

Respiratory syncytial virus-induced exaggeration of allergic airway disease is dependent upon CCR1-associated immune responses

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Severe respiratory syncytial virus (RSV) infection has a significant impact on airway function, and may alter subsequent development of asthma. CCR1 mRNA was significantly up-regulated during primary RSV infection in BALB/c mice, and was also up-regulated during allergen exposure in sensitized mice. Although CCR1^{-/-} mice exhibited similar levels of airway hyperresponsiveness (AHR) as wild-type mice in response to cockroach allergen alone, in animals treated with RSV prior to cockroach antigen (CRA) sensitization and challenge, a significant decrease in exacerbated AHR was observed in the CCR1^{-/-} mice. The reduction in AHR after RSV and allergen challenge in CCR1^{-/-} mice was not associated with changes in peribronchial eosinophilia, but was accompanied by significantly decreased IL-13 levels in the lungs, as well as an absence of mucus cell staining within the airways. When T lymphocyte numbers were compared in animals receiving CRA to animals receiving a combination of RSV and allergen an increase in both CD4 and CD8 T lymphocytes could be detected in wild-type but not CCR1^{-/-} animals. Thus, these data suggest that CCR1-mediated responses have a primary role for inducing severe disease during RSV infection, and may be responsible for altering the lung pathophysiological responses to subsequent allergen challenges via IL-13-mediated mechanisms.

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Introduction

Epidemiological evidence has linked the development of chronic childhood asthma to previous exposure to respiratory syncytial virus (RSV) [1–3]. Severe RSV infection is one of the leading causes of hospitalization in young children [4, 5]. Many of these patients subsequently develop a persistent wheeze and a

significant proportion of the children are later diagnosed with severe asthma. Several recent studies using animal models have attempted to dissect the mechanism by which early exposure to RSV can exacerbate subsequent allergic airway responses. We and others have previously demonstrated that mice infected with human RSV prior to allergen challenge with cockroach antigen (CRA) or ovalbumin (OVA) exhibit elevated levels of airway hyperresponsiveness (AHR) and peribronchial inflammation compared to mice challenged with allergen alone [6–8]. In addition, a crucial role for IL-13 in the pathophysiology of this exacerbated allergic response in animals previously infected with RSV has been identified [8].

A number of clinical studies have suggested that the severity of RSV-induced disease correlates with the influx of leukocytes that lead to the damage in the

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Abbreviations: **RSV:** Respiratory syncytial virus · **AHR:** Airway hyperresponsiveness · **CRA:** Cockroach antigen

airways [9]. In addition, chemokines have been shown to correlate directly to the intensity of the inflammatory response and can be directly induced by viral infection in multiple pulmonary cell populations [10–14]. In particular, a number of studies demonstrated that CXCL8, CCL3 and CCL5 released during the RSV infection correlates to the most severe cases of RSV infection in infants. Two of these chemokines, CCL3 and CCL5, have also been shown to be associated with inflammatory responses in animal models of RSV infection [15–19]. CCL3 has been linked to the severity of primary RSV-induced inflammation and to multiple infections with RSV. Furthermore, Domachoske et al. [20] 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 [21]. At least one study examining CCR1, one receptor for CCL3 and CCL5, has suggested that it has no role in the primary RSV infection [20]. However, this initial study was performed using a C57BL6 mouse, a strain not susceptible to detrimental RSV responses.

In the present study the role of CCR1 in mediating a disease phenotype in primary RSV infection and RSV-induced exacerbated allergen responses in the lung has been examined. The studies have established that a CCR1-mediated mechanism of disease appears to be responsible for the changes in the airway, leading to increased pathophysiological responses that, once established during an RSV infection, can later impact allergen-induced disease. Overall, these studies further demonstrate that one potential consequence of severe RSV-induced disease may be alteration of the lung immune environment, leading to long-term disease parameters. CCR1 may be one potential target to alleviate long-term pulmonary alterations centered upon IL-13-mediated disease.

Results

Expression of CCR1 during RSV infection and allergen challenge

The level of chemokine receptor mRNA expression was determined in lung tissue following RSV infection. CCR1 mRNA expression was elevated by day 4 post infection compared to the day 0 (uninfected) mice, reaching a significantly increased level on day 8 (Fig. 1). This increased CCR1 correlated with the peak in pathophysiological responses previously reported by our laboratory [17, 22]. CCR1 mRNA levels had returned to

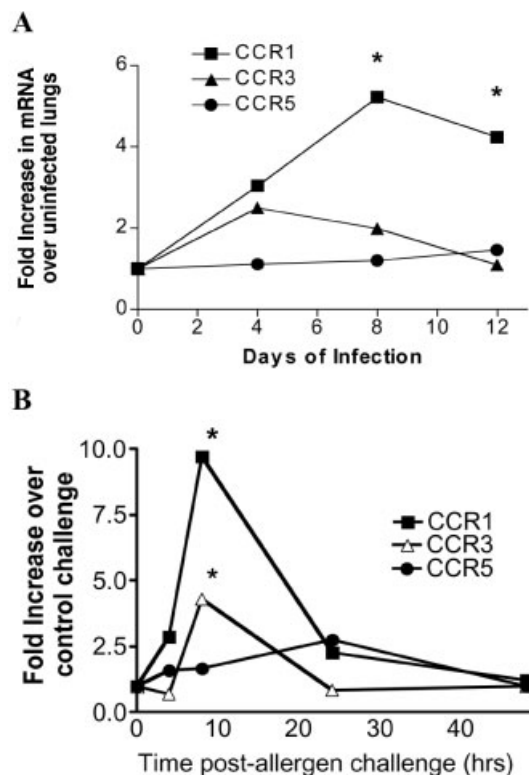


Fig. 1. The expression of chemokine receptors that bind CCL5 in the lungs of RSV-infected mice. (A) Whole lung mRNA was isolated from mice at various times post-virus challenge and reverse transcribed. Quantitative PCR was performed using pre-developed primer/probe analysis and fold increased expression of the individual chemokine receptors was calculated at 4, 8 and 12 days post-infection compared to normal control mice. Only CCR1 expression was significantly up-regulated in the RSV-infected mice. (B) Examining the expression of the same chemokine receptors during CRA only challenge demonstrated that both CCR1 and CCR3 were up-regulated and not CCR5. The data represent the mean from five mice per time point and significance determined at $p < 0.05$ (*).

baseline levels by day 21. In contrast, CCR3 and CCR5 were not increased in the lung during the infection process (Fig. 1A). These data suggested that CCR1 was a chemokine receptor target during RSV-induced disease due to its association with peak disease [17, 18, 21]. To begin to examine the role of CCR1 in allergic disease, we also examined the expression of CCR1 after allergen challenge in sensitized mice over a period of 48 h (Fig. 1B). We also observed a substantial increase in CCR1 after an allergen challenge, which was maximal at 8 h and began returning to background levels by 24–48 h post challenge. Thus, the peak in CCR1 expression preceded peak disease, which occurs at 24 h post-allergen challenge. We observed no further increase in CCR1 expression in animals previously given a RSV infection (data not shown).

Attenuation of RSV-induced AHR exacerbation in CCR1^{-/-} mice following allergen challenge

Previous studies have shown that RSV can predispose mice to more severe allergen responses, and that mice exposed to RSV prior to CRA challenge exhibited significantly elevated AHR compared to mice exposed to allergen alone [21, 22]. Since our previous study [21] suggested that CCL5 has an effect on the development of airway inflammation, the next question was whether removing the primary receptor, CCR1, would completely alter the RSV-induced exacerbated allergic airway responses. Although CCR1^{-/-} mice challenged with CRA alone showed a similar level of AHR as that seen in CCR1^{+/+} mice, the CCR1^{-/-} mice infected with RSV prior to CRA challenge had significantly lower levels of AHR than their wild-type CCR1^{+/+} counterparts (Fig. 2). Interestingly, the AHR in CCR1^{-/-} mice given RSV followed by CRA challenge was actually lower than in CCR1^{-/-} mice given only CRA, potentially suggesting a protective effect in these mice by RSV.

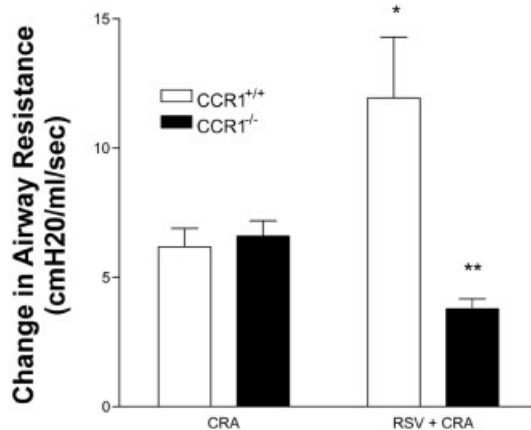


Fig. 2. RSV-infected mice CCR1^{-/-} BALB/c mice have decreased AHR after allergen rechallenged compared to CCR1^{+/+} BALB/c mice. Animals were initially infected by intratracheal injection of 1×10^5 pfu of RSV or UV-inactivated RSV at day 0, followed by a CRA sensitization beginning at day 21 post-RSV infection. After an additional 21 days, the infected and allergic mice were given a final intratracheal challenge and examined 24 h later using direct ventilation, anesthetized plethysmography. After baseline measurements were taken, the mice were challenged with an optimized dose of methacholine (100 μ g/kg) by i.v. tail injection. The peak resistance was monitored and recorded. Naive animals responded with little increase in airway resistance (between 1.0 and 1.8 cmH₂O/ml/s over background). The data represent the mean $\pm$ SE from five to six mice per group. * $p < 0.05$ compared to CCR1^{+/+} CRA; ** $p < 0.05$ compared to CCR1^{+/+} RSV-CRA group.

Th2 cytokine levels and mucus expression following RSV-allergen challenge

Our previous data demonstrated that the increased disease phenotype observed in allergen-challenged mice previously infected with RSV was due to increased levels of IL-13 [8]. In the present studies IL-13 levels were similar in CRA-challenged wild-type and CCR1^{-/-} mice, whereas IL-13 levels were elevated in CCR1^{+/+} mice exposed to RSV prior to CRA challenge (Fig. 3). However, this increase was not detected in the CCR1^{-/-} mice exposed to RSV prior to allergen, which exhibited significantly lower levels of IL-13 than the wild-type mice (Fig. 3). Interestingly, IL-4 levels were similar in CCR1^{+/+} and CCR1^{-/-} mice following both CRA challenge alone and RSV-allergen challenge, suggesting that separate mechanisms govern the regulation of these Th2 cytokines.

One of the primary consequences of overproduction of IL-13 that has been identified during airway responses is hypersecretion of mucus [22–27]. To examine this aspect further, we have performed a histological staining of lungs from CCR1^{+/+} and

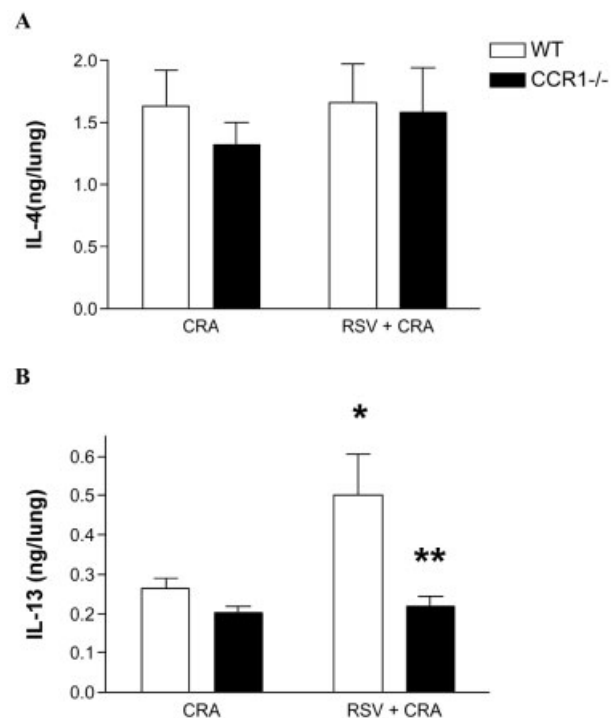


Fig. 3. RSV infection predisposes mice to increased IL-13 production after allergen sensitization in CCR1^{+/+} but not CCR1^{-/-} mice. Lungs from mice in Fig. 2 were subjected to homogenization in 1 ml ELISA buffer to release the total lung cytokines and debris cleared by high-speed centrifugation. The supernatants were assessed for IL-4 (A) and IL-13 (B) levels by ELISA using specific antibodies (R&D Systems). Data represent mean $\pm$ SE from five to six mice per group. * $p < 0.05$ compared to CCR1^{+/+} CRA, ** $p < 0.05$ compared to CCR1^{+/+} RSV-CRA group.

CCR1^{-/-} mice after the final allergen challenge. Our findings clearly indicate that the CCR1^{-/-} mice have a paucity of mucus staining within the airways compared to the CCR1^{+/+} mice (Fig. 4). Thus, it appears that a primary RSV infection not only predisposes mice to increased pathophysiological response, but also to increased development of mucus production. Both of these appear to be CCR1 dependent.

Alteration of leukocyte accumulation in lungs of CCR1^{-/-} mice

To examine specific cell populations during the above responses, the number of CD4⁺ and CD8⁺ T cells in the lungs of mice receiving CRA alone or RSV and CRA were determined on day 43 post-RSV infections. CCR1^{+/+} and CCR1^{-/-} mice challenged with CRA alone had similar numbers of both CD4⁺ (Fig. 5A) and CD8⁺ (Fig. 5B) T cells. However, CD4⁺ T cells were significantly elevated in CCR1^{+/+} mice infected with RSV prior to allergen challenge. This increase was not present in

CCR1^{-/-} mice, in which the number of lung CD4 T cell were similar to those seen in wild-type and knockout mice challenged with CRA alone. A similar pattern was found for the CD8⁺ T cells, with increased cell numbers in wild-type, but not CCR1^{-/-} mice receiving RSV-CRA compared to the allergen challenged control animals that received UV-inactivated RSV. CD4 and CD8 T cell accumulation in the lungs was also investigated earlier on day 22 post infection, but at this time point there was no significant difference in the cell numbers in each group of animals (data not shown). These data demonstrate two important issues: Firstly, RSV-induced exacerbation in allergen-induced disease was associated with increased lymphocyte accumulation and, secondly, the absence of CCR1 during the response abrogates the increased accumulation of both T cell populations. Interestingly, when we investigated the response in the RSV infection only, we found no significant alteration in CD4 or CD8 T lymphocyte accumulation in the lungs of CCR1^{-/-} compared to wild-type mice (data not shown).

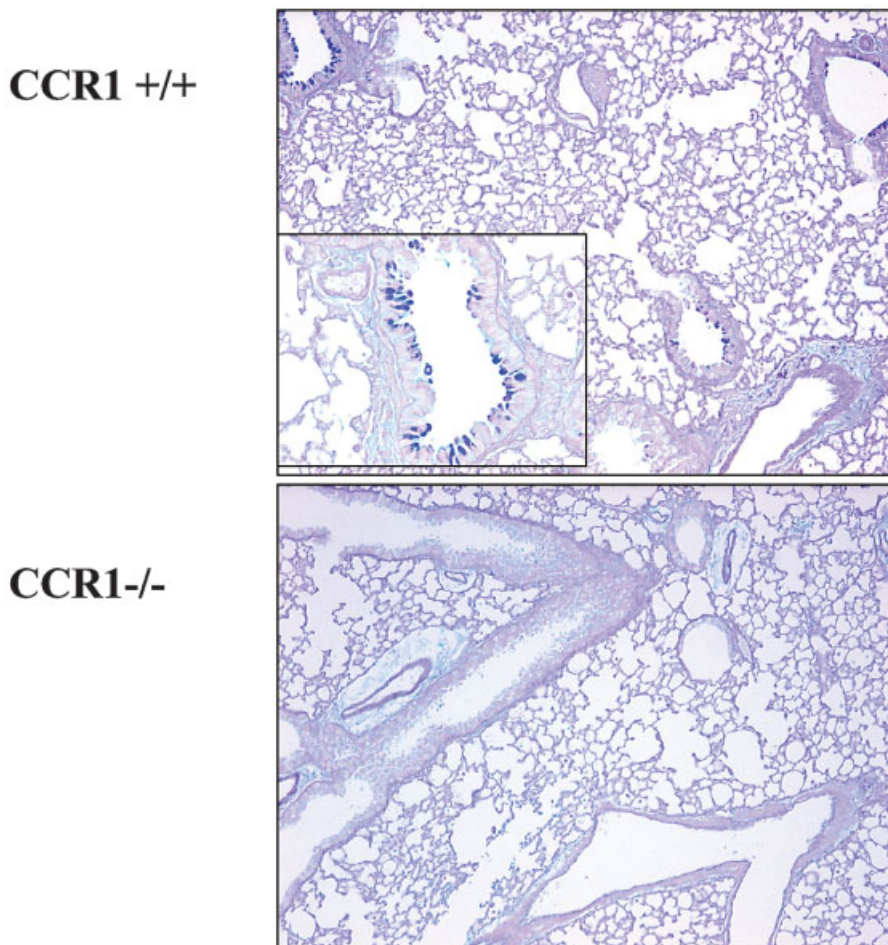


Fig. 4. Lungs from CCR1^{-/-} mice treated with RSV prior to CRA challenge displayed little mucus staining compared to CCR1^{+/+} mice treated in a similar manner. Representative lung sections from CCR1^{+/+} and CCR1^{-/-} mice were stained for mucus production using alcian blue/PAS staining. The sections displayed are representative of those from multiple mice.

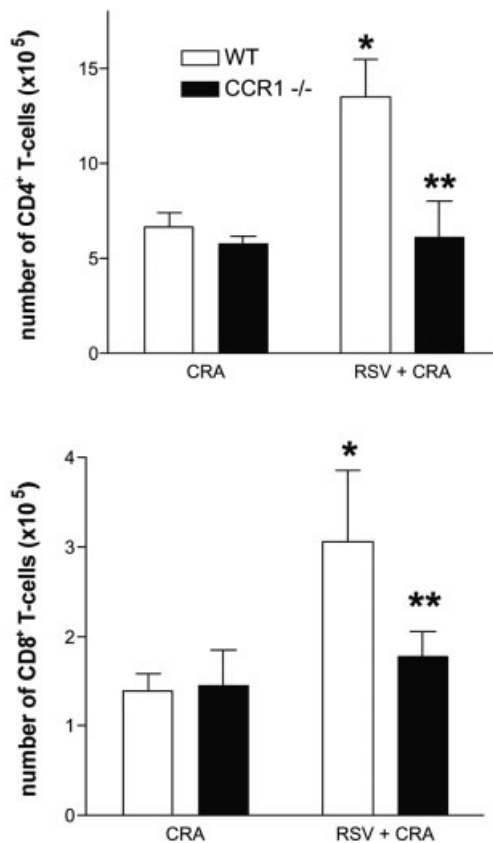


Fig. 5. RSV infection predisposes allergic CCR1^{+/+} but not CCR1^{-/-} mice to increased accumulation of pulmonary CD4 and CD8 T cells after challenge. Lungs infected with either UV-inactivated RSV or RSV followed by CRA challenge were dispersed after collagenase digestion, and the total CD4 and CD8 T cells were assessed by flow cytometry. Data represent the mean $\pm$ SE from five mice per group. * $p < 0.05$ over CRA only, ** $p < 0.05$ decreased compared to CCR1^{+/+} CRA+RSV.

Severity of asthma is often correlated to eosinophil accumulation around and in the airways. However, recent data has suggested that eosinophils may be contributory but not necessary for increased severity. Evaluation of peribronchial eosinophil accumulation in the mice infected with RSV prior to allergen challenge indicated that the absence of CCR1 had no effect in recruitment of eosinophils to peribronchial regions of the lung (Fig. 6). Thus, the eosinophil accumulation was not affected by the absence of CCR1 in the combined model of airway disease.

Discussion

Exposure to infectious agents early in life likely shape the immune responses for subsequent reactions and facilitate the development of severe chronic disease [28–33]. Modeling and characterizing these interrelated responses becomes critical for a better understanding of

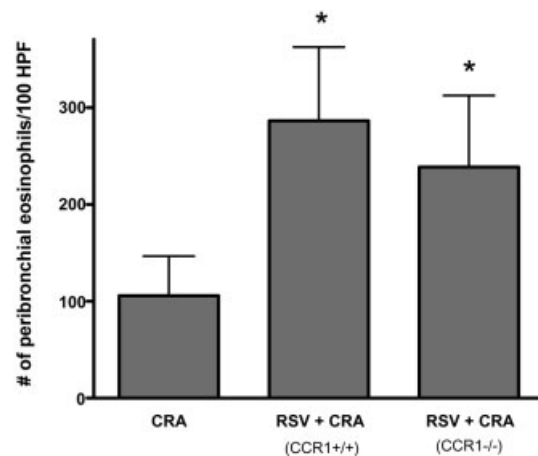


Fig. 6. Enumeration of peribronchial eosinophils induced by allergen challenge demonstrates an increase in eosinophil numbers after an RSV infection and is not altered in the CCR1^{-/-} mice. Since the results for CCR1^{-/-} and CCR1^{+/+} mice challenged with allergen after UV-inactivated RSV were not different, the data here are combined. Data represent the mean $\pm$ SE of peribronchial eosinophils in 100 high power fields (magnification $\times 1,000$) from five mice per group. * $p < 0.05$ over CRA only.

not only the mediators that relate to the disease process, but will also aid in determining the molecules to target during early disease development. In the present studies we have continued to characterize the impact of an initial RSV infection on the emergent allergic responses in susceptible mice. This may model development of immune responses observed in children that have early severe RSV infection and subsequently proceed to develop chronic severe asthma. Although this latter group of asthmatics appears to represent a subset of those patients that develop severe RSV infections, it is important to understand how an initial RSV infection impacts on the severity of the disease process [34–36]. It is necessary to explore specific detrimental mediators that can only be identified in careful temporal studies using animal models, which normally cannot be performed in patient populations. In these studies a single chemokine receptor, CCR1, has been targeted only after establishing the up-regulation of the receptor during the RSV responses. Previous studies performed with these models have demonstrated that CCL5, one of the CCR1 ligands which has been shown to be up-regulated during RSV infection, has an important role in both the development of RSV infection-related pathology, and in RSV-exacerbated allergen responses [10, 11, 17, 21, 37]. In addition, other CCR1 ligands have been implicated in the development of severe RSV-induced responses, especially CCL3 [10, 16, 19]. It appears that RSV-induced pulmonary responses facilitates development of more severe allergic airway responses via a CCR1-mediated mechanism.

The particular question of how CCR1 and its ligands participate in this long-term process is an underlying issue that we have begun to specifically address. The fact that chemokine ligands that bind to CCR1 also bind to other chemokine receptors was not lost in our analysis. When we examined the regulation of the other known receptors within the lungs of RSV-infected mice only CCR1 was up-regulated during the time of severe pathophysiological disease, days 6–12. The other receptors, CCR3 and CCR5, were not significantly up-regulated at similar time points during the infection. Thus, the rationale for examining CCR1 appeared obvious. These studies indicate that the decreased disease in CCR1^{-/-} was linked to the attenuated production of IL-13 observed after the RSV and allergen challenge. These data are consistent with previous data in these models that demonstrated that the detrimental pathophysiological responses are related directly to the overproduction of IL-13 that leads to increased airway hyperreactivity and mucus hypersecretion [8, 22]. The mechanism appears to be related to an increased accumulation of both CD4 and CD8 lymphocytes after the final allergen challenge. We have begun to specifically investigate this latter issue and have found that CD8⁺ T cells produce the majority of IL-13 during a primary RSV infection (data not shown). Thus, the role of CCR1 may be in the recruitment of CD8⁺ T cells that produce IL-13 into the lung and/or lymph nodes, which leads to an altered immune environment and enhances the allergic responses.

It is curious that during RSV-induced amplification of the allergen-induced disease only IL-13, and not IL-4, was related to the altered disease. This reflects the findings in our original analysis using this model [8]. It may be that the RSV infection initiates an altered immune environment that enhances the development of this type of response, but could also indicate a bystander effect that allows reactivation of T lymphocytes during allergen-induced responses. This latter concept has been previously suggested in earlier studies that have utilized transferred CD8⁺ T lymphocytes from RSV-infected mice to exacerbate OVA-induced allergic responses in the lung [38]. In addition, we hypothesize that RSV antigen may persist within the lung and, upon allergen activation, re-initiates the activation of RSV-specific responses. This is supported by studies that have identified the persistence of viral antigen in the lungs of infected animals for up to 100 days post infection [39, 40]. A more recent study has demonstrated that reactivation of RSV can be accomplished 100 days post primary infection by depleting lymphocytes, suggesting that low level virus proliferation may persist in susceptible mice [41]. We have also identified RSV protein and mRNA in the lungs of infected animals at the end of the 6-week protocol used in these studies with a

RSV-specific ELISA to detect the presence of specific RSV proteins and quantitative PCR (data not shown). Additional studies will be performed to explore this latter issue more thoroughly and identify whether it is the response to RSV that augments the allergen-induced responses.

Previous studies utilizing CCR1^{-/-} or CCR1 antagonists have found results that might be compared to those in these studies. In two parallel studies from the same laboratory CCR1 was found to control inflammation and lethality in response to a paramyxovirus of mice (PVM), whereas using similar mice with an RSV infection had no effect on the inflammatory response [42]. In contrast to the latter study that used CCR1^{-/-} on a C57BL/6 mouse background that displays little pathophysiology, our studies utilized a CCR1^{-/-} mouse on a susceptible BALB/c strain that has detrimental responses to RSV [8, 43, 44]. Thus, differences observed between the present and the former studies might be explained by the use of different strains of mice that have different patterns of susceptibility. Other studies have demonstrated that CCR1 is crucial for IL-13-induced inflammation and remodeling responses, and correspond directly with our findings linking IL-13 and CCR1 to chronic diseases [45, 46]. Thus, CCR1 may represent an excellent target for not only this disease process but perhaps for multiple chronic disorders that are impacted by heightened IL-13 production and mucus overproduction.

Materials and methods

Animals

CCR1^{-/-} mice backcrossed onto a BALB/c background were obtained from C. Gerard. 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-matched, wild-type control BALB/c mice (CCR1^{+/+}) were purchased from The Jackson Laboratory (Bar Harbor, ME).

RSV-infection and CRA-sensitization protocols

At 6–8 weeks of age, mice were infected with 1×10^5 pfu human RSV A2 wild-type strain grown from an original University of Michigan hospital isolate. Control mice received inactivated RSV that was previously exposed to UV light for 30 min. For RSV time course experiments, mice were killed 0, 1, 4, 8, 12, and 21 days after infection. In RSV-allergen protocols, mice were immunized i.p. and s.c. with 10 µg CRA (Bayer Corporation, Elkhart, IN) in incomplete Freund's adjuvant 21 days after infection with RSV. On day 35 the

mice received an intranasal challenge of 10 µg CRA to localize the inflammatory response to the airways. Mice were then re-challenged 7 days later (day 42) by intratracheal administration of 10 µg in a 40-µl volume, and parameters of allergic lung disease were analyzed 24 h later.

Assessment of gene expression by real-time RT-PCR

RNA was isolated from lung tissue following homogenization in TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA (5 µg) was reverse transcribed in a 25-µl volume. mRNA expression was determined in 1–2 µl cDNA by Taqman Real time PCR using a Prism 7700 sequence detection system (PE Biosystems, Foster City, CA) using gene specific primers and probes labeled with 5'-6-FAM and 3'-TAMRA. The primers and probes used for the detection of mRNA expression were determined using pre-developed Primer/Probe sets (PE Biosystems, Foster City, CA). Reactions were incubated for 2 min at 50°C, denatured for 10 mins at 95°C, and subjected to 40 two-step amplification cycles with annealing/extension at 60°C for 1 min followed by denaturation at 95°C for 15 s. A pre-developed primer/probe set for murine GAPDH (PE Biosystems) was used as an internal control for quantification of the total amount of cDNA used in the reaction. Results are normalized to GAPDH expression and presented as fold increase in mRNA expression compared to the level detected at day 0.

ELISA analysis

Lung samples were prepared for ELISA analysis by homogenization in buffer containing Complete™ protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and 0.05% Triton X (Sigma Chemical Co, St Louis, MO). Murine cytokines and chemokines concentrations were determined using a standardized sandwich ELISA technique [47]. Briefly, Maxisorp™ ELISA plates (Fisher Scientific, Pittsburgh, PA) were coated with 1–5 µg/ml polyclonal capture antibody (R&D Systems, Minneapolis, MN) in coating buffer (0.6 M NaCl, 0.26 M H₃BO₄, 0.08 M NaOH; pH 9.6) overnight at 4°C and washed with PBS containing 0.05% Tween-20. The antibodies used were the appropriate suggested pairs (R&D Systems). Nonspecific binding sites were blocked with 2% BSA in PBS for 1 h at 37°C. After washing, cell-free lung homogenate or standard was added to each well and incubated for 1 h at 37°C. Plates were subsequently washed and incubated with biotinylated polyclonal detection antibody (3.5 µg/ml) for 45 min at 37°C. After thorough washing, streptavidin-peroxidase (Bio-Rad Laboratories, Richmond, CA) was added to each well for 30 min at 37°C. Plates were washed again, and following addition of the chromogen substrate the absorbance was measured at 492 nm. 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.

Measurement of AHR

AHR was measured using a direct ventilation method on anesthetized trachea cannulated, and ventilated mice with a

Buxco™ mouse plethysmograph (Buxco, Troy, NY) as previously described [8]. Briefly, mice were anesthetized with sodium pentobarbital, intubated via cannulation of the trachea with an 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). Mice were sealed within the plethysmograph for 5 min before baseline airway resistance readings were taken. A dose-response curve to i.v. methacholine was performed to determine the optimal dose required to induce a 1.5- to 2-fold increase in airway resistance in untreated mice. Peak airway resistance was subsequently recorded following injection of 10 µg methacholine/mouse. Data are mean change ± SE in airway resistance (cm H₂O/ml/s) for five animals, and are representative of data obtained in three separate experiments.

Histological analysis of lung inflammation

Bronchoalveolar lavage fluid was collected after lungs were instilled with 1 ml saline. Leukocytes were pelleted by centrifugation, cytospun on to glass slides and stained with hematoxylin and eosin (H&E). Three hundred cells were counted on each slide and the proportion of different leukocyte subtypes calculated. Whole lungs were then dissected and fixed by inflation with formalin. The lungs were maintained in formalin for a further 24 h before being processed into paraffin using standard histological techniques. Lung tissue sections (5 µm thick) were stained with H&E for analysis of peribronchial eosinophil accumulation and alcian blue/periodic acid-Schiff stain for assessment of mucus production. Peribronchial eosinophil counts were analyzed by counting the number of eosinophils surrounding the airways in 50 high power fields (×1,000).

Flow cytometric analysis of lung homogenates

Lung samples were digested in 0.2% collagenase (type IV) in DMEM containing 15% FCS for 45 min at 37°C. Cells were washed in DMEM (15% FCS) and resuspended in DPBS containing 5% FCS and 0.05% sodium azide. The number of dispersed cells in each sample was calculated, and 0.5 × 10⁶ cells were stained with 1 µg anti-CD4 PE or anti-CD8 FITC (BD PharMingen, San Diego, CA) for 20 min at 4°C. Cells were subsequently washed and fixed in 2% paraformaldehyde prior to flow cytometric analysis. The percentage of CD4⁺ or CD8⁺ T cells in the samples was determined by flow cytometry. The total number of each of these cell types in the lung homogenate was then calculated.

Statistical analysis

All results are expressed as mean ± SE. Statistical significance was calculated by ANOVA or unpaired Student's *t*-test to calculate the two-tailed *p* value. Significance was determined as values of *p* < 0.05.

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