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CCL20/CCR6 blockade enhances immunity to RSV by impairing recruitment of DC

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Chemokines are important mediators of the immune response to pathogens, but can also promote chronic inflammatory states. Chemokine receptor 6 (CCR6) is found on immature DC and effector/memory T cells, and binds a single ligand, CCL20, with high affinity. Here, we investigated the role of CCL20 and CCR6 in a pulmonary viral infection caused by RSV, a ubiquitous virus that can cause severe pulmonary complications. Neutralization of CCL20 during RSV infection significantly reduced lung pathology and favored a Th1 effector response. CCR6-deficient animals recapitulated this phenotype, and additionally showed enhanced viral clearance when compared with WT mice. No differences were observed in migration of T cells to the lungs of CCR6$^{-/-}$ animals; however, a significant reduction was observed in numbers of conventional DC (cDC), but not plasmacytoid DC, in CCR6$^{-/-}$ mice. A pathogenic phenotype could be reconstituted in CCR6$^{-/-}$ mice by supplying cDC into the airway, indicating that mere number of cDC dictates the adverse response. Our data suggest that blockade of the CCL20/CCR6 pathway provides an environment whereby the attenuated recruitment of cDC alters the balance of innate immune cells and mediates the efficient antiviral response to RSV.

Key words: Chemokines · DC · Mucosal immunity

Introduction

RSV is a pervasive virus that is the most common cause of hospitalization in children under the age of 2 [1]. RSV can also adversely affect the elderly and immunocompromised individuals, causing severe lower respiratory tract infection [2]. Although both Th1 and Th2 effector responses may be generated, Th2 immunity is responsible for RSV-associated pathology, including airway damage and mucus hypersecretion [3]. RSV represents a recurrent problem throughout life because immunologic memory never fully develops [4]. Furthermore, studies have demonstrated a correlation between early exposure to RSV and the later development of asthma [5, 6]. No vaccine currently exists, and early attempts to develop a vaccine proved detrimental, as individuals inoculated with a formalin-inactivated form of virus demonstrated enhanced pulmonary eosinophilia and Th2 responses [7]. Clearly, further investigation is needed to clarify the fine balance between immune protection and pathology during RSV infection.

Chemokines are key mediators of leukocyte recruitment during pathogenic insult, and also play a prominent role in homeostasis [8]. Most chemokines are promiscuous in that they can bind multiple receptors. CC chemokine receptor 6 (CCR6) is unique in the latter regard in that it binds a single chemokine, CCL20 [9]. CCL20 is a homeostatic chemokine, with a prominent role in organizing lymphoid tissue in the gut [10], but is also upregulated upon pro-inflammatory stimulation [11]. This dual function of CCL20 is evident in the cells expressing its corresponding receptor, CCR6, and contributes to a role for these cells in various immune settings.

CCR6 is found on immature DC, B cells, effector/memory T cells and T regulatory cells [12–15]. Studies have identified CCR6 as contributing to the pathology of inflammatory conditions such as

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asthma [16], autoimmune disorders such as rheumatoid arthritis [17] and graft *versus* host disease [18]. However, studies examining the effect of CCR6 deficiency on pathogen clearance showed defective DC recruitment, resulting in reduced T-cell-mediated control of infection [19, 20]. Thus, CCR6 contributes to both immune pathogenesis and appropriate immunity to pathogens.

Herein, we investigated the impact of CCR6 deficiency on appropriate immunity to RSV, and the contribution of DC and T cells to the response. We used a blocking Ab to CCL20 in conjunction with CCR6-deficient mice to show that RSV-induced pathology was attenuated in these animals, and a predominant Th1 effector response was generated. $CCR6^{-/-}$ mice had a significant decrease in conventional DC (cDC) in the lungs, however, plasmacytoid DC (pDC), which have been demonstrated to limit viral replication and modulate immunopathology following RSV infection [21, 22], were recruited equally. Several studies have elucidated the differential roles played by cDC and pDC in the lung, and suggest a critical balance between these DC subsets in order for appropriate responses to occur to both innocuous and pathogenic stimuli [21, 23]. Our data support this concept, suggesting that appropriate immunity to RSV involves an altered balance of DC subsets, where reduced recruitment of cDC promotes a beneficial Th1-based environment.

Results

Treatment with anti-CCL20 alters the immune response to RSV infection

Previous studies demonstrated a pathological role for CCR6 in mouse models of disease [16, 24, 25]. To determine whether CCR6/CCL20 played a role during RSV-induced disease, we infected mice intratracheally with 5×10^4 PFU RSV and assayed for production of CCL20 in BALF. We found increased levels of CCL20 at days 1 and 2 post-RSV challenge (Fig. 1A), indicating

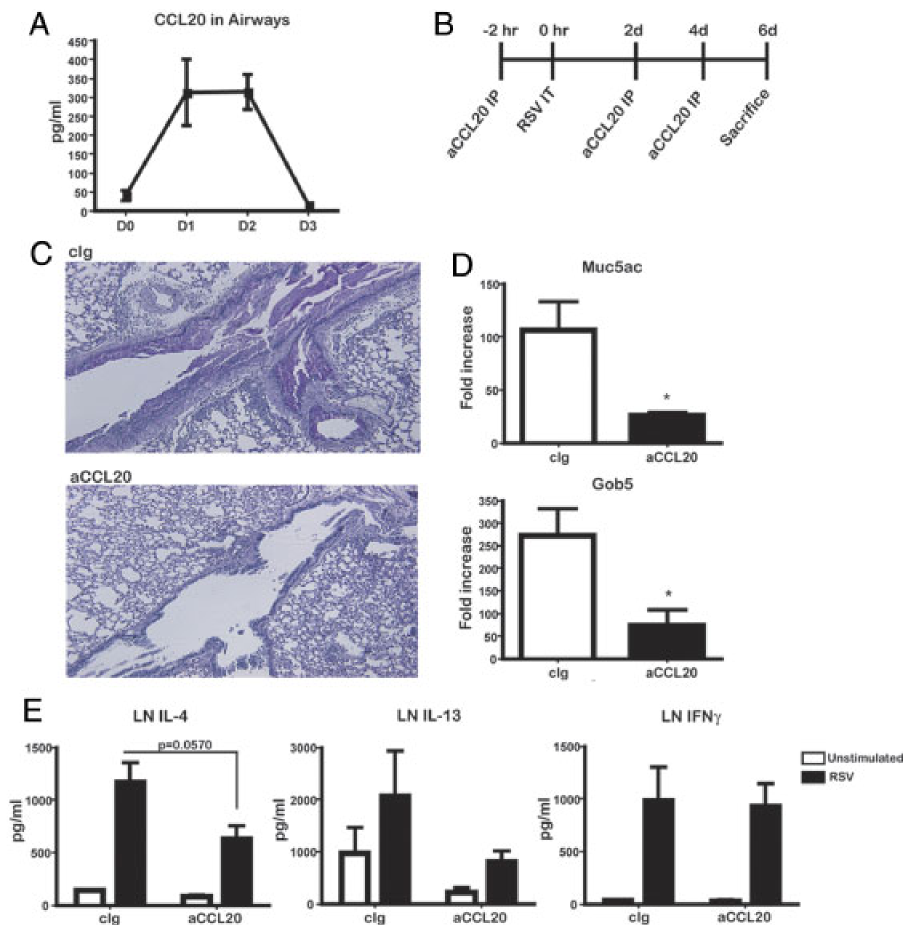

Figure 1. Anti-CCL20-treated mice have reduced lung pathology and lower Th2 cytokines. Mice were infected with 5×10^4 PFU of RSV/mouse *via* intratracheal injection. Lungs and lymph nodes were harvested at day 6 post-infection for analysis. (A) Protein levels of CCL20 were determined by ELISA in BALF of BALB/c mice after RSV infection. Data show mean ± SE from five mice/timepoint. (B) Schematic of anti-CCL20 Ab administration to BALB/c mice. (C) Histology of mucus production in Ab-treated mice. Data are representative of samples from four mice/group, magnified 100×. (D) Expression of *Muc5ac* and *Gob5* was assessed in lung samples by RT-PCR. Data are expressed as fold increase over uninfected mice and show mean+SE from five mice/group. *$p<0.05$. (E) RSV-specific T-cell cytokines were determined by Bioplex in lymph node cultures restimulated with RSV. Data show mean+SE from five mice/group. *$p<0.05$. All data shown are representative of two independent experiments.

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that CCL20 is important in mediating leukocyte recruitment early upon infection with RSV.

To determine whether CCL20 had a pathogenic role, BALB/c mice were treated with a neutralizing Ab to CCL20 prior to and during infection with RSV (Fig. 1B). Histological examination of lungs revealed a decrease in mucus production in mice treated with anti-CCL20 (Fig. 1C). Consistent with this, anti-CCL20-treated mice had significantly reduced expression of the mucus-associated genes, *Muc5ac* and *Gob5* (Fig. 1D). RSV-specific T-cell responses were next assessed by restimulating lymph node cultures with RSV. Mice treated with anti-CCL20 exhibited reduced expression of the Th2 cytokines, IL-4 and IL-13, but showed no difference from control-treated mice in the Th1 cytokine, IFN-γ (Fig. 1E). Together, these studies indicate that CCR6-CCL20 plays a pathological role during RSV infection, and influences the nature of the T-cell response.

Differential expression of CCR6 on leukocytes during RSV infection

To investigate the contribution of leukocytes during RSV infection, the expression pattern of CCR6 on subsets of DC and T cells was examined in the lung by flow cytometry. A significant increase was seen in numbers of CCR6$^+$ MHC II$^+$ CD11b$^+$ CD11c$^+$cDC on day 2 following RSV infection (Fig. 2A). An insignificant increase was seen in CCR6$^+$ CD11c$^+$ B220$^+$pDC (Fig. 2B). When T-cell subsets were assessed, a significant increase was found in numbers of CCR6$^+$ CD69$^+$(activated) CD4$^+$ T cells at day 6 post-infection

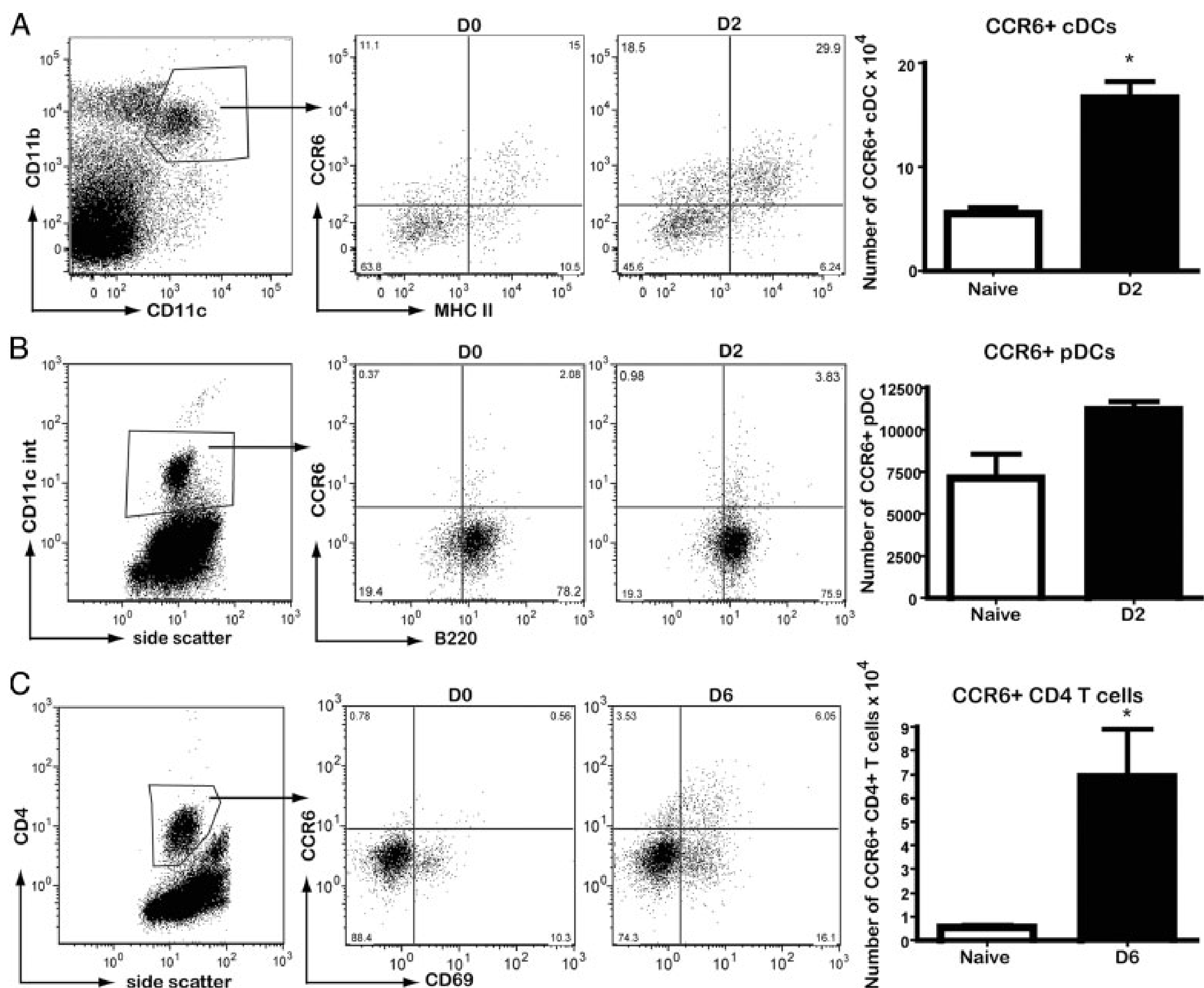

Figure 2. CCR6$^+$ cDC and CCR6$^+$ CD4 T cells are increased in lung after RSV infection. Mice were infected with 5×10^4 PFU of RSV/mouse *via* intratracheal injection. Lungs were harvested at day 6 post-infection for analysis. (A) CCR6$^+$ cDC were assessed in lung tissue by flow cytometry. Autofluorescence was gated out and cells were gated on the CD11b$^+$CD11c$^+$ population and analyzed for MHC class II and CCR6 expression. Graph shows total numbers of cDC in each sample and represents the mean+SE from five mice/group. *$p<0.05$. (B) CCR6$^+$ pDC were assessed in lung tissue by flow cytometry. Samples were gated on the lymphocyte cell population and the CD11c^{int} population was analyzed for CCR6 and B220 expression. Graph shows total numbers of pDC in each sample and represents the mean+SE from five mice/group. (C) CCR6$^+$CD69$^+$ CD4$^+$ T cells were assessed in lung tissue by flow cytometry. Samples were gated on the lymphocyte population and the CD4$^+$ population was analyzed for CCR6 and CD69 expression. Graph shows total numbers of T cells in each sample and represents the mean+SE from five mice/group. *$p<0.05$. All data shown are representative of two independent experiments.

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(Fig. 2C), but not CCR6$^+$ CD69$^+$ CD8$^+$ T cells (data not shown). These data indicate that CCR6-expressing cDC and CD4 T cells are increased in the lung after RSV infection, and thus may play a role in the anti-viral immune response.

CCR6$^{-/-}$ mice display reduced pathology but control RSV more efficiently

To assess the mechanism and specific cells involved in the altered pathogenic phenotype seen in mice treated with anti-CCL20, mice deficient in CCR6 were used in our RSV model. Like anti-CCL20-treated mice, CCR6$^{-/-}$ mice demonstrated reduced mucus production in the lungs, as shown histologically (Fig. 3A), and by significantly lower expression of *Gob5* (Fig. 3B). CCR6$^{-/-}$ mice also exhibited significantly lower airway resistance compared with WT mice (Fig. 3C).

To determine whether the decreased pathology observed in CCR6$^{-/-}$ animals affected their ability to control infection with RSV, whole lungs were isolated at day 3 to determine viral titer. CCR6$^{-/-}$ mice had significantly fewer plaques than WT mice, suggesting that CCR6$^{-/-}$ animals were better able to control viral replication (Fig. 3D). To insure that both strains were infected equally, transcript levels of the RSV G protein were measured by RT-PCR, with no differences found at either day 1 or day 2 post-infection (Fig. 3E). Because CCR6$^{-/-}$ animals had more efficient handling of RSV, we examined early recruitment of NK cells, which have been shown to participate in RSV clearance prior to T-cell involvement [26]. Paradoxically, a 50% reduction in NK cells was seen in CCR6$^{-/-}$ mice at day 2, suggesting that other innate cells are involved in the early anti-viral response to RSV in these animals (data not shown). Thus, CCR6$^{-/-}$ mice appear to have a decreased pathological response to RSV coincident with more efficient viral clearance.

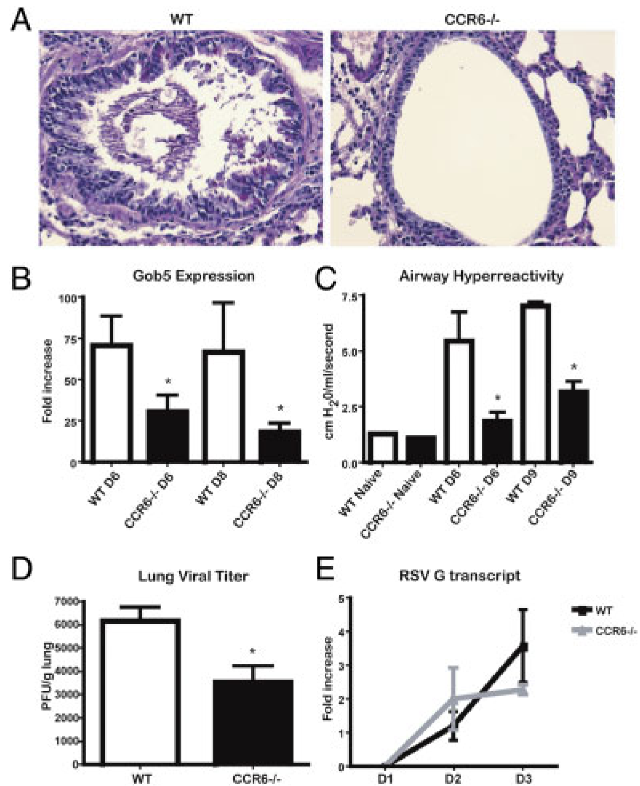

Figure 3. CCR6$^{-/-}$ mice have reduced lung pathology and more efficient viral clearance. Mice were infected with 5×10^4 PFU of RSV/mouse *via* intratracheal injection. Lungs were harvested at days 1, 2 and 3 to assess viral transcript levels and titer, and at days 6, 8 and 9 for analysis of mucus and airway hyperreactivity. (A) Mucus production in the lung was assessed in CCR6$^{-/-}$ mice by histology. Data are representative of samples from four mice/group, magnified $200\times$. (B) *Gob5* gene expression was assessed in lung samples by RT-PCR. Data are expressed as fold increase over uninfected mice and represent the mean+SE from four mice/group/experiment; data are pooled from two experiments. *$p<0.05$. D6 and D8 represent days 6 and 8 post-infection, respectively. (C) Airway hyperreactivity was determined using whole body plethysmography. Data represent the mean$\pm$SE from four mice/group. *$p<0.05$. D6 and D8 represent days 6 and 8 post-infection, respectively. (D) RSV viral titer was determined by plaque assay at day 3 post-infection. Data represent the mean+SE from five mice/group. *$p<0.05$. (E) RSV protein G transcript levels were measured in lung samples by RT-PCR. Data are expressed as fold increase over WT-challenged mice on day 1, and represent the mean$\pm$SE from four mice/group. All data shown are representative of three independent experiments.

Altered T-lymphocyte response in CCR6$^{-/-}$ mice

To examine the T-cell phenotype in CCR6$^{-/-}$ animals after RSV infection, migration of CD69$^+$ CD4 T cells into the lungs was assessed by flow cytometry. No differences were seen between WT and CCR6$^{-/-}$ mice in numbers of activated CD4$^+$ T cells at days 6 or 8 (Fig. 4A). We also assayed for this subset of T cells in the lymph nodes and likewise found no differences between the groups (data not shown).

RSV-specific effector T-cell responses were next assessed by lymph node restimulation with RSV. CCR6$^{-/-}$ mice produced significantly less IL-4 and IL-13, but exhibited no difference from WT mice in IFN-γ production (Fig. 4B, left panel). In support of this data, lymph node cells stimulated with anti-CD3/anti-CD28 also demonstrated Th2 skewing by WT animals (Fig. 4B, right panel). Recent work has identified CCR6 to be a primary receptor on Th17 cells [15]. In our model, we found RSV-specific induction of IL-17, but no difference in this induction, or in the recruitment of Th17 cells, by CCR6$^{-/-}$ mice (data not shown). This is not surprising, as studies have shown that Th17 development is inhibited by Type I IFN [27, 28]; furthermore, Hashimoto *et al.* found augmented IL-17 in mice deficient for STAT1, the transcription factor responsible for IFN-α/β expression, after infection with RSV [29]. Thus, IL-17 does not appear to play a role in influencing the phenotype of CCR6$^{-/-}$ animals after RSV infection.

Effector cytokine expression was then examined in the lungs of WT and CCR6$^{-/-}$ mice by RT-PCR. Similar to the response generated in the lymph node, CCR6$^{-/-}$ mice expressed significantly lower levels of IL-4 and IL-13 (Fig. 4C). However, reduced expression of IFN-γ was also seen in CCR6$^{-/-}$ mice (Fig. 4C). As we saw similar numbers of CD69$^+$ CD4 T cells in the lungs of WT

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and CCR6$^{-/-}$ animals, we examined the intracellular cytokine profile of lung CD4 T cells. Consistent with the expression data, CCR6$^{-/-}$ animals displayed reduced numbers of IL-4^{+} and IFN-γ^{+} CD4 T cells in the lung (Fig. 4D). Intracellular cytokine staining in the lymph node likewise reflected our Bioplex data, with CCR6$^{-/-}$ animals showing reduced numbers of IL-4^{+} CD4 T cells but similar numbers of IFN-γ^{+} CD4 T cells compared with WT animals (data not shown). Together, these data indicate that after infection with RSV, CCR6$^{-/-}$ mice exhibit an altered Th cytokine phenotype in lungs and lymph nodes, with a less pathogenic profile of cytokines in the lung.

To further examine the cytokine milieu, BALF was collected early after RSV infection and chemokines associated with a Th1 immune environment were assessed [30]. Significantly, higher production of CXCL10 and CCL5 was found in the BALF of CCR6$^{-/-}$ mice; CXCL9, which binds the same receptor as CXCL10, was also elevated, but was not significantly different in CCR6$^{-/-}$ mice (Fig. 4E). Thus, the early milieu in CCR6$^{-/-}$ animals may influence the subsequent response by providing a more favorable environment for activation of appropriate subsets of effector cells.

cDC promote Th2 pathology upon RSV infection

Because CCR6$^{-/-}$ animals cleared virus more efficiently and showed an overall decrease in Th effector cytokine production in the lung, we hypothesized that innate cells, such as macrophages and DC, were influencing the early antiviral response to RSV. Macrophage recruitment was first assessed by flow cytometry, with no difference found between the strains in macrophage numbers (data not shown). Interestingly, a recent study demonstrated that macrophage depletion had no effect on T-cell responses to RSV infection [31]. DC, on the other hand, both participate in activating RSV-specific T cells (cDC) [32], as well as promote direct anti-viral immunity through production of IFN-α (pDC) upon RSV stimulation [33]. When cDC and pDC subsets were assessed, CCR6$^{-/-}$ mice had significantly fewer cDC recruited into the lungs at day 2 post-infection (Fig. 5A). However, no difference was found in numbers of pDC (Fig. 5B). Consistent with the latter data, no difference was found in the production of IFN-α in whole lung and BALF of WT and CCR6$^{-/-}$ mice (data not shown). To further confirm that the defective trafficking of cDC in CCR6$^{-/-}$ animals was due to the

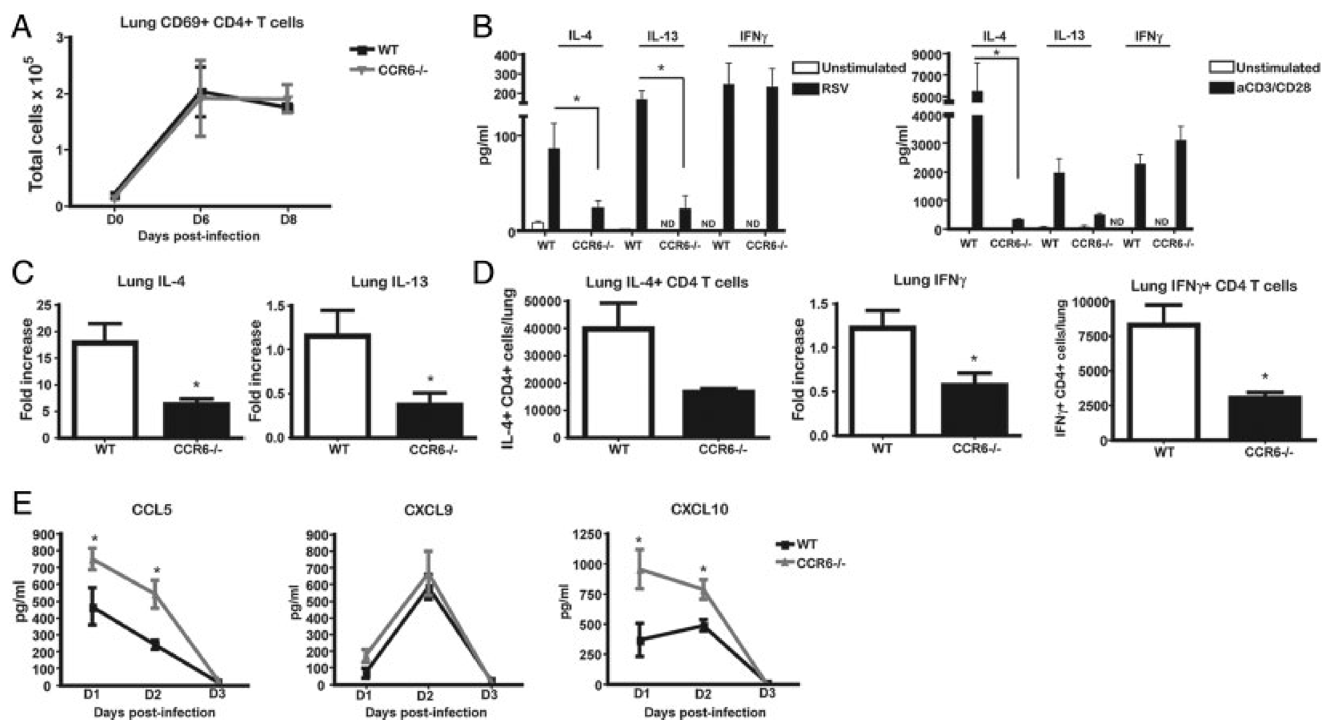

Figure 4. CCR6$^{-/-}$ mice have an altered T-cell response following RSV infection. Mice were infected with 5×10^{4} PFU of RSV/mouse *via* intratracheal injection. Lungs were harvested at days 6 and 8 to assess T-cell recruitment, and both lungs and lymph nodes were harvested at day 6 for cytokine analysis. (A) Recruitment of CD69^{+} CD4^{+} T cells to the lungs was determined by flow cytometry. Data represent the mean$\pm$SE from four mice/group. (B) RSV-specific T-cell cytokines were assessed by Bioplex in lymph node cultures at day 6 post-RSV infection. Left panel: lymph node cultures restimulated with RSV. Data represent the mean+SE from four mice/group. *$p<0.05$. Right panel: lymph node cultures restimulated with anti-CD3/anti-CD28. Data represent the mean+SE from five mice/group. *$p<0.05$. (C) Cytokine gene expression was determined by RT-PCR in lung samples at day 6 post-RSV infection. IL-13 and IFN-γ are expressed as fold increase over WT-challenged samples, and IL-4 expressed as fold increase over unchallenged samples. IL-4 and IL-13 data represent the mean+SE from four mice/group; IFN-γ data represent the mean+SE from four mice/group/experiment, data are pooled from three experiments. *$p<0.05$. (D) Intracellular cytokine staining was assessed by flow cytometry in lung samples at day 6 post-RSV infection. Total IL-4^{+} CD4^{+} and IFN-γ^{+} CD4^{+} cells are shown. Data represent the mean+SE from five mice/group. *$p<0.05$. (E) Th1-associated chemokines were assessed by Bioplex in BALF. Data represent the mean+SE from four mice/group. *$p<0.05$. Data shown in (A–C) are representative of three independent experiments; data in (D) and (E) are representative of two independent experiments.

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absence of CCR6, we assayed for $CCR6^+$ cDC in animals treated with anti-CCL20 and infected with RSV. We found significantly reduced numbers of $CCR6^+$ cDC in mice that were administered anti-CCL20 (Fig. 5C). Together with our T-cell data, these studies suggest that the absence of early recruitment of cDC alters the immune response to RSV in $CCR6^{-/-}$ animals, potentially by abrogating the Th2 response while maintaining effective IFN-γ production.

To test whether cDC administration to $CCR6^{-/-}$ animals could recapitulate the lung pathology and Th2-biased responses seen in WT mice, 5×10^5 WT BMDC were transferred intratracheally [34–36] into $CCR6^{-/-}$ animals immediately prior to intranasal administration of RSV. Upon histological analysis, $CCR6^{-/-}$ mice receiving either WT or $CCR6^{-/-}$ cDC had increased mucus production compared with $CCR6^{-/-}$ mice not receiving cDC (Fig. 5D). Measurement of *Muc5ac* and *Gob5* gene expression supports the histology (Fig. 5E). When RSV-specific cytokine responses were assessed, $CCR6^{-/-}$ mice receiving either WT or $CCR6^{-/-}$ cDC produced elevated levels of IL-4 and IL-13, but showed no difference in IFN-γ compared with $CCR6^{-/-}$ mice not receiving cDC (Fig. 5F). While the Th2 cytokine responses were not statistically significant, the trends along with the pathology suggest that merely supplying additional cDC, regardless of CCR6 expression, partially reconstitutes the response. This supports a role for cDC in promoting the Th2-biased pathology after RSV infection in WT mice, and that CCR6 mediates the recruitment of this subset of DC into the lung.

$CCR6^{-/-}$ cDC have competent antigen presenting function

The above data demonstrates that $CCR6^{-/-}$ cDC are capable of priming a Th2 response when transferred into the lungs of $CCR6^{-/-}$ animals. To further confirm the functional capacity of $CCR6^{-/-}$ cDC *in vitro*, cDC ability to restimulate a primed RSV T-cell response was examined. BM-derived cDC were stimulated with RSV and co-cultured with $CD4^+$ T cells isolated from lymph nodes of WT and $CCR6^{-/-}$ mice at day 8 post-RSV infection. In agreement with our lymph node restimulation data, $CCR6^{-/-}$ T cells were skewed toward a Th1 phenotype, irrespective of origin of DC in the culture (Fig. 6A). Thus, $CCR6^{-/-}$ cDC are capable of antigen presentation to the same extent as WT cDC.

Next, cDC contribution to a primary T-cell response was examined using DO11.10 mice. cDC were pulsed with OVA peptide and co-cultured with $CD4^+$ T cells isolated from the spleens of DO11.10 mice. Upon analysis of cytokine production, $CCR6^{-/-}$ cDC were equally able to elicit Th1 and Th2 cytokine production from OVA-specific T cells (Fig. 6B). Together, these studies suggest that cDC from $CCR6^{-/-}$ animals are capable of priming both Th1 and Th2 responses, but upon RSV infection, $CCR6^{-/-}$ cDC are skewed by their *in vivo* environment, allowing them to preferably prime a Th1 response.

Discussion

This study investigated the role of CCR6 and its ligand, CCL20, in a model of pulmonary viral infection induced by RSV. Previous studies demonstrated this receptor to be essential for pathogen clearance in the gut, such as in models using *Salmonella typhimurium* [19] and murine enteric rotavirus [37], as well as in the lung, using *Aspergillus fumigatus* [38]. However, CCR6 has also been implicated in the pathology of diseases such as asthma, inflammatory bowel disease and psoriasis [39–41]. In our studies, mice treated with an Ab to CCL20 and infected with RSV exhibited significantly decreased mucus production in the lung and generated a T-cell response favoring IFN-γ-production. Further investigation using $CCR6^{-/-}$ mice showed that these animals likewise had attenuated RSV-induced pathology and a predominant Th1 effector response when compared with WT mice. Examination of leukocyte trafficking to the lungs of $CCR6^{-/-}$ animals revealed no differences in T-cell accumulation, but significant decreases in early cDC recruitment. Effector T-cell cytokines were reduced in $CCR6^{-/-}$ animals; however, viral clearance was enhanced. Together, these data suggest that the early pulmonary environment, characterized by a reduction in cDC recruitment, is key in shaping the subsequent immune response to RSV by $CCR6^{-/-}$ mice.

Several recent studies have demonstrated the importance of pulmonary DC in mediating the immune response to RSV. Different subsets of cDC, including $CD103^+$ and $CD103^-$ populations, migrate to the lymph nodes and activate RSV-specific CD4 and CD8 T cells [32], while pDC mobilize directly to mucosal tissue upon RSV infection [32, 42]. Interestingly, RSV has been shown to interfere with cDC and pDC function, resulting in reduced cytokine production and impaired T-cell activation [43, 44]. In the latter study, T-cell activation was assessed using proliferation assays, whereas our studies examined RSV-specific effector cytokine production. It is likely that these pathways are differently regulated and therefore account for the differences seen in T-cell activation. The DC studies suggest that subversion of DC function may impact on the inadequate long-term immunity to RSV infection. Thus, DC clearly play a part in shaping the anti-viral response.

To further investigate the role of DC during RSV infection, we infected mice that were deficient in the mucosa-specific chemokine receptor, CCR6, which mediates cDC migration to the lung upon inflammatory stimuli [45]. When stimulated with RSV, cDC become activated and upregulate the costimulatory molecules CD40, CD80 and CD86, and when pulsed with OVA and RSV, prime OVA-specific Th1 and Th2 responses [33]. In our studies, $CCR6^{-/-}$ cDC were defective in their ability to traffic to the lung, but were not deficient in their capacity to prime either Th1 or Th2 responses using an *in vitro* co-culture assay with OVA-specific T cells. *In vivo*, however, $CCR6^{-/-}$ mice generated a predominant Th1 response, and reconstitution of these mice with either WT or $CCR6^{-/-}$ cDC reverted the phenotype to the mixed Th1/Th2 response, and enhanced lung pathology, seen in WT mice. These latter data indicate that the alteration observed in the

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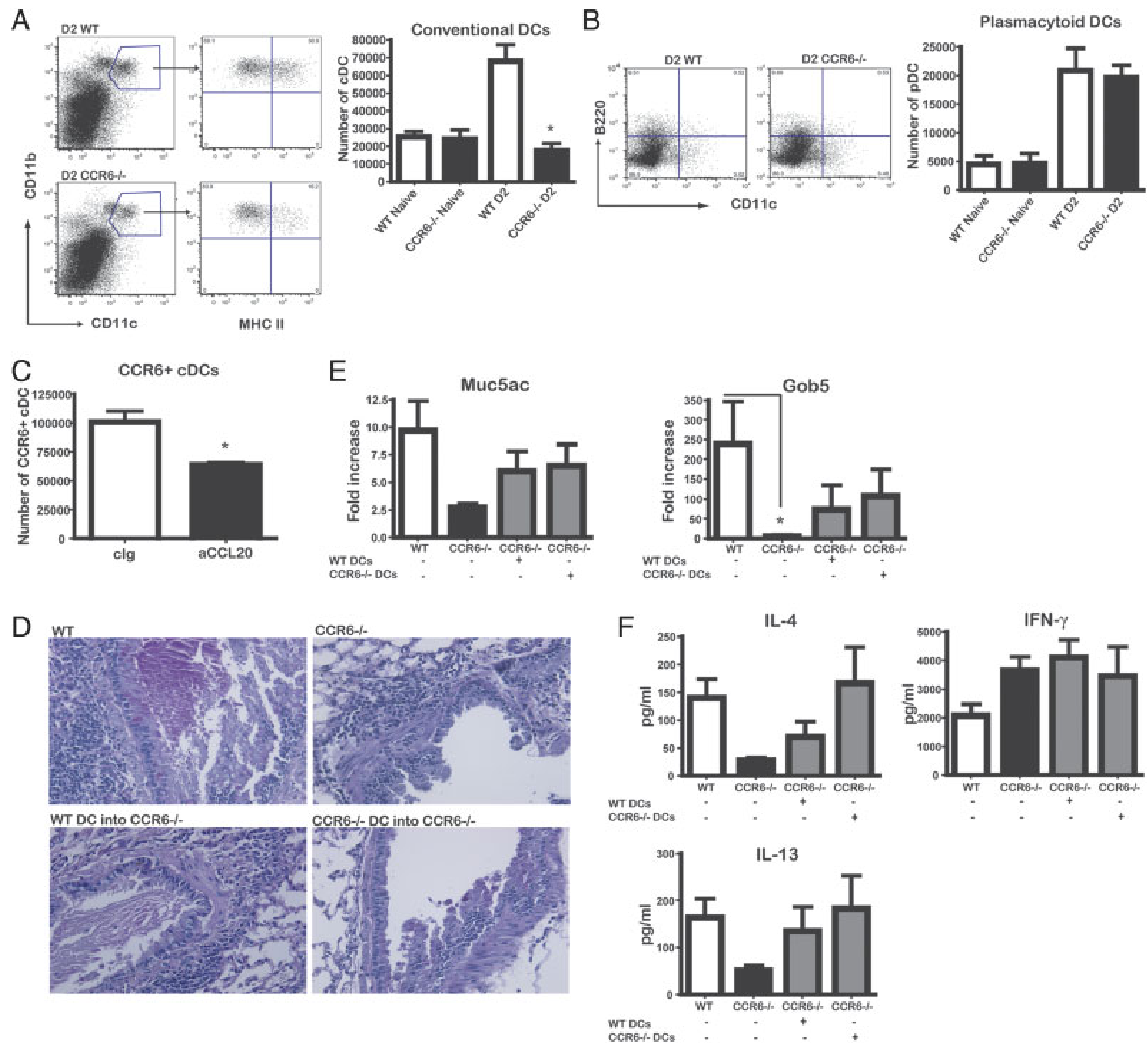

Figure 5. CCR6$^{-/-}$ mice have reduced cDC recruitment, and reconstitution of animals with cDC promotes Th2 pathology. (A) Lung cDC recruitment was assessed by flow cytometry in WT and CCR6$^{-/-}$ mice. Lung cells were gated on CD11b and CD11c expression and analyzed for MHC class II expression. Graph shows mean number of cDC+SE from four mice/group. *$p<0.05$. (B) Lung pDC recruitment was assessed by flow cytometry in the same mice as (A) gating on CD11c$^+$B220$^+$ cells. Data represent the mean+SE from four mice/group. (C) CCR6$^+$ cDC recruitment was assessed by flow cytometry in RSV-infected animals treated with anti-CCL20. Data represent the mean+SE from five mice/group. *$p<0.05$. (D) Mucus production was assessed by histology in WT and CCR6$^{-/-}$ animals reconstituted with cDC. Shown are representative samples from five mice/group, magnified 400 ×. (E) Mucus-associated gene expression was determined in DC transfer groups by RT-PCR, and represented as fold increase over unchallenged samples. *x*-axis indicates recipient animals; cDC transferred are indicated below the *x*-axis. Data represent the mean+SE from five mice/group. *$p<0.05$. (F) RSV-specific T-cell cytokines were measured by Bioplex in lymph node cultures of DC transfer groups. Data represent the mean+SE from five mice/group/experiment; data are pooled from three experiments. Data shown in (A) and (D–F) are representative of three independent experiments; data in (B) and (C) are representative of two independent experiments.

CCR6$^{-/-}$ mice is related to the number of cDC migrating to the lung early in the response.

Alternatively, we found no defect in accumulation of pDC in the lungs of CCR6$^{-/-}$ mice after RSV infection, and this difference in migratory behavior between cDC and pDC highlights the distinct role these DC subsets play in the lung. For example, cDC activated in the lung have been shown to preferentially induce Th2 effector responses [46], and only under particular stimulatory conditions will induce a Th1 response, such as upon exposure to Type I IFN [47]. pDC, on the other hand, produce IFN-α upon stimulation with RSV [33], and this Type I environment likely influences the activational state of cDC. The influence of pDC during RSV infection has been verified by both depletion [21] and repletion [22] protocols, and demonstrates a beneficial role for pDC on viral clearance and lung pathology. Together with the current data, it is our view that the balance between pDC and

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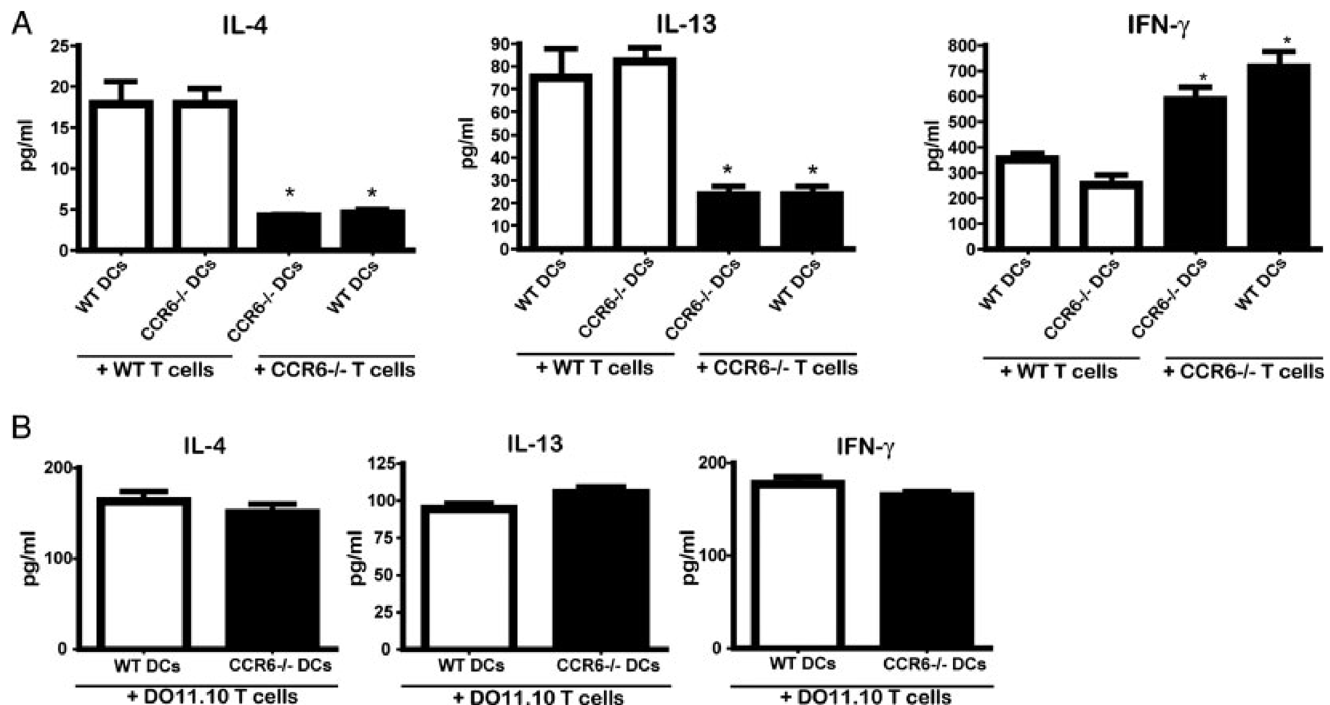

Figure 6. CCR6$^{-/-}$ cDC can prime both Th1 and Th2 T-cell responses. (A) T cells from RSV-challenged mice were co-cultured with RSV-primed CCR6$^{-/-}$ and WT DC to assess the T-cell response as determined by IL-4, IL-13 and IFN-γ secretion. Data represent the mean+SE from triplicates/group. *$p<0.05$. (B) OVA-specific, primary T-cell responses as determined by IL-4, IL-13 and IFN-γ secretion were measured after co-culture with OVA-primed CCR6$^{-/-}$ and WT DC. Data represent the mean+SE from three replicates/group. *$p<0.05$. All data shown are representative of three independent experiments.

cDC is critical for RSV clearance and the nature of the subsequent T-cell response.

In support of cDC promoting a pathogenic response are numerous studies demonstrating that they alone induce a skew toward Th2-associated disease. An original study that isolated cDC subsets indicated that the response to allergen was skewed toward a Th2 response exclusively by a subset of lung DC [48]. Subsequently, cDC have been classified into DC1 and DC2 subsets based on their ability to activate T cells into Th1 and Th2 effectors, respectively [49, 50]. Although this study has not characterized these subsets, it appears that CCR6 influences the recruitment of cDC in general and it may be the mucosal environment that dictates the ability of cDC to promote a pathogenic Th2 response. The nature of the response and environment can be influenced by specific mediators, including epithelial cell-derived thymic stromal lymphopoietin, which promotes the upregulation of OX40 on cDC and skews T cells toward a Th2 phenotype [51]. Other mediators, such as CXCL9, CXCL10 and CCL5, have been associated with promoting a Th1-skewed environment [30], and this study shows elevated production of these chemokines in BALF of CCR6$^{-/-}$ mice. Thus, the early cytokine environment along with the absence of a Th2-promoting cDC subset may favor generation of a predominant Th1 response.

Overall, using this infection model, WT mice generated IL-13 and IFN-γ Th effector responses to RSV, but in the absence of the CCL20/CCR6-associated immune pathway, mice instead initiated an IFN-γ-dominated antiviral response. cDC recruitment was impaired in CCR6$^{-/-}$ mice, yet the recruitment and function of pDC remained intact. When cDC, whether WT or CCR6$^{-/-}$, were provided into the lungs, the more pathogenic Th2 response was partially reestablished. This suggests a model whereby a cytokine milieu established by the altered balance of cDC and pDC in CCR6$^{-/-}$ mice favors the generation of a Th1-based, efficient antiviral response without the concurrent Th2-based pathology promoted by cDC.

Materials and methods

Mice

BALB/c and DO11.10 mice were purchased from Jackson Laboratories. CCR6$^{-/-}$ mice were provided by Dr. Sergio Lira. All animal work was performed in accordance with the University of Michigan Committee on Use and Care of Animals policy.

RSV

Our laboratory uses the antigenic subgroup A strain of RSV, referred to as Line 19, which induces RSV-associated disease [52].

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Ab administration

50 μg *per* mouse of rat anti-mouse CCL20 Ab, clone 114908 (R&D Systems, Minneapolis, MN, USA), was administered either intratracheally at the time of RSV infection, and 1 day after, until day 2, or intraperitoneally 2 h prior to infection, and every other day thereafter until day 6.

Histology

Left lobes were removed and perfused with 10% formalin. Tissue was paraffin-embedded and 5 μm sections were stained with periodic acid schiff to detect mucus production.

Real-time taqman PCR

Lung RNA was obtained using Trizol reagent *per* the Invitrogen protocol. Detection of cytokine mRNA was determined using predeveloped primer/probe sets (PE Biosystems, Foster City, CA) and analyzed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Gene expression was normalized to GAPDH.

Flow cytometry

Lung tissue was digested and dispersed to obtain single-cell suspensions. Cells were Fc-blocked, stained with fluorophore-conjugated Ab and fixed in 4% formalin. For intracellular cytokine staining, cells were first stimulated for 6 h with 50 ng/mL of PMA and 10 mM of ionomycin, with inclusion of protein transport inhibitor. Analysis was conducted using FlowJo software.

RSV-specific T-cell response

Mediastinal and cervical lymph nodes were harvested and single-cell suspensions obtained. Samples were plated at 1×10^6 cells *per* well and restimulated for 24 h with either 4×10^4 PFU RSV or 1 μg/mL of anti-CD3 and anti-CD28.

Airway response

Airway hyperreactivity was determined using whole body plethysmography with ventilation of anesthetized mice as described previously [52].

Viral plaque assay

Whole lungs were harvested and ground with sand using a mortar and pestle. Supernatants were serially diluted onto Vero cells and incubated for 5 days. Viral plaques were determined using a goat anti-human RSV polyclonal Ab (Chemicon International).

DC transfer

BM was cultured for 10 days in 10 ng/mL of murine GM-CSF (R&D Systems) to generate DC. Briefly, 5×10^5 DC were transferred intratracheally.

In vitro co-cultures

BMDC were pulsed for 2 h with either 4×10^4 PFU RSV or 1 μg/mL of OVA peptide (323–339, Peptides International) and incubated with $CD4^+$ T cells isolated from lymph nodes or spleen by MACS® cell separation (Miltenyi Biotec).

Statistical analysis

Data were analyzed using Prism GraphPad software. Unless otherwise specified, data shown are representative of two or more experiments. Statistical significance in all experiments was determined by one-way ANOVA followed by a Newman–Keuls *post hoc* test. Significant differences were regarded as $p<0.05$.

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Abbreviations: **cDC:** conventional DC · **pDC:** plasmacytoid DC · **RSV:** respiratory syncytial virus

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