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Blockade of CCL2/CCR2 signaling pathway prevents inflammatory monocyte recruitment and attenuates OVA-Induced allergic asthma in mice

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ABSTRACT

Recent studies have reported recruitment of inflammatory monocytes by cytokines including chemokine (C-C motif) ligand 2 (CCL2) are critical in allergic responses. We aimed to investigate the role of inflammatory monocytes and CCL2 in mouse model with ovalbumin (OVA)-induced allergic asthma. Mice were sensitized with OVA to induce allergic asthma. The proportion of inflammatory cells in bronchoalveolar lavage fluid (BALF) and peritoneal lavage fluid (PLF) were measured by flow cytometry. The expression of CCL2 and CCL2 receptor (CCR2) were determined by qPCR and western blot. The concentrations of Type 1 helper T (Th1) and Type 2 helper T (Th2) cytokines in PLF were detected by ELISA. Inflammatory monocytes are recruited in PLF, and expression of CCL2 and CCR2 were elevated in OVA-induced mice. In addition, transfer of CCR2 knockdown inflammatory monocytes decreased the levels of allergic asthma biomarkers. Injection of anti−CCL2 or anti−CCR2 antibody decreased the proportion of eosinophils and inflammatory monocytes in BALF. Blockade of CCL2/CCR2 signaling pathway suppressed the allergen-induced Th2 cytokines and enhanced the levels of Th1-associated cytokines. Blockade of CCL2/CCR2 signaling pathway in sensitization-recruited inflammatory monocytes exhibits protective effects in mouse model of OVA-induced allergic asthma by inhibiting the Th2 inflammatory responses.

1. Introduction

Bronchial asthma (referred to as asthma) is a common and frequent chronic respiratory disease affecting approximately 300 million people worldwide [1]. The main symptoms conclude paroxysmal wheezing, chest tightness, shortness of breath, and cough [2]. In recent years, the prevalence of asthma has been increasing year by year, seriously affecting the physical and mental health of patients and bringing a heavy economic and social burden to the society [3]. Therefore, in-depth research on the pathogenesis of asthma is of great significance for the control and treatment of asthma.

Allergic asthma is the most common type of asthma and is often associated with allergies and allergens [4], producing a range of allergen exposure associated symptoms including chest tightness, cough, and wheezing. It is an airway chronic inflammatory disease involving a variety of inflammatory cells (such as lymphocytes, eosinophils, mast cells, macrophages, airway epithelial cells, *etc.*) and inflammatory mediators [5], often characterized by the increase of biomarkers such as eosinophils, serum Immunoglobulin E (IgE), and Type 2 helper T (Th2) cell type cytokines (such as interleukin [IL] 4, IL-5, and IL-13) [6–9]. IL-4 promotes the production of IgE, which binds to and activates the surface receptors of mast cells to release a variety of inflammatory factors, leading to bronchial smooth muscle spasm and luminal stenosis [10,11]. IL-5 regulates the growth, differentiation, recruitment and survival of eosinophils, which plays an important role in asthma inflammation and airway remodeling [12]. IL-13 is critical in airway hyperactivity (AHR) and airway remodeling, mucus hypersecretion, and eosinophilic inflammation [13]. Therefore, research related to Th2 type cytokines has become a focus area of allergic asthma research [14,15]. However, in recent years, with the further study of asthma, the role of other immune cells (such as macrophages, inflammatory monocytes, *etc.*) in allergic asthma has gradually been revealed [16,17].

Recent studies have shown that inflammatory monocytes play a decisive role in the development of ovalbumin (OVA)-induced allergic asthma [16]. Therefore, reducing the number of inflammatory monocytes in the abdominal cavity might be an effective treatment strategy for allergic asthma. The chemokine (C-C motif) ligand 2 (CCL2) is a small cytokine that belongs to the CC chemokine family, which functions in the recruitment of monocytes to inflammation sites [18]. Previous studies have demonstrated that either blocking the CCL2 receptor

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C-C chemokine receptor type 2 (CCR2) or neutralizing CCL2 could effectively block the recruitment of inflammatory monocytes to inflammatory tissues [19]. Our present study aimed to investigate the role of inflammatory monocytes and CCL2 in mouse model with OVA-induced allergic asthma.

2. Methods

2.1. Mouse model of OVA-induced allergic asthma

Mice were initially sensitized by peritoneal administration at day 0 and day 7 with 100 μg of OVA (grade V; Sigma-Aldrich, St. Louis, MO) in 100 μL of sterile saline and adsorbed in 50 μL of Imject alum (Thermo Scientific, Waltham, MA USA). Mice were subsequently challenged by intranasal administration with 100 μg of OVA in 50 μL of sterile saline after anesthetized at day 14, 15, and 16. Mice were euthanized at day 21. Animal study was reviewed and approved by Yuhuangding Hospital.

2.2. Collection of BALF

Before the collection of BALF sample, mice tracheas were cannulated and lavaged with cold PBS. After being centrifuged, the supernatants of samples were stored at −80 °C for detection of cytokines and cell pellets were resuspended for cell counting.

2.3. Flow cytometry

Cell types were characterized as $CD45^+SiglecF^+CD11b^+CD11c^-$ (eosinophils), $CD45^+CD11b^+Ly6C^{hi}$ (inflammatory monocytes), $CD4^+CD11b^-$ (T helper cells). For surface staining, after blocking the Fc receptor, single cells were incubated with fluorescein-labeled monoclonal antibodies for 30 min at 4 °C, then washed twice and analyzed. The expressions of CCR2 on $CD3^+$ T cells, $CD19^+$ B cells, $CD11b^+Ly6G^+$ neutrophil, $CD11b^+F4/80^+$ and $CD11b^+Ly6C^+$ monocyte were examined *via* FACS. For the examination of cytokines in T cells, T cells were stimulated for 4 h with 30 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) and 1 μM ionomycin (Sigma) in the presence of 2.5 μg/ml monensin (eBioscience). After stimulation, cells were stained for surface markers, fixed and permeabilized. Fixed cells were stained with antibodies to IL13. All samples were acquired on an FACSCalibur (BD Biosciences) and were analyzed using FlowJo software (Tree Star).

2.4. RNA extraction and qRT-PCR

Trizol reagent (Invitrogen Life Technologies) was applied to extract the total RNA from cells according to the manufacturer's instructions. A reverse transcription kit (Fermentas) was used for cDNA synthesis by adding approximately 2 μg of RNA. Quantitative analysis of CCL2 mRNA levels mentioned in this paper was examined by SYBR-Green Master mix (Life Technologies).

2.5. Western blot

Western blot was performed as the standard method. Anti−CCR2 (12199, 1: 1,000) and anti-β-Actin (3700, 1: 2,000) were purchased from Cell Signaling Technology.

2.6. Inflammatory monocytes transfer

100 μg OVA was diluted in 100 μL sterile saline and then adsorbed in 50 μL Imject alum before it is used in the immunization of mice. The peritoneal lavage fluids (PLF) were collected by washing the peritoneal cavity with 1 ml sterile, and inflammatory monocytes in PLF were purified by FACSAria II cell sorter (BD Biosciences) after stained with anti-Ly6C-PE antibody. 2 h post sensitization, monocytes-depleted recipient mice were transferred with siCCR2 transfected monocytes for two weeks. Recipient mice were transferred with monocytes twice and treated with OVA one week after transfer. One week after OVA treatment, recipient mice samples were collected. Inflammatory monocytes were depleted by the monoclonal antibody anti−CCR2 in the recipient mice.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Lung was perfused by PBS with 5 mM EDTA to collect BALF sample. ELISA kits purchased from Dakewe Biotech were used to measure IL-4, IL-5, and IgE levels in serum and BALF.

2.8. Histology

Before being embedded with paraffin, lung tissues were fixed by 10% formalin longer than one day. Hematoxylin and eosin staining were used to show the histological features in lung tissue sections. The degree of lung inflammation was scored based on staining data. 4: more than four cells deep inflammatory cell ring; 3: two to four cells deep inflammatory cell ring; 2: one cell deep inflammatory cell ring; 1: few inflammatory cells; 0: normal tissue.

2.9. Statistical analysis

SPSS version 17.0 was employed to perform the statistical analysis. Data were shown as mean ± SD. The differences were evaluated by Student's two-tailed *t*-test or one-way ANOVA analysis followed by a Tukey's post hoc test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results

3.1. Mouse model of OVA-induced allergic asthma

To explore the underlying mechanism and efficacious treatment of allergic asthma, we first generated the OVA-induced mouse model (Fig. 1A). Mice were initially sensitized by peritoneal administration with OVA/alum at day 0 and day 7, and subsequently challenged by intranasal administration with OVA protein at day 14, 15, and 16 to induce allergic asthma. In order to verify that the model was successfully built, we collected the bronchoalveolar lavage fluid (BALF) from normal and asthmatic mice and measured the proportion of eosinophils in BALF by flow cytometry. As shown in Fig. 1B and C, our results showed an increasing number of eosinophils in OVA-induced mice (41.2%) compared with normal control (0.63%), proving that we successfully induced allergic asthma with OVA in mice.

3.2. Inflammatory monocytes are recruited after sensitization and up-regulate the expression of CCL2 and CCR2

Monocytes are produced in the bone marrow and mainly reside in the peripheral blood and spleen. When an inflammatory reaction occurs in a certain part of the body, monocytes are rapidly recruited to the inflammation sites. Thus, we collected the peritoneal lavage fluid (PLF) and measured the ratio of inflammatory monocytes in normal mice and asthmatic mice by flow cytometry. Our results demonstrated that the proportion of monocytes in PLF was significantly increased in OVA-induced mice compared with the normal mice (Fig. 2A and B). Next, we detected the expression of CCL2 by qPCR and western blot in cells isolated from PLF. Our results revealed that both the mRNA levels (Fig. 2C) and the protein levels (Fig. 2D) of CCL2 were remarkably elevated in asthmatic mice compared with normal mice. Moreover, the expression of CCR2 in different immune cells was examined by flow cytometry. As shown in Fig. 2E, we demonstrated that CCR2 was highly expressed specifically in inflammatory monocytes. Taken together, our

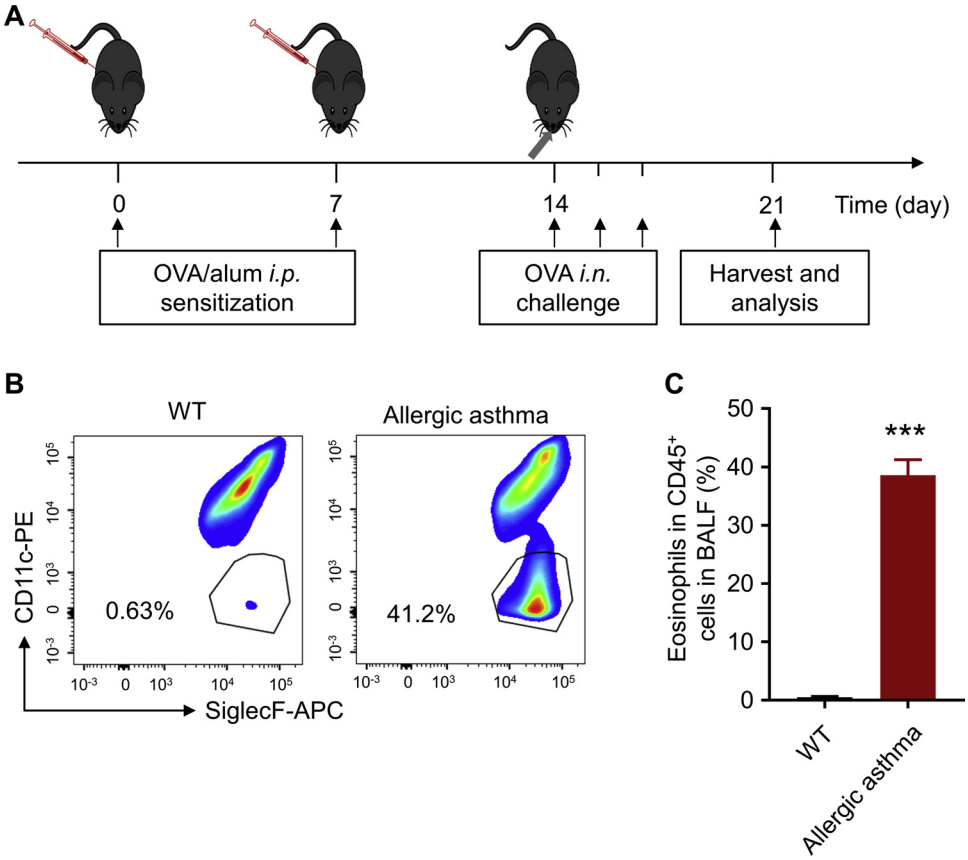

Fig. 1. Establishment of mice model of ovalbumin (OVA)-induced allergic asthma. (A) Procedure of OVA-induced allergic asthma in mice. Six-weeks old mice were treated intraperitoneally (*i.p.*) with OVA/alum for sensitization at day 0 and 7 and challenged intranasally (*i.n.*) with OVA at day 14, 15 and 16. All mice were harvested and analyzed 1 week after OVA challenge. (B) FACS analysis of the abundance of eosinophils in bronchoalveolar lavage fluid (BALF) from asthmatic and control mice. Eosinophils were identified as $CD45^+SiglecF^+CD11b^+CD11c^-$ cells. (C) Bar graph shows the percentage of eosinophils in $CD45^+$ cells in BALF from asthmatic and control mice. Data represent means ± SD, n = 8, $^{***}p < 0.001$.

results revealed that the inflammatory monocytes are recruited in PLF and the expression of CCL2 and CCR2 were elevated.

3.3. Knockdown of CCR2 in inflammatory monocytes decreases the production of allergic asthma biomarkers

To further explore the role of CCR2 in the effects of inflammatory monocytes on the symptoms of allergic asthma, we transfected the sorted inflammatory monocytes with siCCR2 and transferred the cells into monocytes-depleted recipient mice (Fig. 3A). Western blot analysis showed that the protein levels of CCR2 were significantly down-regulated after transfection of siCCR2 in inflammatory monocytes (Fig. 3B). Subsequently, we measured the concentrations of IgE, IL-4 and IL-5 in PLF by ELISA. Our results demonstrated that knockdown of CCR2 in inflammatory monocytes remarkably decreased the levels of IgE (Fig. 3C), IL-4 (Fig. 3D) and IL-5 (Fig. 3E), suggesting that adoptive transfer of CCR2 depletion inflammatory monocytes could attenuate allergic symptoms.

3.4. Blockade of CCL2/CCR2 signaling pathway exerts protective function in OVA-induced allergic asthma

To investigate whether CCL2/CCR2 signaling pathway could mediate the recruited inflammatory monocytes, we injected the anti−CCL2 or anti−CCR2 antibody into OVA-induced mice (Fig. 4A). The abundance of eosinophils in BALF and the frequency of inflammatory monocytes in PLF were analyzed by flow cytometry. Our results revealed that the proportion of eosinophils (Fig. 4B) or inflammatory monocytes (Fig. 4C) was significantly decreased by injection of anti−CCL2 or anti−CCR2 antibody. In addition, lung tissues from each group were obtained for further histological examination. As shown in Fig. 4D and E, administration of either anti−CCL2 or anti−CCR2 antibody suppressed the inflammatory infiltrates into the perivascular connective tissues and the peribronchiolar tissues. Taken together, these results suggested that blockade of CCL2/CCR2 signaling pathway decreased the abundance of inflammatory cells and suppressed the inflammatory infiltration in lung.

3.5. Blockade of CCL2/CCR2 signaling pathway inhibits the Th2 inflammatory responses

Proliferating Helper T cells that develop into effector T cells differentiate into two major subtypes of cells known as Th1 and Th2 cells. Previous studies have demonstrated that allergic asthma is an allergen-induced Th2 inflammatory disease. Therefore, we examined the frequency of Th2 cells in total CD4 + T cells from lung following administration of anti−CCL2 or anti−CCR2 antibody. Our results demonstrated that blocking CCL2 or CCR2 signaling pathway significantly decreased the proportion of Th2 cells (Fig. 5A and B). Next, we detected the levels of Th1-type cytokines and Th2-type cytokines in PLF by ELISA. We found that injection of anti−CCL2 or anti−CCR2 antibody remarkably increased the levels of Th1-associated cytokines, including interferon (IFN)-γ (Fig. 5C), tumor necrosis factor (TNF)-α (Fig. 5D), and interleukin (IL)-6 (Fig. 5E). Furthermore, blocking CCL2 or CCR2 significantly decreased the levels of Th2-associated cytokines, including IL-13 (Fig. 5F), IL-4 (Fig. 5G), and IL-5 (Fig. 5H). Therefore, our data indicate that blockade of CCL2/CCR2 signaling pathway in recruited inflammatory monocytes could suppress the allergen-induced Th2 inflammatory responses and upregulate the levels of Th1-associated cytokines.

4. Discussion

Currently, symptoms of allergic asthma can be prevented by avoiding exposure to allergens and irritants and inhaling corticosteroids, but there is no cure for allergic asthma [20]. Therefore, in-depth

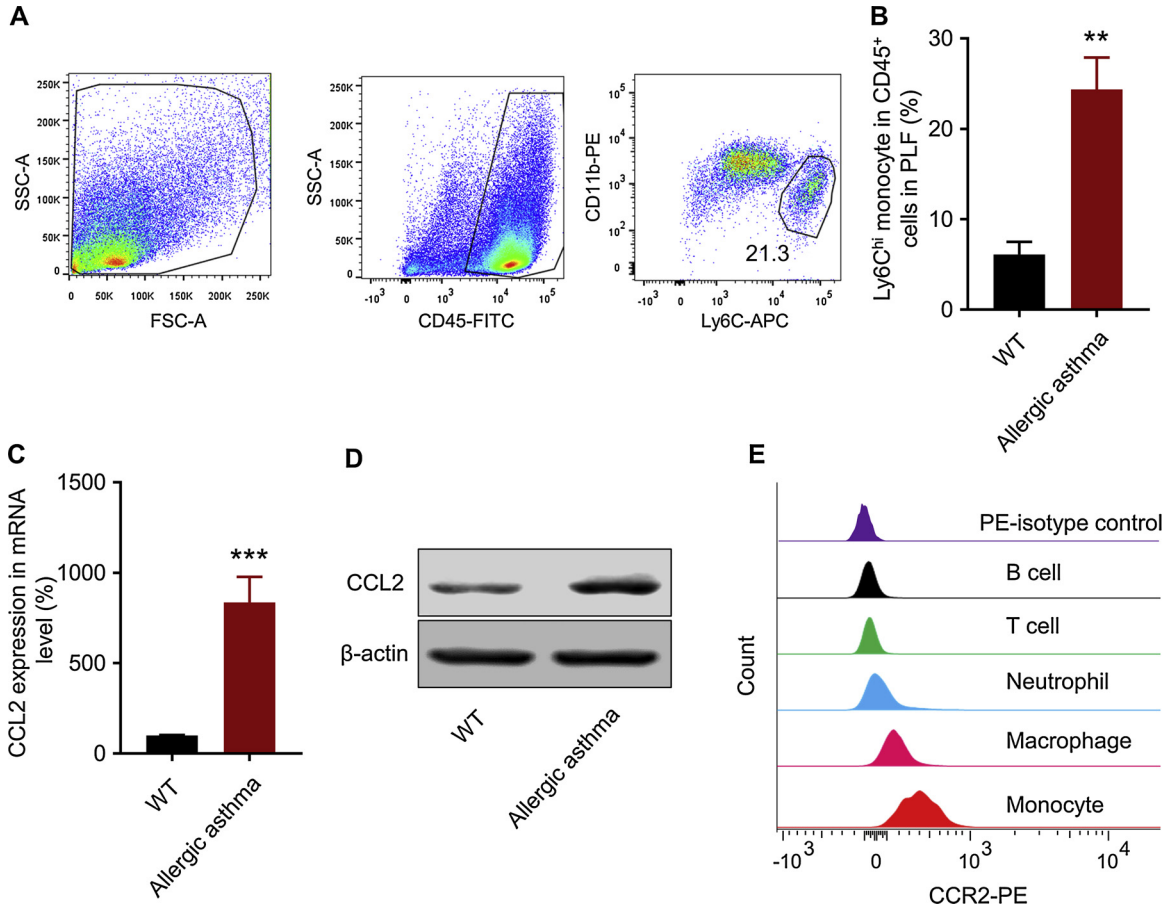

Fig. 2. Inflammatory cells are recruited in peritoneal lavage after sensitization in asthmatic mice. (A) FACS analysis of the abundance of Ly6C^{hi} inflammatory cells in CD45^{+} immune cells in peritoneal lavage fluids (PLF) from asthmatic and control mice. Inflammatory cells were identified as CD45^{+}CD11b^{+}Ly6C^{hi} cells. (B) Bar graph shows the percentage of inflammatory cells in CD45^{+} cells in PLF from asthmatic and control mice. Data represent means ± SD. n = 8, ***$p < 0.001$. CCL2 expression in mRNA (C) and protein level (D) in cells isolated from PLF. n = 8, ***$p < 0.001$. (E) CCR2 expression in different immune cells (including B cells, T cells, neutrophil, macrophage and monocytes) examined by FACS.

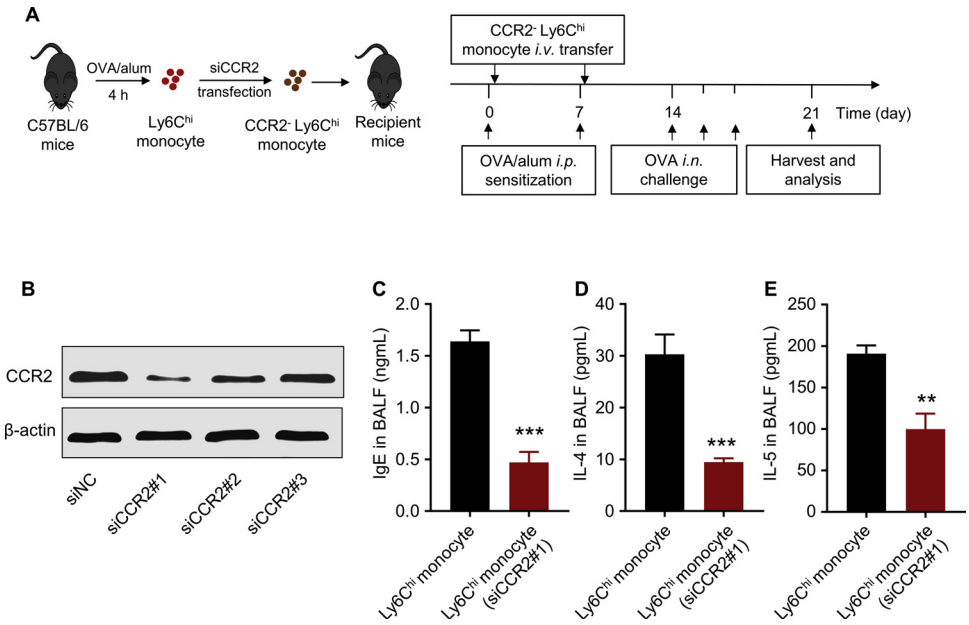

Fig. 3. Transfer of CCR2 downregulated inflammatory monocytes protects mice against OVA-induced allergic asthma. (A) C57BL/6 mice were treated *i.p.* with OVA/alum, and PLF were harvested 4 h after treatment to purify inflammatory monocytes by sorting. Sorted monocytes were transfected with siCCR2 and then 1.5×10^5 transfected cells were transferred *i.v.* into monocytes-depleted recipient mice 2 h post sensitization for two weeks. The recipient mice were challenged *i.n.* with OVA 1 week after the second transfer. All recipient mice were harvested and analyzed 1 week after OVA challenge. (B) Protein levels of CCR2 in sorted monocytes transfected with siNC (negative control siRNA) or siCCR2 detected by western blot. β-actin was used as loading control. The siCCR2#1 could effectively downregulate target gene. (C–E) The levels of IgE, IL-4 and IL-5 in BALF were analyzed by ELISA. Data represent means ± SD. n = 8, **$p < 0.005$, ***$p < 0.001$.

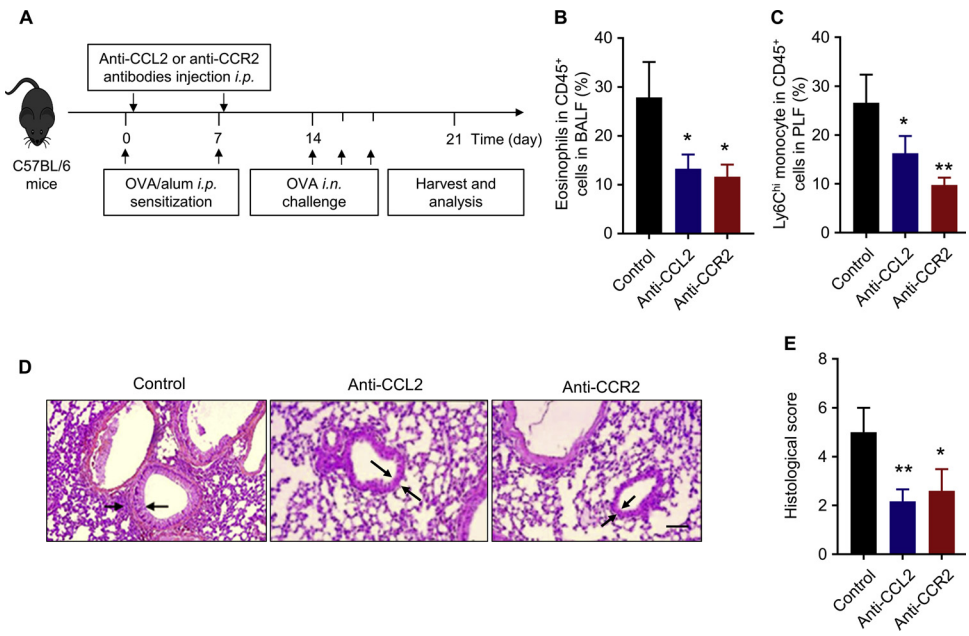

Fig. 4. Blockade of CCL2/CCR2 signaling pathway exerts protective function in OVA-induced allergic asthma. (A) Six-weeks old mice were treated intraperitoneally (*i.p.*) with OVA/alum for sensitization at day 0 and 7, and *i.p.* injected with anti−CCL2 or anti−CCR2 antibodies (100 μg per mouse) 2 h later, and then challenged *i.n.* with OVA at day 14, 15 and 16. All mice were harvested and analyzed 1 week after OVA challenge. The abundance of eosinophils in BALF (B) and the frequency of inflammatory monocytes in PLF (C) were analyzed by FACS. Data represent means ± SD. n = 8, $*p < 0.05$, $**p < 0.005$ *vs* control group. (D) Histologic sections of lungs from each group were analyzed by H&E. Pictures show representative samples of 8 mice/group. Scale bar = 100 μm. (E) Histological scores for assessment of lung injury were shown. Data represent means ± SD. n = 8, $*p < 0.05$, $**p < 0.005$ *vs* control group.

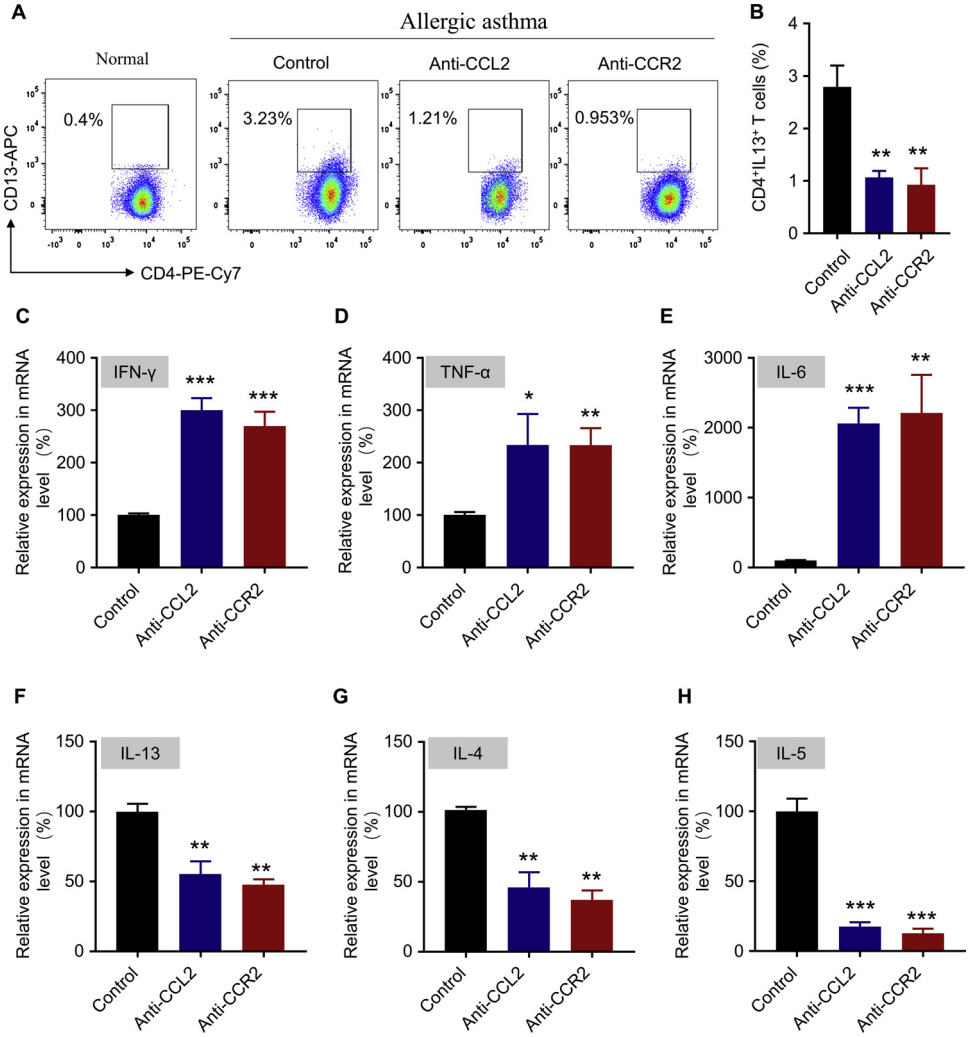

Fig. 5. Blockade of CCL2/CCR2 signaling pathway attenuates OVA-induced allergic asthma by increasing Th1 cytokines. (A) FACS analysis of the frequency of $IL13^+$ Th2 cells in total $CD3^+CD4^+$ T cells from lungs. (B) Bar graph shows the percentage of Th2 cells. Data represent means ± SD. n = 8. $***p < 0.001$. (C–H) Relative expression of Th1-type cytokines (IFN-γ, TNF-α and IL-6) and Th2-type cytokines (IL-13, IL4 and IL-5) in PLF analyzed by real-time PCR. Data are the mean ± SD. n = 8. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, *vs* control group.

research to elucidate the pathogenesis of allergic asthma is of great significance for the control and treatment of this disease. In order to explore the underlying mechanism and efficacious treatment, we first generated the OVA-induced mouse model. The OVA-induced mice exhibited an increase of eosinophils in BALF compared with normal mice, which was consistent with the previous results. Recent studies have shown that inflammatory monocytes play a decisive role in the development of ovalbumin-induced allergic asthma, and suggest that reducing the number of inflammatory monocytes in the peritoneal cavity might be an effective treatment strategy for allergic asthma [16]. Based on our mouse model, we confirmed that the proportion of inflammatory monocytes in PLF was remarkably enhanced in OVA-induced mice. Our experiment provides a basis for treating allergic asthma by blocking the recruitment of inflammatory monocytes to inflammatory tissues.

CCL2 is also referred to as monocyte chemoattractant protein 1 (MCP1), which exhibits a chemotactic activity for basophils and monocytes [21]. CCL2 is involved in the pathogenesis of various inflammatory disorders characterized by monocyte infiltration, including atherosclerosis, rheumatoid arthritis, allergic asthma and psoriasis [22,23]. CCL2 recruits dendritic cells, memory T cells, and monocytes to inflammation sites caused by infection or injury [24]. Therefore, we examined the expression of CCL2 by qPCR and western blot in cells isolated from PLF and found that both the mRNA levels and the protein levels of CCL2 were significantly increased in OVA-induced asthmatic mice. Our results further verified the crucial role of CCL2 in recruiting the inflammatory monocytes, suggesting that blockade of CCL2 might be a potential target for alleviating the inflammatory responses in allergic asthma mice.

CCR2 and CCR4 are two cell surface receptors that bind CCL2 [25]. CCR4 is commonly expressed on leukemic cells in cutaneous T-cell lymphoma (CTCL) [26]. A recent research has reported that the level of CCL2 was increased in BALF and lung tissues in mice with asthma [27]. The CCL2/CCR2 signaling pathway is critical in regulating the airway inflammation [27]. Therefore, we analyzed the expression of CCR2 in different immune cells isolated from PLF (including B cells, T cells, neutrophil, macrophage and monocytes). We found that CCR2 was highly expressed specifically in inflammatory monocytes in OVA-induced mice. Taken together, we speculate that the CCL2/CCR2 axis might be crucial in allergic asthma by mediating the recruitment of inflammatory monocytes to specific inflammation sites.

To further verify our conjecture, we transferred the CCR2 knockdown inflammatory monocytes into monocytes-depleted recipient mice and tested the levels of some allergic asthma biomarkers. Our results showed that the concentrations of IgE, IL-4 and IL-5 were significantly decreased after transfection of siCCR2 in inflammatory monocytes. IL-4 regulates critical pro-inflammatory procedures in allergic asthma, such as production of vascular cell adhesion molecule 1 (VCAM-1) and Th2 cytokines, mucus secretion, induction of isotype rearrangement of IgE, and eosinophilic transmigration [28]. IL-5 is involved in various allergic diseases such as asthma and allergic rhinitis, which exhibits elevated abundance of eosinophils in circulating and airway tissues [29]. Therefore, downregulating the levels of IgE, IL-4 and IL-5 by depletion of CCR2 is of great significance in ameliorating the symptoms of allergic asthma.

Our previous results proved that blocking the CCL2/CCR2 axis decreased the production of IL-4, which induces the differentiation of Th2 cells and promotes the expression of Th2 cytokines. These findings lead us to conjecture that the CCL2/CCR2 signaling pathway might also regulate the levels of Th1 and Th2 cytokines. The Th cells, also called CD4 cells, are a type of T cell that are crucial in the immune system [30]. Proliferating Th cells that develop into effector T cells differentiate into two major subtypes of cells known as Th1 and Th2 cells [31]. Stimulated by signals of proliferation and activation, Th cells will develop to Th0 cells, which secrete interferon gamma (IFN-γ), IL-4 and IL-2 [32]. Whether Th0 cells will differentiate into Th1 or Th2 cells depends on the cytokine environment [33]. IL-4 and IL-10 suppress the Th1 cell production while IFN-γ promotes the Th1 cell production [34]. Conversely, IFN-γ inhibits Th2 cells and IL-4 drives Th2 cell production [34]. The excessive activation of Th2 cells could induce Type1 IgE-mediated allergy and hypersensitivity, such as allergic asthma, atopic dermatitis, and allergic rhinitis [35]. In addition, Th2 cell type cytokines play a major role in the pathogenesis of allergic asthma [35]. Therefore, we examined the levels of Th1-type cytokines and Th2-type cytokines in our OVA-induced mouse model. We found that injection of anti − CCL2 or anti − CCR2 antibody significantly increased the production of Th1-associated cytokines and decreased the levels of Th2-associated cytokines. Macrophages and monocytes in tissues have been identified as a critical source of CCL1 and CCL22, which play a crucial role in the recruitment of CD4 + Th2 cells. As our data showed, blockade of CCL2/CCR2 signaling reduces the abundance of monocytes in lung. Thus, we speculate that these responses might decrease the release of CCL1 and CCL22, thereby weaken the Th2 response and increase Th1 response. Taken together, our data suggest that blockade of CCL2/CCR2 signaling pathway could alleviate the inflammatory responses in allergic asthma by inhibiting the allergen-induced Th2 activation.

5. Conclusion

In conclusion, we successfully constructed a mouse model of allergic asthma induced by OVA and found that the number of inflammatory monocytes in PLF of asthmatic mice was significantly higher than that of normal mice. Down-regulation of CCR2 can significantly reduce the recruitment of inflammatory monocytes into the peritoneal cavity and alleviate the inflammatory response in mice. Blocking CCL2/CCR2 significantly reduced the expression of T2 cytokines and increased the expression of Th1 cytokines. Our study suggests that blocking CCL2/CCR2 to prevent the recruitment of inflammatory monocytes into the abdominal cavity might be a potential and useful treatment for allergic asthma.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

None.

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