

CCL2/CCR2-Dependent Recruitment of Th17 Cells but Not Tc17 Cells to the Lung in a Murine Asthma Model

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Key Words

Asthma · CC chemokine ligand 2 · Tc17 cells · Th17 cells ·
Airway inflammation

Abstract

Background: Interleukin (IL)-17 has been implicated in the pathogenesis of asthma and the progression of airway inflammation. Here, we used a model of allergic asthma and found that the frequencies of IL-17-secreting T helper (Th)17 and CD8 (Tc)17 cells were both significantly increased, as was the expression of the CC chemokine receptor (CCR2) on the surface of these cells. CC chemokine ligand 2 (CCL2) has been shown to mediate the activation and recruitment of inflammatory cells in asthma, which are also skewed after ovalbumin (OVA) challenge. However, the role of CCL2 on Th17 cells and Tc17 cells in asthma has not been illuminated. **Methods:** Mice that were sensitized and challenged with OVA received anti-CCL2 antibody (Ab; 5 µg/day intratracheally) or CCR2 antagonist (RS504393, 2 mg/kg/day intraperitoneally) prior to the challenge. Some mice received an isotype control Ab or vehicle alone. We then assessed the effects of allergic asthma and anti-CCL2 Ab or CCR2 antagonist treatment on the levels of IL-17 and CCL2, the Th17 and Tc17 cell frequencies and lung tissue inflammation. **Results:** We demonstrated that CCL2 and IL-17 levels and the frequency

of Th17 and Tc17 cells in lung tissues and bronchoalveolar lavage fluid increased in the asthma group compared with the normal control mice. Blocking the CCL2/CCR2 axis greatly reduced the Th17 but not the Tc17 cell frequency, and revealed a suppressive effect on airway inflammation. **Conclusion:** These findings indicate a role for the CCL2/CCR2 axis in mediating Th17 but not Tc17 cell migration during acute allergic airway inflammation.

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Introduction

Allergic asthma is a chronic inflammatory disease that is characterized by airway hyperreactivity, limited reversible airflow and airway inflammation. Many cell types are involved in its pathogenesis, including eosinophils, T cells, neutrophils, mast cells and dendritic cells as well as various inflammatory mediators. Numerous clinical and experimental observations have suggested that asthma is a heterogeneous disease which includes complexities that extend beyond the T helper type 2 (Th2) cell paradigms of increased secretion of interleukin (IL)-4, IL-5, IL-9 and IL-13. These observations also revealed that non-Th2-associated factors, such as IL-17, are frequently detected in the lungs of patients with asthma. These factors can de-

velop independently and mediate complex heterologous interactions in allergic asthma [1].

CCL2, also known as monocyte chemoattractant protein (MCP-1), is a member of the CC family of chemokines [2] that can be secreted by many cell types, including macrophages, lymphocytes, basophils, epithelial cells, airway smooth-muscle cells and neutrophils [3–8]. Accumulating evidence [9–12] indicates that CCL2 plays an important role in asthma. Asthma patients have significantly higher serum CCL2 levels than normal controls in the asymptomatic phase of the disease and also during an acute asthma attack [13]. In atopic-asthma patients, high levels of CCL2 were inversely correlated with forced expiratory volume in 1 s and forced expiratory flow at 50% vital capacity [14, 15]. In *Schistosoma mansoni*-challenged mice, CCL2 production has been found to increase significantly and be accompanied by an influx of leukocyte migration into the lung and subsequent airway hyperreactivity [16]. CCL2 mediates the activation and recruitment of inflammatory cells into the lung by binding to its specific receptor, CCR2 [3, 17]. Some studies have explored the relationship between the CCL2/CCR2 axis and inflammatory cells. Mellado et al. [17] found that neutralization of CCR2 attenuated macrophage and eosinophil accumulation in the bronchoalveolar lavage fluid (BALF) of asthmatic monkeys. Lukacs et al. [16] reported that neutralization of CCL2 significantly reduced total leukocyte migration, especially CD4+ and CD8+ lymphocytes, although it was not clear which T-cell subsets were involved.

Th17 cells, a proinflammatory subset of CD4+ T cells, were named for their production of IL-17. They have been shown to play a vital role in asthma by enhancing Th2-mediated eosinophilic airway inflammation and inducing neutrophil recruitment and airway hyperreactivity in Th17 cell-dependent neutrophilia [18–21]. Similar to Th17 cells, CD8+ T cells can differentiate into noncytotoxic, IL-17-producing cells in the presence of TGF- β and IL-6 in mixed lymphocyte cultures; they are categorized as the T noncytotoxic 17 (Tc17) cell subset [22]. Tc17 cells are another source of IL-17 and have been studied in asthma [23]. However, the precise role of CCL2 in Th17-cell-mediated or Tc17-cell-mediated inflammation in asthma has not been described. In this study, we found a direct association between increased CCL2 levels and Th17 cell recruitment to inflammatory sites in a murine asthma model. We found that CCL2 levels in the BALF and CCR2+ Th17 cell numbers in the lung were significantly increased. IL-17 levels and Th17 cell frequencies were reduced

when mice were treated with anti-CCL2 antibody (Ab) or a specific antagonist for CCR2. Furthermore, inflammatory cell infiltration in peribronchial and perivascular tissues was also diminished. However, after blockade of the CCL2/CCR2 pathway, we found no change in the percentage of Tc17 cells.

Materials and Methods

Mice

Female BALB/c mice (age: 6–8 weeks; weight: 16–22 g) were purchased from the Animal Experimental Center of Hubei Province. All mice were housed in specific pathogen-free-grade conditions at the Centers for Disease Control and Prevention of Hubei Province (animal certificate No. H20110338). All experiments conducted in this study were in accordance with the Animal Care and Use Committee of the Tongji Medical College, Huazhong University of Science and Technology.

Induction of Airway Inflammation and Administration of CCL2 Neutralizing Antibody or CCR2 Antagonist

The asthma model was constructed as before with minor modifications [24]. Briefly, mice were sensitized with 100 μ g ovalbumin (OVA; grade V, Sigma-Aldrich, St. Louis, Mo., USA) conjugated with 100 μ l aluminum hydroxide by intraperitoneal injection on days 0, 7 and 14. After 10 days, the mice were anesthetized with 10 mg/ml ketamine and 1 mg/ml xylazine diluted in sterile phosphate-buffered saline (PBS), and were then challenged with 200 μ g OVA or PBS (5 μ g/ μ l, dissolved in normal saline) intratracheally every other day for 3 consecutive doses. Some mice received anti-CCL2 Ab (R&D Systems Inc., Minneapolis, Minn., USA) or isotype control rat IgG2b Ab (5 μ g/mouse; R&D Systems) dissolved in PBS by intratracheal injection 1 h before challenge, as previously reported [25, 26]. For CCR2 receptor blocking, some mice received 2 mg/kg RS504393 (Tocris Bioscience, Ellisville, Mo., USA), a selective chemical antagonist of CCR2 [27] or a DMSO vehicle control by intraperitoneal injection prior to the challenge (fig. 1a).

BALF Analysis

BALF samples were obtained as before [28]. Mouse lungs were flushed via the trachea cannula with 0.8 ml PBS 3 times following the final OVA challenge. BALF was centrifuged at 200 g for 5 min. Supernatants were stored at -80°C for subsequent cytokine measurements. The total cell suspension was re-suspended in 1 ml PBS. Cells were counted using a hemocytometer. Differential cell counts were carried out after Diff-Quick staining.

Lung Homogenate Preparation and Inflammatory Mediator Measurements

Lung tissue suspensions were generated as before [29]. In brief, the right lung was dissected, then quickly frozen in liquid nitrogen and stored at -80°C . After thawing, lung tissues were homogenized in PBS and centrifuged at 1,000 g for 15 min at 4°C to remove sediment; supernatants were then harvested for the measurement of IL-17 levels. Levels of CCL2 in the BALF and IL-17

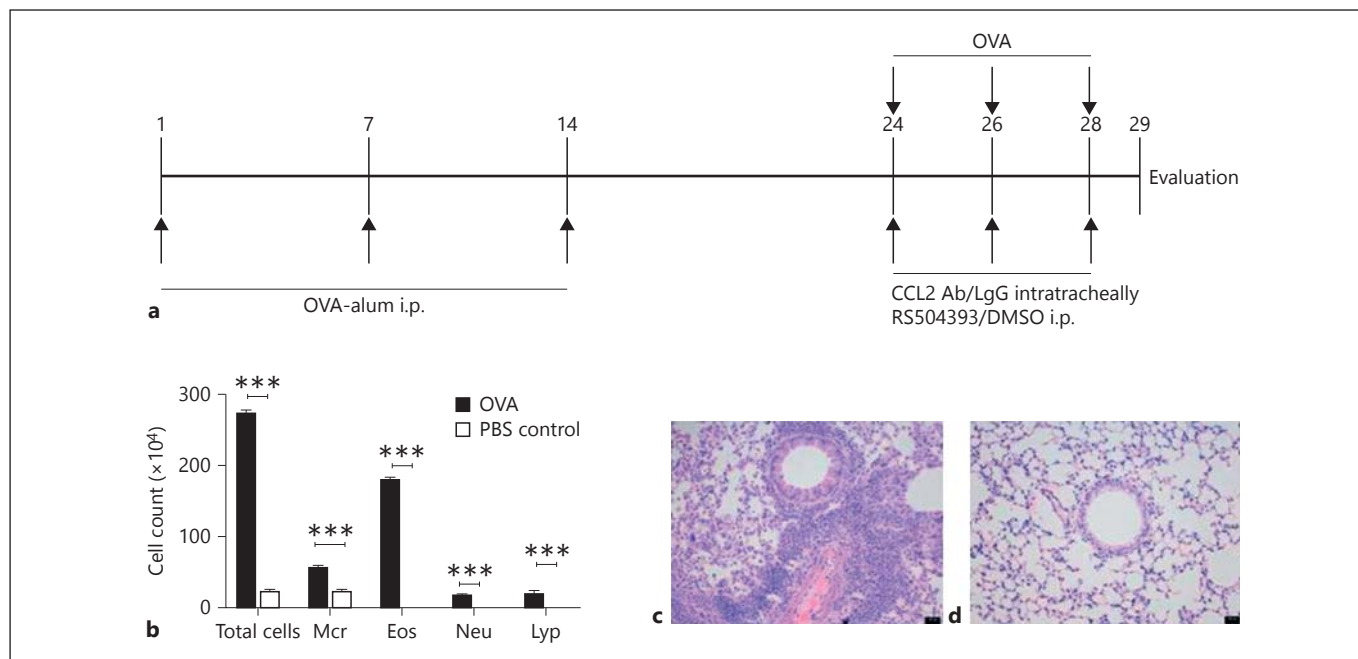


Fig. 1. OVA-induced airway inflammation in BALB/c mice: an outline of the protocol used for our OVA-induced asthma model. Mice were sensitized 3 times by intraperitoneal injection of OVA and alum on days 1, 7 and 14, followed by challenge with OVA solution (intratracheal) on days 24, 26 and 28. For some experiments, before each provocation, mice were treated with CCL2 Ab, isotypic control Ab, RS504393 or DMSO vehicle control. **a** Airway

inflammation was evaluated 24 h after the final OVA exposure. **b** The number of total cells, macrophages (Mcr), eosinophils (Eos), neutrophils (Neu) and lymphocytes (Lyp) in the BALF from OVA-challenged and control mice. **c, d** Lung tissue sections from OVA-challenged and control mice. HE. $\times 100$. Scale bars: 50 μm . *** $p < 0.0001$, compared with the respective control group by unpaired t test. Data indicate the means \pm SEM for groups of 4–6 mice.

in lung tissue homogenates were determined using ELISA kits (Neobioscience, Beijing, PR China) according to the manufacturer's instructions.

Lung Morphology and Immunofluorescence

Lung morphology examinations were performed as before [30]. Left lobes were filled with 4% paraformaldehyde and embedded in paraffin. Next, 5 μm -thick longitudinal sections were deparaffinized and stained with hematoxylin and eosin (HE). The severity of peribronchovascular inflammation was graded semiquantitatively as before [31]. The distribution of Th17 and Tc17 cells around bronchioles and peribronchovascular vessels was analyzed by immunofluorescence, as previously described [32].

Flow Cytometric Analysis of IL-17 and Chemokine Receptor Expression

BALF cells and lung tissues were stimulated with phorbol ester (50 ng/ml; Sigma) and ionomycin (1,000 ng/ml; Sigma) for 5 h. Brefeldin A (50 ng/ml; Sigma) was used to block cytokine secretion. Next, the cells were incubated with an appropriate concentration of PE-Cy5-conjugated anti-CD3 mAb and FITC-conjugated anti-CD8 mAb (both from Biolegend, San Diego, Calif., USA) for 30 min at 4°C in the dark. For CCR2 staining, APC-conjugated mAb was added to some samples. Cells were then fixed and permeabilized with cytofix/cytoperm solution (BD Biosciences, San Jose, Calif., USA) for intracellular cytokine IL-17A staining. Flow

cytometry was performed on a BD LSR-II cytometer to quantify the frequencies of CCR2⁺ Th17 cells or Tc17 cells against the totals of these cells, respectively.

Statistical Analysis

The mean and SEM were calculated for each parameter measured in this study. Statistical significance between groups was tested by one-way ANOVA using the Newman-Keuls analysis for multiple-group comparisons and the unpaired Student t test for comparisons between 2 groups. We used a threshold for statistically significant differences of $p < 0.05$. Data were plotted and analyzed using Prism5 (GraphPad, La Jolla, Calif., USA).

Results

Adaptive Airway Inflammation in the Lung after OVA Challenge

Airway inflammation was evaluated. Total cell numbers, particularly of eosinophils, macrophages and lymphocytes in the BALF from OVA-challenged mice were higher than those in control mice ($p < 0.0001$; fig. 1b). Our analysis of the HE-stained lung tissues supported these observations (fig. 1c, d).

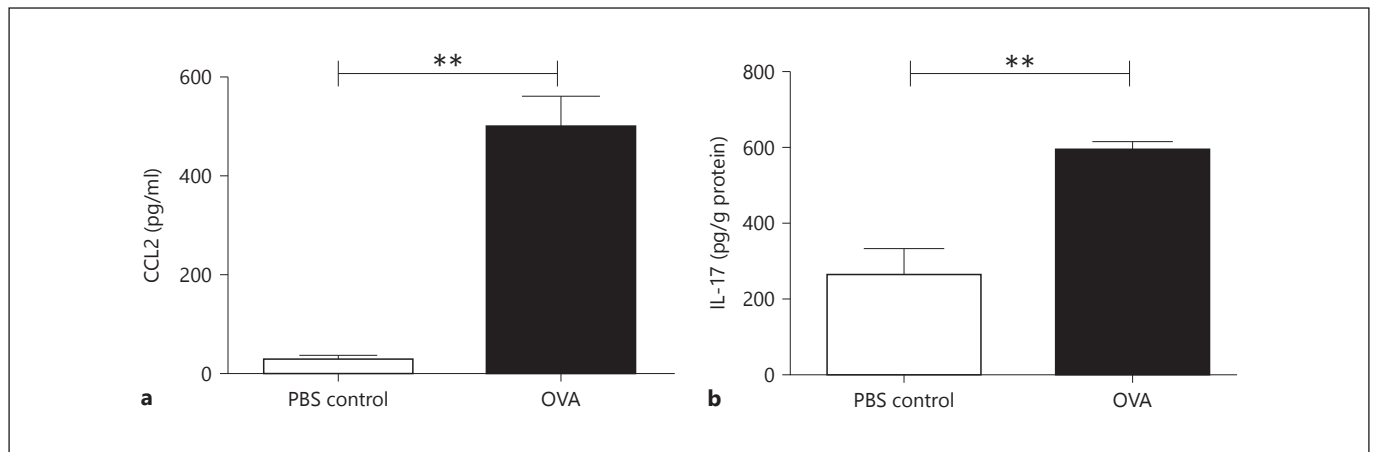


Fig. 2. a, b IL-17 levels in lung homogenates and CCL2 levels in BALF were measured by ELISA from OVA-challenged and control mice. ** $p < 0.01$, compared with the respective control group by unpaired t test. Data shown indicate the means \pm SEM for groups of 4–6 mice.

CCL2 and IL-17 Secretion upon OVA Sensitization and Challenge

CCL2 levels in the BALF and IL-17 levels in lung tissues were measured. We found that the concentration of CCL2 and IL-17 was significantly elevated after antigen challenge compared with the control group ($p < 0.01$; fig. 2a, b).

Th17 and Tc17 Cell Abundance in Mice Increases after OVA Challenge

By comparing the intracellular expression levels of IL-17 in CD4⁺ Th17 cells and CD8⁺ Tc17 cells from OVA-challenged and control mice, we found that the frequency of both Th17 cells and Tc17 cells was higher in the BALF of OVA-challenged mice ($p < 0.001$ and $p < 0.01$, respectively; fig. 3a). As BALF data may not always reflect inflammatory processes that occur in the lung tissues [33], IL-17-secreting T cells in the lung tissues were further quantified by flow cytometry. We also found that the OVA-challenged mice exhibited increased Th17 and Tc17 cell frequencies ($p < 0.001$ and $p < 0.01$, respectively; fig. 3b). We also analyzed the distribution of Th17 cells and Tc17 cells in the lung, which showed increased accumulation around the bronchioles and vessels (fig. 3c).

Increased Surface Expression of CCR2 on Th17 and Tc17 Cells

To investigate the potential involvement of CCL2 in the recruitment of Th17 or Tc17 cells to the lung, we confirmed the CCR2 expression levels on the surface of the Th17 and Tc17 cells in the BALF by flow cytometry. The frequency of

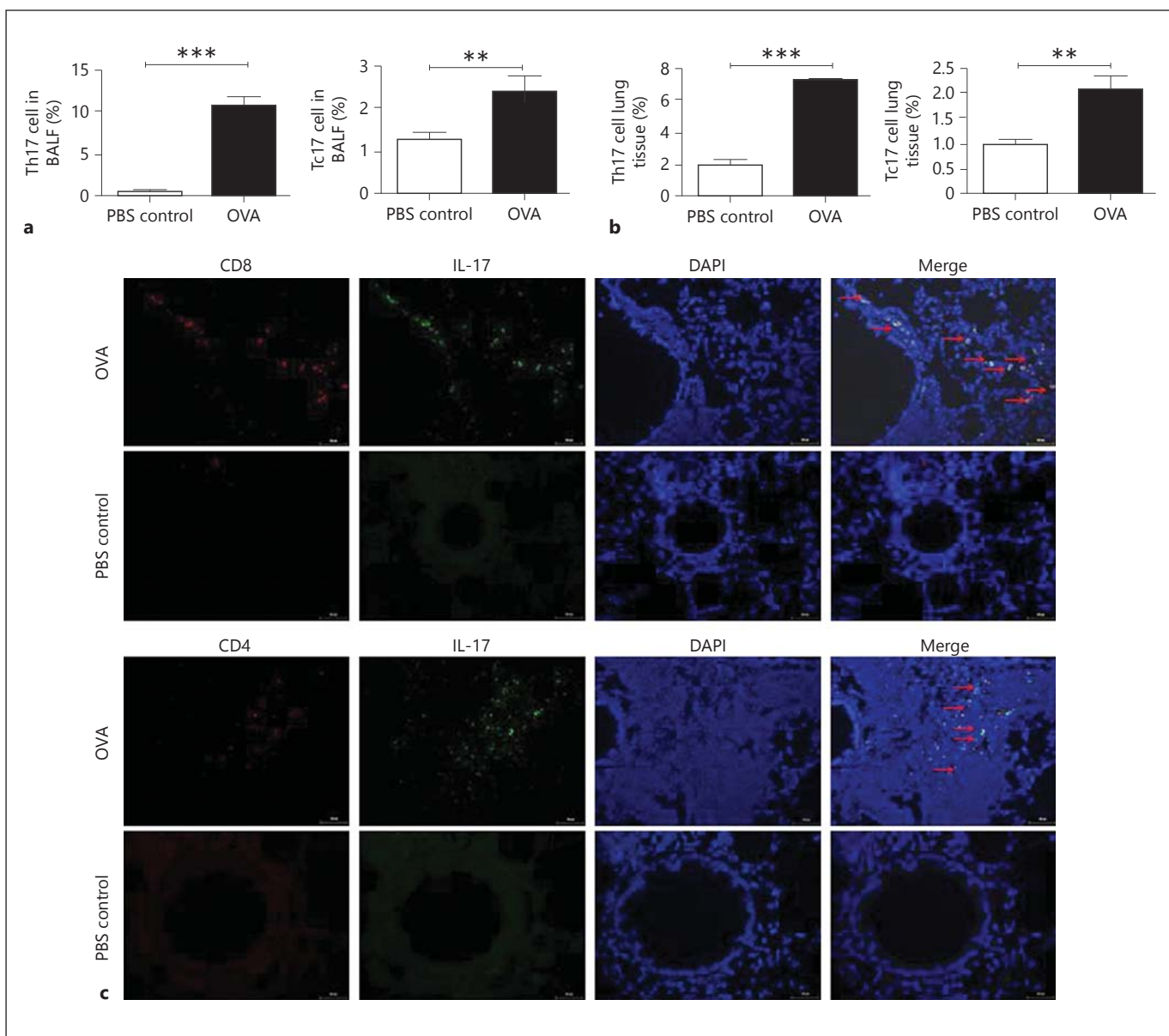
CCR2⁺ Th17 cells against the total of Th17 cells in the OVA-challenged mice was significantly increased compared with the control mice ($p < 0.05$; fig. 4a). However, the frequency of CCR2⁺ Tc17 cells against total Tc17 cells was only mildly increased and this difference did not reach our threshold for statistical significance (fig. 4b).

The Effect of Blocking the Inflammatory CCL2/CCR2 Chemokine Pathway on the Recruitment of Th17 and Tc17 Cells

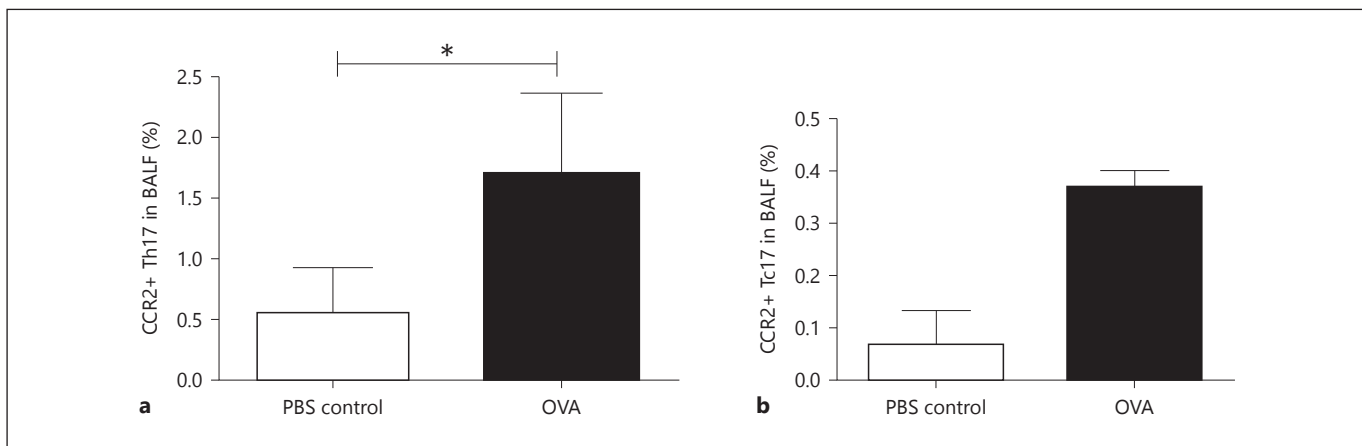
There were significantly fewer Th17 cells ($p < 0.05$), but not Tc17 cells ($p > 0.05$), in the lung tissues of the mice that received anti-CCL2 Ab compared with the IgG-treated mice (fig. 5a, b), indicating a selective effect of CCL2 on the recruitment of Th17 cells. Compared with the DMSO-treated mice, the recruitment of Th17 cells, but not Tc17 cells, into the lung was significantly reduced after RS504393 administration ($p < 0.01$ and not significant, respectively; fig. 5a, b). In addition, consistent with the reduced frequency of IL-17-secreting Th17 cells in the lung tissue detected by flow cytometry, fewer CD4⁺ and IL-17⁺ cells were detected by immunofluorescence in the lung tissues from the mice treated with anti-CCL2 Ab and RS504393 (fig. 5c). By contrast, we observed only minor changes in the infiltration of Tc17 cells to inflammatory sites (fig. 5d).

The Effect of Blocking the Inflammatory CCL2/CCR2 Chemokine Pathway on IL-17 and CCL2 Secretion

Next, we measured IL-17 levels in lung homogenates (we also measured IL-17 levels in BALF, but detected very low levels) and CCL2 levels in the BALF. Compared



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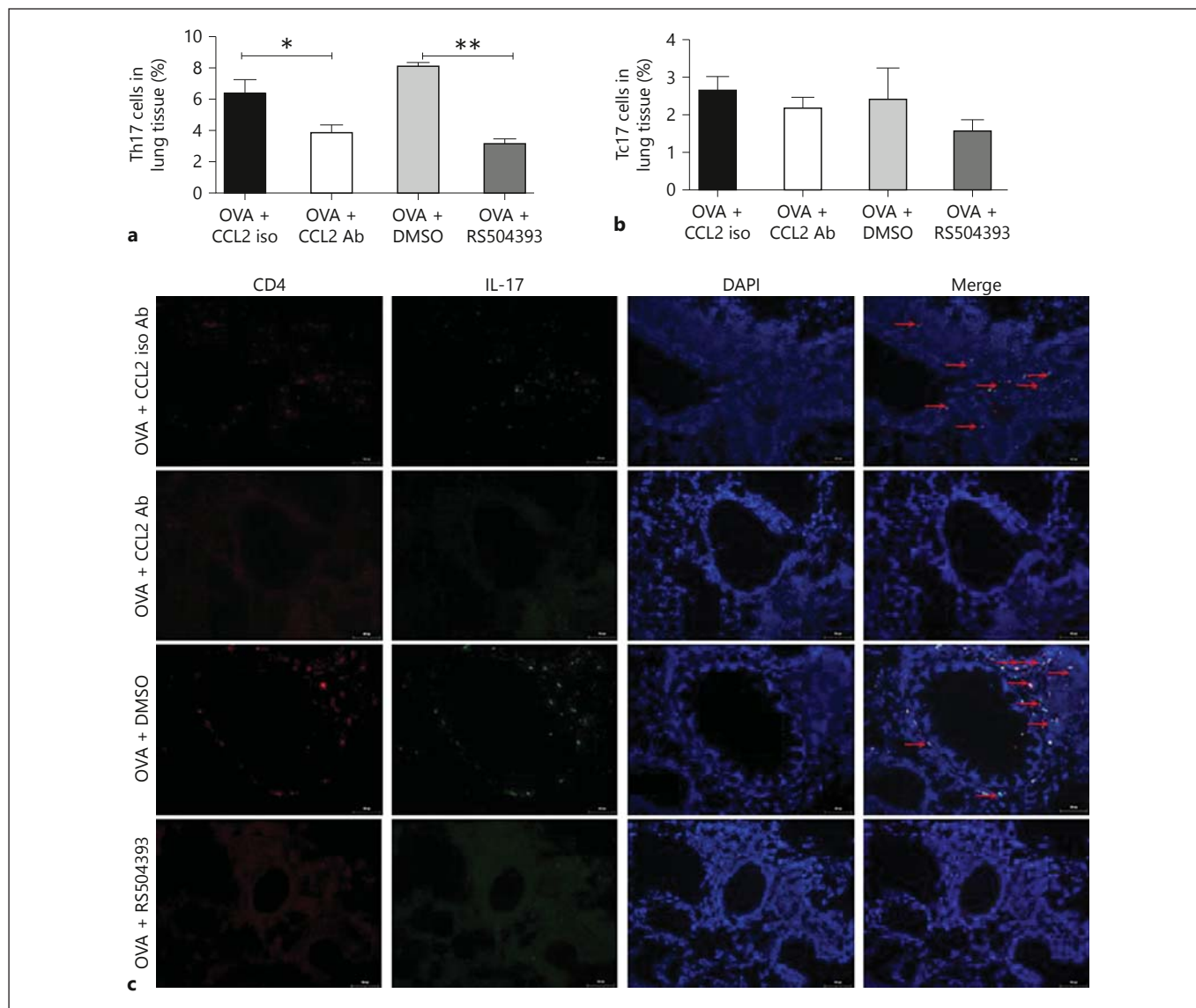


Fig. 5. a, b Blockade of the CCL2/CCR2 pathway significantly reduces the recruitment of allergen-specific Th17 but not Tc17 cells to the lung. Th17 and Tc17 cell frequencies in lung tissues from isotype control Ab-, CCL2 Ab-, DMSO- or RS504393-treated mice. **c, d** Colocalization of CD4 or CD8 with IL-17A in the

lungs of OVA-challenged and control mice; Th17 and Tc17 cells (arrows) were visualized. $\times 200$. Scale bars: 50 μm . ** $p < 0.01$, * $p < 0.05$, as compared among the indicated groups by one-way ANOVA. Data indicate the means \pm SEM for groups of 4–6 mice. iso Ab = Isotype control Ab.

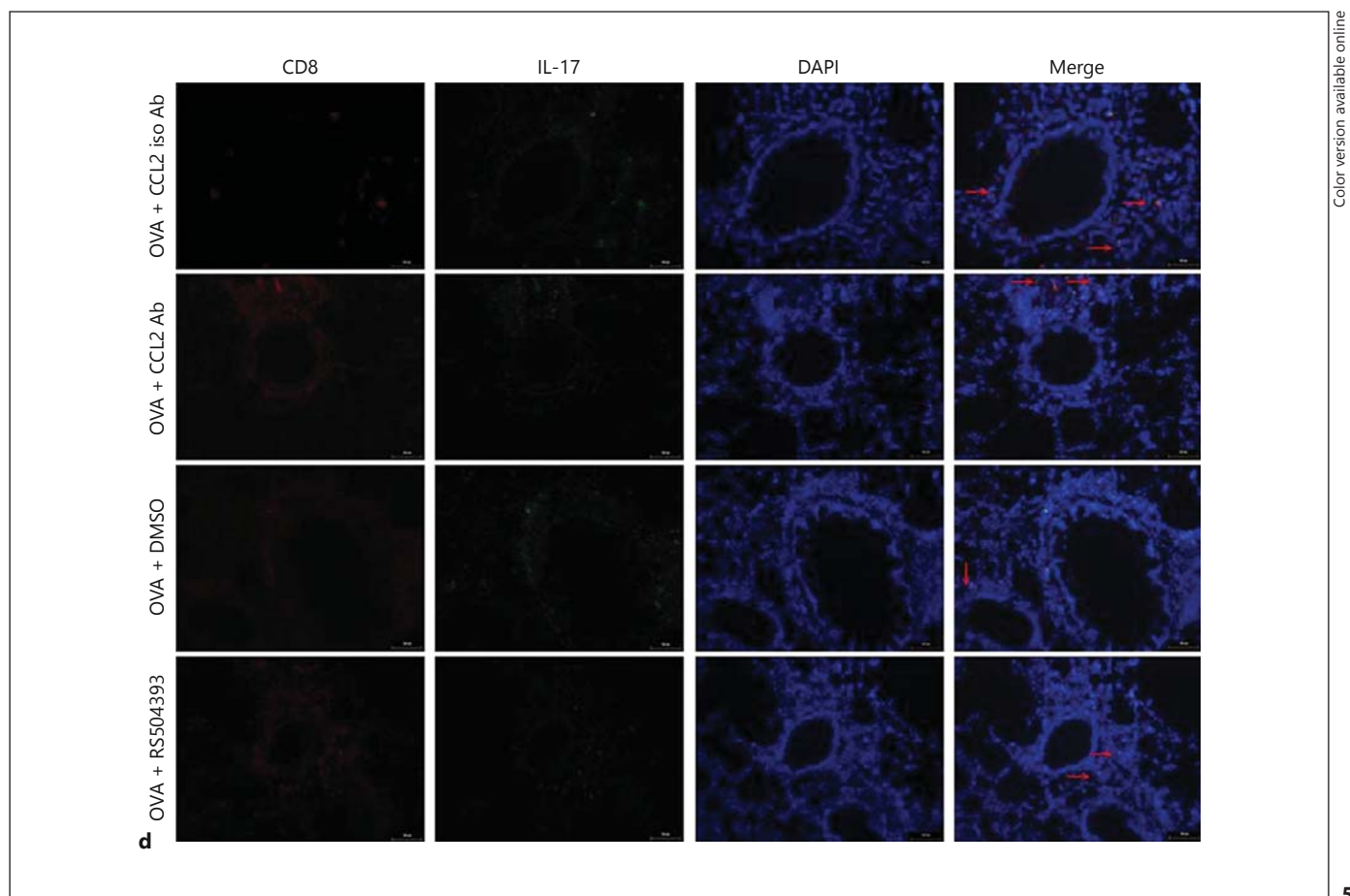
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Fig. 3. a, b Th17 and Tc17 cell frequencies increased during the development of allergen-specific adaptive responses. Data for Th17 and Tc17 cell frequencies in BALF and lung tissues from OVA-challenged and control mice were measured by flow cytometry. **c** Colocalization of CD4 or CD8 with IL-17A in the

lungs of OVA-challenged and control mice; Th17 and Tc17 cells (arrows) were visualized. $\times 200$. Scale bars: 50 μm . *** $p < 0.001$, ** $p < 0.01$, compared with the respective control group by unpaired t test. Data indicate the means \pm SEM for groups of 4–6 mice.

Fig. 4. a, b Increased CCR2 expression on the surface of Th17 and Tc17 cells. Bars indicate the frequencies of CCR2+ Th17 cells and CCR2+ Tc17 cells against the total Th17 cells or total Tc17 cells, respectively, in BALF from OVA-challenged and control

mice, as quantified by flow cytometry. * $p < 0.05$, compared with the respective control group by unpaired t test. Data indicate the means \pm SEM for groups of 4–6 mice.



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with IgG-treated mice, mice that received anti-CCL2 Ab showed a significant reduction in IL-17 levels ($p < 0.01$). In contrast to DMSO-treated mice, IL-17 levels were also moderately reduced in the RS504393-treated mice, but the difference was not statistically significant (fig. 6a). Notably, reduced CCL2 levels were also detected after RS504393-treatment when compared with DMSO-treated vehicle control mice ($p < 0.01$; fig. 6b).

The Effect of Blocking the Inflammatory CCL2/CCR2 Chemokine Pathway on Airway Inflammation

After the final challenge, lung pathology and BALF cellularity were assessed. Compared with the mice treated with isotype control Ab or DMSO, the mice that received anti-CCL2 Ab or RS504393 exhibited alleviated inflammation in both the peribronchial and perivascular areas of the lung, indicated by both HE staining and semiquantitative inflammatory scoring ($p < 0.01$; fig. 7a–d). Furthermore, the total of inflammatory cells and BALF eosinophils was significantly reduced in both the anti-CCL2

Ab-treated and RS504393-treated groups ($p < 0.05$). A significant reduction in lymphocyte numbers was observed in the RS504393-treated mice ($p < 0.001$) and a moderate reduction in macrophage and neutrophil numbers, though the differences were not significant (fig. 7e).

Discussion

In this study, we established an asthmatic model that resulted in increased eosinophil and Th17 cell infiltration to the lung, accompanied by increased levels of CCL2 and IL-17. We showed that Th17 and Tc17 cells accumulated in the lung, and that CCR2 expression levels also increased on the surface of these cells. The CCL2/CCR2 chemokine pathway can promote Th17 cell recruitment to the lung in vivo, but may have no effect on Tc17 cell recruitment.

CCL2, which was initially identified as a monocyte chemotactic protein, is also a potent chemoattractant for macrophages, mast cells and eosinophils via their CCR2

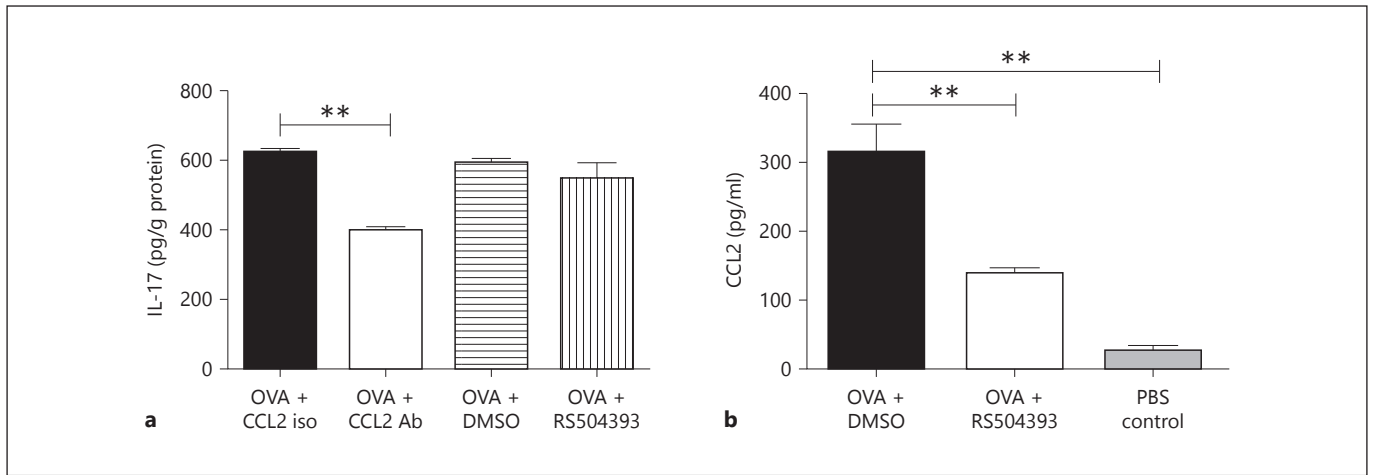


Fig. 6. a, b Cytokine secretion declined after blockade of the inflammatory CCL2/CCR2 chemokine pathway. IL-17 production in lung homogenates and CCL2 levels in BALF were measured by ELISA.

** $p < 0.01$, as compared among the indicated groups by one-way ANOVA. Data indicate the means \pm SEM for groups of 4–6 mice. iso = Isotype.

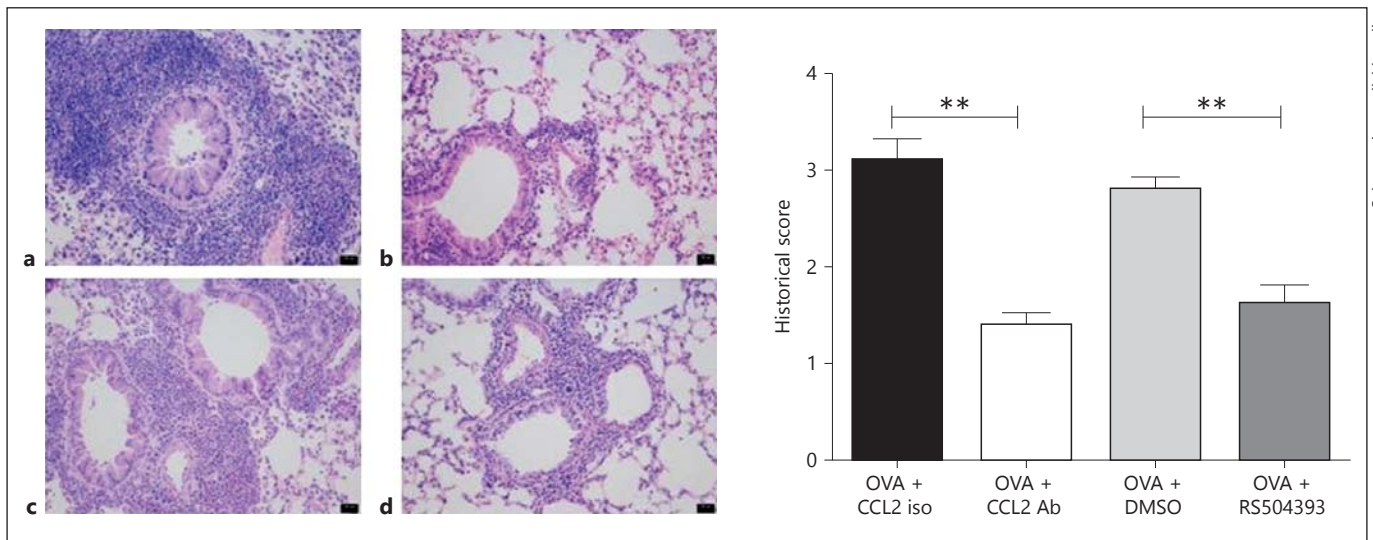
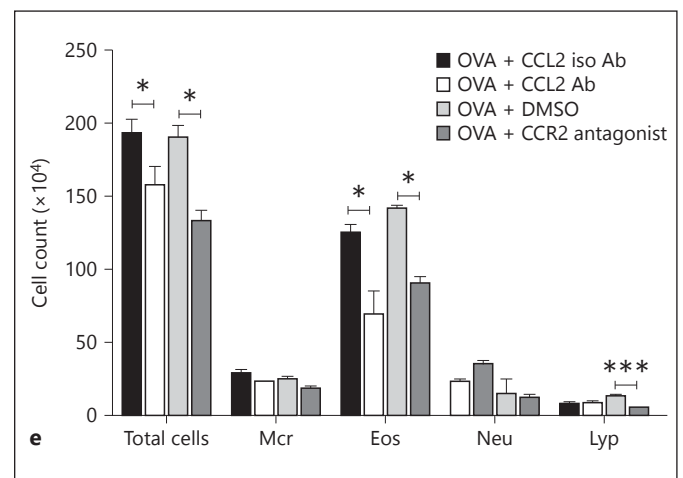


Fig. 7. Development of airway inflammation was significantly alleviated after CCL2/CCR2 pathway blockade. HE-stained lungs from isotype (iso) control Ab- (a), CCL2 Ab- (b), DMSO- (c) or RS504393-treated mice (d) 24 h after the final challenge with OVA. HE. On the right, quantification of the histological images is shown. Images were scored by at least two pathologists and the mean score was used as the inflammation index, which represents the severity of peribronchial inflammation. e The number of total cells, macrophages (Mcr), eosinophils (Eos), neutrophils (Neu) and lymphocytes (Lyp) in the BALF 24 h after the final challenge with OVA. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, compared with the respective control group by unpaired t test. Data shown indicate the means \pm SEM for groups of 4–6 mice.



[34–36]. In research on the pathogenesis of asthma, Mellado et al. [17] found that the neutralization of CCR2 attenuated macrophage and eosinophil accumulation in the BALF of asthmatic monkeys. Our study also found that the number of eosinophils and lymphocytes was reduced after the treatment with anti-CCL2 Ab or CCR2 antagonist, and although the differences were not significant, a moderate reduction in macrophage numbers was also observed. This supports the finding by Mellado et al. [17]. Lukacs et al. [16] reported that neutralization of CCL2 significantly reduced total leukocyte migration, especially of CD4+ and CD8+ lymphocytes; our study also found a significant reduction in lymphocyte numbers in the RS504393-treated mice. The elevated levels of CCL2 and the increased numbers of lymphocytes in BALF were observed in OVA-challenged animals and were significantly reduced when the mice were treated with CCR2 antagonist, leading to the assumption that, in asthma, a large portion of CCL2 is released from lymphocytes.

On flow cytometry, we detected a significant increase in the frequency of Th17 and Tc17 cells that migrated to the BALF and lung tissue. The location of both the Th17 and Tc17 cells in the lung was characterized by a peribronchial and perivascular distribution visible by immunofluorescence. These data suggest that the accumulation of both IL-17-secreting Th17 and Tc17 cells in the lung represents one of the drivers of immunopathology following OVA stimulation.

Th17 cells are known to express the chemokine receptor CCR2 *in vitro* [37]. The CCL2/CCR2 axis is relevant in various autoimmune diseases, such as multiple sclerosis, autoimmune encephalomyelitis and autoimmune arthritis [38–40]. However, the role of the CCL2/CCR2 axis in the migration of Th17 and Tc17 cells to the lung in allergic asthma has not yet been reported. We thus investigated the expression of CCR2 on Th17 and Tc17 cells in asthmatic mice and found that the frequency of CCR2 expression in both cell subsets was increased. Using anti-CCL2 Ab for CCL2 neutralization or RS504393 for CCR2 blockade *in vivo*, in both treatments, we observed a significant reduction in the frequency of Th17 cells and a change in their distribution, compared with mice treated with isotype control Ab and DMSO, on flow cytometry and by immunofluorescence. This result was accompanied by a reduction in the IL-17 expression and in lung inflammation. All of these findings indicated that the CCL2/CCR2 interaction plays an important role in the OVA-induced migration of Th17 cells and airway inflammation. Taken together, treatment with anti-CCL2 or CCR2 antagonist reduced

lung inflammation which was due, at least in part, to the reduced infiltration of Th17 cells.

In addition, for the first time, we emphasized the role of CCL2 on the migration of Th17 and Tc17 cells to the lung and the subsequent effects on airway inflammation. However, compared with the mice treated with isotype control Ab or DMSO, the reduction of Tc17 cells was not as remarkable as the effect on the Th17 cells that we observed in both the CCL2 Ab-treated and RS504393-treated mice, suggesting that the CCL2/CCR2 cytokine signaling pathway might be not essential for Tc17 cell recruitment during inflammation. A previous study of inflamed liver tissue suggested that Tc17 cells express high levels of CXCR3, CCR6 and CXCR6, and that the increasing expression of CXCR3 mediates Tc17 cells recruitment [41]. The small amount of Tc17 cells and difficulty in their detection might be another reason for the discordant results. Thus, characterizing the precise mechanisms used for Tc17 cell recruitment to inflammatory sites will require further research.

The elevated levels of CCL2 and IL-17 in BALF and lung homogenates were observed in OVA-challenged animals. After CCR2 blockade, CCL2 levels also showed a significant reduction in BALF that paralleled the reduced IL-17 levels. This finding may imply that a regulatory loop exists between CCL2 and Th17 cells. The potential mechanisms involved, shown *in vitro* by Wong et al. [42], include the IL-17-dependent enhancement of ICAM-1 expression on the surface of primary bronchial epithelial cells and BEAS-2B cells via intracellular signaling events that result in the activation of NF- κ B and p38 MAPK. Subsequently, ICAM-1/CD18 interactions between bronchial epithelial cells and basophils induce the activation of ERK, p38 MAPK, JNK and NF- κ B, which might provide sufficient stimulation to cause the release of CCL2. Thus, cross-talk between CCL2 and IL-17 in allergic asthma might play an important role in perpetuating pulmonary inflammation.

In summary, we provide compelling evidence for a direct association between CCL2 and Th17 cells *in vivo*. This study provides new insights into our understanding of the role of the CCL2/CCR2 pathway in asthma through the recruitment Th17 cells to the lung. This may aid the development of novel asthma treatment strategies.

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