found in the lungs of RSV-infected mice, relative to uninfected lungs (Fig. 5A). CXCL10 neutralization had no effect on the accumulation of pDC in the lungs of RSV-infected mice. While the gate used to define pDC (CD45+, low FSC, low SSC, CD11c int, B220+) may include some NK DC (*20% DX5+ in both control and CXCL10-neutralized mice, Table 2), 50% of the CD11c+B220+ cells co-express Gr-1 (Table 2). These data confirm that these cells are predominantly pDC, and CXCL10 neutralization did not affect pDC trafficking to the lungs. This result was unexpected, however, as pDC in the lungs expressed CXCR3 at higher levels than did mDC (Fig. 4B). CXCL10 neutralization did not affect the recruitment of NK cells or monocyte/macrophages (Fig. 5A). These results demonstrate that CXCL10 promotes recruitment of mDC to the lungs during RSV infection, but since mDC expressed low levels of CXCR3, relative to pDC, this effect may be indirect.

Pathogen recognition by DC via TLR and other pattern recognition receptors results in the up-regulation of MHC class II and costimulatory molecules, collectively referred to as “maturation”. To determine whether CXCL10 plays a role in modulating DC maturation, we determined the expression of MHC class II and costimulatory molecules by DC from the lungs of anti-CXCL10-treated and control RSV-infected mice. The expression of MHC class II was up-regulated in control RSV-infected mice, relative to controls (Fig. 5B and C). There was a trend toward increased CD80 and CD86 expression following RSV infection, but this did not reach statistical significance (data not shown). The up-regulation of MHC class II by mDC was significantly impaired in CXCL10-neutralized mice (Fig. 5B and C). These results suggest that CXCL10 promotes pulmonary DC activation/maturation during RSV infection.

Table 2. Expression of pDC and NK associated markers by low FSC/SSC CD45+CD11c+B220− in the lungs of RSV infected mice; day 8 post infection

<table>
<thead>
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<th></th>
<th>Ctrl RSV</th>
<th>Anti-CXCL10</th>
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<tr>
<td>Gr-1(%)</td>
<td>52.0 ± 3.7%</td>
<td>46.1 ± 5.2%</td>
</tr>
<tr>
<td>DX5 (%)</td>
<td>19.2 ± 2.6%</td>
<td>21.1 ± 2.9%</td>
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Pulmonary T cell numbers

We next wanted to determine whether CXCL10 neutralization would impact T cell responses during RSV infection. To this end, RSV-infected mice were treated with control or CXCL10-neutralizing antibodies and the numbers of lung CD4, CD8, and RSV M82–90 Tetramer-specific CD8 T cells were determined from whole lungs. CXCL10 neutralization resulted in significantly decreased numbers of CD4, CD8, and RSV M82–90-specific CD8 T cells in the lungs of RSV-infected mice (Fig. 6). These data demonstrate that CXCL10 promotes RSV-specific T cell responses in the lungs.

CXCR3 blockade and RSV-induced pathophysiology

CXCR3 is the only known receptor for CXCL10 in mice. To determine whether blockade of CXCR3 altered pathophysiology in a manner similar to CXCL10 neutralization, we administered anti-CXCR3 antibodies during RSV infection. Similar to anti-CXCL10, neutralization of CXCR3 resulted in increased AHR (Fig. 7A), again suggesting that this receptor-ligand system has an important role in activation of the appropriate anti-viral response. Additionally, CXCR3-neutralized mice had significantly increased Muc5ac expression and a trend toward increased Gob5 (Fig. 7B), indicating that the overproduction of mucus corresponded with the changes in lung function (AHR). These data...
provide supporting evidence that the protective effects of CXCL10 during pulmonary RSV infection are mediated via CXCR3.

**CXCL10 promotes Ifnα4 induction and limits viral replication in CXCR3-expressing DC**

Our in vitro data demonstrated that CXCL10 neutralization altered lung DC numbers in the lungs of RSV-infected mice. Our next objective was to determine whether CXCL10 plays a direct role in modulating DC. Bone marrow-derived DC (BMDC) were infected with RSV (MOI of 1) and treated with medium alone or CXCL10. BMDC were CD11b+, consistent with published reports, and expressed moderate levels of CXCR3 (Fig. 5A). RSV infection of BMDC induced dramatic increases in Ifnα4, and CXCL10 treatment of RSV-infected BMDC augmented IFN-α4 mRNA expression (Fig. 5B). Interestingly, RSV replication/transcription, as assessed by RSV G protein expression, was significantly decreased in CXCL10-treated BMDC, compared to DC treated with medium alone (Fig. 5B). The addition of CXCL10 did not directly promote RSV-induced IL-12 production or MHC class II up-regulation. These data suggest that CXCL10 promotes an “anti-viral state” in CXCR3-expressing DC, but does not directly influence DC maturation. Taken together with in vivo data, these results demonstrate that CXCL10 promotes Ifnα4.
The production of chemokines during the initial phases and throughout the infectious process in the lung provides a key role in immunity against and regulating pathogenesis of RSV. In the present study the role of CXCL10 and CXCR3 induced during RSV infection has been carefully explored using a murine model of disease. The neutralization of either CXCL10 or CXCR3 led to a similar exacerbation of disease pathogenesis with significant increases in AHR and mucus gene expression. These data correlated with the reduction in DC accumulation and viral specific CD8+ T cell numbers. The increased presence of virus gene expression after blocking this mediator cascade indicates that the activation event induced by this chemokine-receptor pair is critical to the function of the anti-viral responses. Furthermore, these results demonstrate that CXCL10 plays a role in modulating the host response to pulmonary RSV infection via promoting leukocyte recruitment, and has a role in the trafficking and function of DC. The expression of CXCL10 has been observed in several models of respiratory virus infection, including Sendai, influenza, SARS-CoV, and adenovirus [20–23], as well as in neurogenic viral responses [24–28]. Thus, the responses induced by CXCL10 during the RSV-induced disease appear to be a general mechanism for responding to and/or clearing viral infections.

Neutralization of CXCL10 resulted in impaired type I IFN and DC maturation in the lungs of RSV-infected mice. A number of studies have shown that type I IFN promotes DC maturation and costimulatory molecule expression [29–33]. Recent studies from our laboratory have shown that, in the absence of type I IFN signaling, RSV-infected DC do not mature [34]. Thus, considerable evidence links type I IFN to DC maturation. Taken together, these results suggest that a likely mechanism for the DC maturation defect observed in CXCL10-neutralized mice (and consequently impaired T cell response) is a defect in the induction of type I IFN. We suggest that CXCL10 promotes type I IFN in CXCR3-expressing cells (likely pDC in vivo), which in turn promotes mDC maturation via type I IFN.

Clinical studies have suggested that the severity of RSV-induced disease correlates with the influx of leukocytes that lead to the damage in the airways [3]. In particular, studies demonstrated that a number of other chemokines released within the airways during RSV infection, including CXCL8 (IL-8), CCL3 (MIP-1α), and CCL5 (RANTES), correlate to the most severe cases of RSV infection in infants [8–10, 35, 36]. Two of these chemokines, CCL3 and CCL5, have also been shown to be associated with inflammatory responses in animal models of RSV infection. CCL3 has been linked to the severity of primary RSV-induced inflammation as well as with multiple infections with RSV. Furthermore, Domachowske et al. [37] demonstrated that infection of mice with paramyxovirus induces increased CCL3 related to eosinophil and PMN recruitment. Likewise, studies examining CCL5 indicate a significant impact on the pathophysiological responses in primary RSV infection as well as the pattern of leukocyte recruitment and leukotriene release in the lungs during RSV-induced allergen exacerbated disease [13, 38]. Previous studies have also found that the overproduction of mucus and development of airway hyperreactivity is directly related to the expression and activation of CXCR2 (an IL-8 receptor homolog) in mice [39]. At the same time the overproduction of CXCL10, a chemokine clearly associated with viral clearance and disease resolution, is also highly induced by RSV [14, 40, 41]. The overexpression of CXCR3 and CXCL10 ligand appears to mediate a protective response to RSV, and therefore this represents a balance for the CCL5/CCR1-induced pathophysiology. In contrast, using a combination of reagents to block CCL5 or CCR1/−/− mice, numerous studies have shown that this highly expressed chemokine/receptor pathway corresponds to a detrimental pathological consequence [13, 37, 38, 42, 43]. Thus, it appears that the appropriate activation and recruitment of CXCR3+ DC and T cell populations mediate a less pathogenic immune environment, while blocking the CCR1/CCL5-mediated pathway would provide a potential therapeutic strategy.

Several pathways may induce the production of CXCL10 in the airways during viral infection. One well-characterized mechanism via TLR-mediated activation of CXCL10 has been demonstrated in several viral systems including RSV [12, 44–46]. Initial studies...
using poly-I:C to activate CXCL10 production demonstrated that it was one of the highest expressed cytokines during the activation of multiple cell populations including epithelial cells, fibroblasts and astrocytes [44, 47–51]. More recently, the activation of other dsRNA-mediated pathways have also been identified in the overproduction of CXCL10, as well as other IFN-inducible chemokines, including RIG-I and PKR [52–54]. Additionally, these pathways promote the activation of type I IFN production that provides additional increases in CXCL10 production, thus further augmenting its production within the system and enhancing the anti-viral environment.

The expression of CXCR3 on distinct immune cell populations may be key for understanding the mechanism of RSV clearance in the lung. Studies originally identified CXCR3 expression on Th1 CD4⁺ T cells but not on Th2 T cell populations, indicating a clear dichotomy in recruitment and function [55, 56]. Subsequent studies have identified that CD8⁺ cytotoxic T cells can also differentially express chemokine receptors, depending upon their state of activation [57, 58]. While there is still a paucity of data that fully define the receptor patterns on CD8⁺ T cells, CXCR3 appears to be defining for efficient recruitment of cytotoxic T cells for viral clearance in several infection models [25, 26, 59–62]. A mechanistic picture of the importance of CXCR3 in effective Th1 type anti-viral immunity continues to be developed, but also includes the appropriate recruitment of DC populations [21, 63–66]. In particular, the migration of pDC into the lymph node from the site of viral infection was found to depend upon CXCR3 providing activation signals for lymph node DC to properly activate efficient anti-HSV immunity [21]. The latter studies suggested that CXCR3-recruited pDC provided type I IFN signals in the lymph node that allowed the activation of the T cell populations. In the present studies we have also observed that pDC preferentially express CXCR3. Furthermore, previous studies have demonstrated a key role of pDC in the clearance of RSV and the maintenance of an effective, non-pathogenic response to RSV [67, 68]. Overall, our studies identify a key chemokine receptor system that initiates and maintains the proper immune environment for anti-viral responses.

Figure 8. Effect of CXCL10 on type I IFN production and viral replication/transcription in vitro. BMDC were prepared as described in the Materials and methods. (A) BMDC were stained with either anti-CXCR3 (shaded) or isotype control (hollow line) antibodies and analyzed by flow cytometry. One representative histogram is shown. Similar results were obtained in two independent experiments. (B) Effect of recombinant CXCL10 on RSV-induced expression of Ifna4 mRNA and RSV G transcript. BMDC were infected with RSV (MOI of 1) alone, or in the presence of 100 ng/ml CXCL10, and the levels of Ifna4 mRNA and RSV G protein transcript were assessed by real-time PCR 24 h after infection. Bars represent the Mean of three replicates + SEM. *p<0.05 vs. control. Similar results were obtained in two independent experiments. (C) Effect of CXCL10 on RSV-induced IL-12 p70 and MHC class II expression. BMDC were cultured as in (B), IL-12p70 was measured in the supernatant at 24 h post infection. The expression of MHC class II was determined by flow cytometry.
Materials and methods

Mice

Female BALB/c mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Mice were housed under pathogen-free conditions in enclosed filter-topped cages. Clean food and water were given ad libitum. The mice were handled and maintained using microisolator techniques, with daily veterinarian monitoring. Bedding from the mice was transferred weekly to cages of uninfected sentinel mice that were subsequently bled at weekly intervals and found to be negative for antibodies to mouse hepatitis virus, Sendai virus, and Mycoplasma pulmonis. The University Committee on Use and Care of Animals (UCUCA) at the University of Michigan approved all studies involving mice.

RSV and infection

RSV A strain was derived from a clinical isolate at the University of Michigan and was propagated in Hep2 cells. After adsorption, medium was added to the flask, and the infection was allowed to proceed until syncytia were observed. The cells were frozen at –80°C overnight, and the supernatant was harvested, clarified, and aliquoted. Viral titers were determined by plaque assay. For infections, mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). An incision was made over the trachea and mice were infected with ~1 × 10^5 PFU RSV in a volume of 40 μL via intratracheal injection. The surgical wound was closed with surgical staples and mice recovered with minimal visible trauma.

Generation of CXCL10- and CXCRL3-neutralizing antibodies

Rabbit anti-murine CXCL10 was generated by immunization of rabbits with recombinant protein. Rabbit anti-CXCR3 was generated by immunizing rabbits with a 16-mer peptide (PYDYGENESDFSDSPP) corresponding to the N terminus of murine CXCR3, and has been shown to neutralize CXCR3 in vivo [26, 69]. CXCR3 expressing T cells and DC were detected in anti-CXCR3-treated mice, demonstrating that these antibodies are neutralizing, but not depleting. Sera from immunized rabbits were titered by ELISA and verified for minimal cross-reactivity to a panel of other chemokines and cytokines. Antibodies were purified from total serum by protein A-based affinity columns and the concentration of antibody was determined by spectrophotometry. Neutralizing antibodies (3.5 mg/mouse) were administered via intraperitoneal injection 1 day prior to RSV infection and then every 3 days. The efficacy of anti-CXCL10 antibody neutralization in the lungs is shown in Table 1.

Airway hyperreactivity

AHR was assessed as previously described [15]. Briefly, mice were anesthetized with sodium pentobarbital, intubated via cannula of the trachea, and ventilated with a Harvard pump ventilator (~0.3 mL tidal volume; 120 breaths/min). AHR was measured using a Buxco mouse plethysmograph and software for calculation of the measurements. Mice were administered a single optimized intravenous dose of methacholine (150 mg/kg), and the peak airway resistance was recorded.

RNA and protein assays

The upper left lobe of mouse lungs was used for protein analysis and RNA was isolated from the lower left lobe. Lung samples were snap-frozen in liquid nitrogen and stored at –70°C until use. For protein assays, lungs were homogenized in buffered saline containing Complete™ protease inhibitors (Roche Applied Science, Indianapolis, IN). The concentration of protein was determined by sandwich ELISA using commercially available paired antibodies (R&D Systems, Minneapolis, MN) RNA was isolated from lower left lobes of lungs using TRIZol (Invitrogen, Carlsbad, CA), and ~5 μg total RNA was reverse transcribed in a volume of 25 μL. Real-time PCR was performed on cDNA using commercially available primers for GAPDH, and custom designed primers for RSV G protein transcript, GOb-5, and Muc5ac, as previously described [39]. Ifnλ4 expression was determined using SYBR green I dye, using the following primer sequences: Ifnλ4-F: CCT GTGTGATGC AGG AAC C, Ifnλ4-R: TCA CCT CCC AGG CAC AGA. Gene expression was normalized to GAPDH before the fold change was calculated.

Histology

Right lobes of the lungs were removed and immediately fixed in 10% neutral buffered formalin. Lung samples were subsequently processed, embedded in paraffin, sectioned, and placed on L-lysine-coated slides. PAS staining was done to identify mucus and mucus-secreting cells.

Lung and lymph node leukocyte isolation

Lung leukocytes were isolated from enzyme-dispersed lung tissue. Right lungs from each mouse were excised, washed in PBS, minced and digested enzymatically for 45 min in 15 mL of digestion buffer [RPMI, 5% FBS, 1 mg/mL collagenase (Roche Applied Science), and 30 μg/mL DNase (Sigma-Aldrich, St Louis, MO)]. Lung-associated lymph nodes were dispersed similarly to lungs, but using only 5 mL digestion buffer. Following erythrocyte lysis using ammonium chloride (NH4Cl) buffer, cells were washed, and resuspended in media (RPMI, 5% FBS). Total lung leukocyte numbers were assessed in the presence of trypan blue using a hemocytometer; viability was >85%.
Flow cytometry

Leukocytes were washed and resuspended at a concentration of 10^7 cells/mL in Flow Buffer (PBS + 1% FBS + 0.1% NaN3; Sigma). Fc receptors were blocked by the addition of anti-CD16/32 (Fc block; BD Biosciences, San Jose, CA). Following Fc receptor blocking, 10^6 cells were stained, in a final volume of 120 μL in 12 x 75 mm polystyrene tubes (BD Biosciences) for 20 min at 4°C. Leukocytes were stained with the following mAb, according to the manufacturer’s instructions: CD4 (RM4–5), CD8a (5H10–1), CD45 (30-F11), CD45R/B220 (RA3–6B2), CD11b (M1/70), CD11c (HL3), CD19 (1D3), NK (DX5), I-Ad (AMS-32.1), Gr-1 (RB6–8C5) (BD Biosciences), and CXCRI3 (R&D Systems). Cells were washed twice with Flow buffer, resuspended in 100 μL and 200 μL 4% formalin was added to fix the cells. A minimum of 75 000 events was acquired on a dual-laser Cytoomics FC500TM flow cytometer (Beckman Coulter, Fullerton, CA) and analyzed using FlowJo™ software (Treestar, Ashland, OR). Classical mDC were defined by the following parameters: low forward scatter, low side scatter, CD45R/B220 ^+, M82–90 tetramer (NIH Tetramer Core, Emory University, Atlanta, GA). RSV M82–90 has been previously shown to be the dominant CD8 ^+ epitope during RSV infection in BALB/c mice [70].

BMDC culture

DC were grown from mouse bone marrow using minor modification of an establish method [71]. Bone marrow cells were flushed from the femurs and tibias of naïve BALB/c mice. Bone marrow cells were seeded at 2 x 10^5/mL in 30 mL BMDC media (RPMI + 10% FBS, 1% Pen-Strep, 1% nonessential amino acids, 1% sodium pyruvate, 1% L-glutamine, 5 x 10^{-5} M 2-mercaptoethanol) with 10 ng/mL recombinant GM-CSF (R&D) in T150 flasks. At day 2, another 15 mL BMDC media + GM-CSF was added to the culture. At days 4, 6, and 8, half of the media was removed, centrifuged and the cell pellet added back to the culture with fresh media + GM-CSF. Nonadherent cells were harvested at day 10, and the resulting cells were > 85% CD11c ^+. In some experiments, BMDC were infected with virus at an MOI of 1, and/or stimulated with recombinant CXCL10 (R&D).

Materials and methods

For airway responses and cytokine levels, p values were calculated using the two-tailed student’s t-test, assuming equal or unequal variance, as dictated by F test. For quantitative real-time PCR, statistical comparisons were made by the same methods, except that p values were calculated based on cycle numbers normalized to GAPDH controls, before conversion to fold increases. p values of <0.05 were considered statistically significant.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

References


Recent studies have focused on the role of chemokine receptors and their ligands in the immune system. For instance, CCR9 is expressed on a subset of T cells that are able to enter the brain and spinal cord (T-helper 17 cells) and play a role in multiple sclerosis. The chemokine ligand CCL19 interacts with CCR9 on these cells, facilitating their migration into the CNS. Furthermore, CCR9 and CCL19 have been shown to be upregulated in the brains of animal models of multiple sclerosis, suggesting a potential therapeutic target.

Influenza A virus infection is another area of interest. The chemokine CXCL10 (IP-10) is induced in response to influenza infection and plays a role in immune cell trafficking to the lungs. CXCR3-expressing cells, such as NK cells and CD8+ T cells, are attracted to sites of infection by CXCL10, facilitating the immune response. CXCR3 is also upregulated in response to the viral infection, further supporting the role of chemokines and their receptors in the immune response to respiratory infections.

These findings highlight the importance of chemokines and their receptors in the immune response to various pathogens. Dysregulation of chemokine expression and receptor activity has been implicated in the pathogenesis of several diseases, including multiple sclerosis and influenza infection. Understanding the mechanisms underlying chemokine expression during infection will be crucial for the development of new therapeutic strategies.

References:


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Abbreviations: AHR: airway hyperresponsiveness; mDC: myeloid DC; pDC: plasmacytid DC; RSV: respiratory syncytial virus; Tc1: cytotoxic T cell type 1

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