CXCR4 inhibitor attenuates ovalbumin-induced airway inflammation and hyperresponsiveness by inhibiting Th17 and Tc17 cell immune response

HUILONG CHEN[1], XIANGQIN XU[1], JIEMING TENG[1], SHENG CHENG[1], HANSVIN BUNJHOO[1], YONG CAO[1], JIN LIU[1], JUNGANG XIE[1], CONGYI WANG[2], YONGJIAN XU[1] and WEINING XIONG[1]

[1]Department of Respiratory and Critical Care Medicine and [2]Center for Biomedical Research, Tongji Hospital, Key Laboratory of Pulmonary Diseases of the Health Ministry, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, P.R. China

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Abstract. Accumulating evidence suggests that chemokine (C-X-C motif) ligand 12 (CXCL12) and its receptor chemokine (C-X-C motif) receptor 4 (CXCR4) may contribute to the pathogenesis of allergic asthma. However, the underlying molecular mechanisms remain to be fully understood. T-helper 17 cells (Th17) and T-cytotoxic 17 cells (Tc17) have been implicated in the development of several allergic disorders, including asthma. The present study aimed to explore the association between CXCL12 signaling and Th17/Tc17 cells in the development of asthma. Ovalbumin (OVA)-sensitized BALB/c mice were treated with AMD3100, a specific CXCR4 antagonist, prior to OVA challenge. Following the final allergen (OVA) challenge, airway responsiveness to methacholine, influx of inflammatory cells to the airway, and cytokine levels in the bronchoalveolar lavage fluids (BALF) and lung homogenate were assessed. Interleukin (IL)-17-expressing $CD3^+CD8^-$ lymphocytes (Th17 cells) and $IL\text{-}17^+CD3^+CD8^+$ lymphocytes (Tc17 cells) isolated from lung tissue samples were detected by flow cytometry. The results of the present study demonstrated that administration of AMD3100 significantly decreased airway responsiveness to methacholine, attenuated the influx of inflammatory cells to the airway and reduced the levels of IL-4, IL-5 and IL-13 in the BALF. Furthermore, AMD3100 significantly reduced the increased number of lung Th17 and Tc17 cells as well as the levels of IL-17 in the lung homogenate induced by OVA challenge. In conclusion, the CXCR4 inhibitor suppresses the asthmatic response, which is associated with attenuation of the Th17 and Tc17 cell immune response.

Correspondence to: Professor Weining Xiong, Department of Respiratory and Critical Care Medicine, Tongji Hospital, Key Laboratory of Pulmonary Diseases of the Health Ministry, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan, Hubei 430030, P.R. China
E-mail: xiongdoctor@hotmail.com

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Introduction

Asthma is a chronic disease characterized by airway inflammation, airway hyperresponsiveness (AHR) and reversible airway obstruction. Although the precise mechanism underlying this response remains to be fully elucidated, it is now generally believed that asthma arises as a result of dysregulated immune responses in which T-helper (Th)2 cells have a central role in the pathogenesis and pathology (1).

Chemokine (C-X-C motif) ligand 12 (CXCL12), also known as stromal cell-derived factor-1, is a member of the chemokine family, which consists of low molecular-weight proteins (8-15 kD) produced by various types of cells involved in allergic inflammation (2). CXCL12 binds to chemokine (C-X-C motif) receptor 4 (CXCR4) and attracts a variety of cells, including resting T lymphocytes, monocytes, $CD34^+$ stem cells and mature eosinophils (3). CXCL12 and its receptor CXCR4 have been demonstrated to be involved in Th2-type allergic airway responses, and inhibition of CXCL12 or CXCR4 leads to reduced airway inflammation and AHR (4-6). Furthermore, another study demonstrated that the levels of CXCL12 were significantly higher in bronchoalveolar lavage fluids (BALF) of asthmatic patients compared with healthy individuals, and the concentration of CXCL12 was correlated with inflammatory cell numbers in the BALF (7). These results suggested that CXCL12 may contribute to inflammatory cell recruitment in asthma.

Interleukin (IL)-17 is a pro-inflammatory cytokine that is expressed in the airways of patients with asthma (8), and its expression is correlated with the severity of asthma (9). The concentration of IL-17 was significantly increased in the BALF, sputum and blood of patients with asthma (8,10,11). Furthermore, IL-17 or IL-17R-deficient mice exhibit reduced allergic airway inflammation (10,12). These results demonstrate the importance of IL-17 in the induction of allergic airway inflammation.

IL-17-producing $CD4^+$ T cells (Th17 cells) are a distinct subset of T helper cells similar to Th1 and Th2 cells (13-16). It has been demonstrated that Th17 cells enhance Th2 cell-mediated eosinophilic airway inflammation in mice (13). A corresponding subset of IL-17-secreting $CD8^+$ T cells [T-cytotoxic 17 (Tc17) cells] similar to the Th17 cells also exists (17). Our previous study demonstrated an increased proportion of Tc17 cells in the peripheral blood of patients with asthma compared with healthy controls, as well as in the spleen cells and lung tissue samples of asthmatic mice (18). These data support both Th17 and Tc17 cells may have a role in the regulation of allergic airway inflammation.

Although both experimental and clinical data support that CXCL12/CXCR4 signaling and Th17/Tc17 cells are involved in the pathogenesis of asthma, their association during the course of asthmatic responses however remains unknown. It is proposed that blockade of CXCR4 may suppress the asthmatic response associated with the attenuation of Th17 and Tc17 cell infiltration in the lung. Therefore, the anti-inflammatory effect of a CXCR4 antagonist, AMD3100, on Th2-type cytokines, inflammation cell infiltration and AHR using an ovalbumin (OVA)-induced murine asthma model, was investigated. In addition, the effects of AMD3100 on the percentage of Th17 and Tc17 cells in the lung was investigated.

Materials and methods

Mice. A total of 18 female BALB/c mice (weight, 18-21 g; age, 5-6 weeks) were obtained from the Laboratory Animal Center of the Hubei Province (Wuhan, China). All mice were housed in a specific pathogen-free facility in microisolator cages, an provided with autoclaved food and acidified water under a 12 h light/dark cycle. All experimental animal care and treatment protocols followed the guidelines established by the Institutional Animal Care and Use Committee of the Tongji Hospital (Wuhan, China). The ethics committee of Tongii Hospital.

Generation of asthmatic model and treatment. To generate an asthmatic model, mice were sensitized and challenged with OVA (Sigma-Aldrich, St. Louis, MO, USA) as previously reported (19). Briefly, the mice were sensitized by intraperitoneal injection with 100 μg OVA and 1 mg aluminium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 200 μl saline on days 0, 7 and 14. In the OVA group, the sensitized mice were then challenged by intranasal administration of 1 mg OVA in 50 μl saline on days 21, 22 and 23. In the control group, mice were challenged with 200 μl saline alone. In the OVA + AMD3100 group, AMD3100 (Cayman chemical company, Ann Arbor, MI, USA) was freshly dissolved in saline and administered intraperitoneally at a dose of 10 mg/kg in 200 μl saline 1 h prior to every challenge on days 21, 22 and 23.

Determination of AHR. AHR to inhaled methacholine (Sigma-Aldrich) was measured using FlexiVent (SCIREQ Scientific Respiratory Equipment, Inc., Montréal, QC, Canada) (20). The mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) (Wuhan Sanjiang Space Guge Biotech Co., Ltd., Wuhan, China), tracheotomized, and connected to the FlexiVent. Baseline airway resistance was measured in each mouse following nebulization (SCIREQ Scientific Respiratory Equipment, Inc.) of phosphate-buffered saline (PBS; vehicle for methacholine) for 10 sec using an Aeroneb ultrasonic nebulizer (SCIREQ Scientific Respiratory Equipment, Inc.). Following baseline measurements, the mice were first exposed to nebulized saline, followed by increasing doses (3, 6, 12 and 25 mg/ml) of nebulized methacholine for 3 min each. Breathing indices were read for 3 min following each nebulization, and the enhanced pause values were determined.

Collection of BALF and histological analysis. The mice were sacrificed 24 h by cervical dislocation following the last OVA or saline challenge. The lungs were lavaged 3 times with 0.8 ml saline, and the collected cells were centrifuged at 300 x g for 10 min at 4˚C. The total number of cells in BALF was counted by a hemacytometer. Eosinophils, lymphocytes, neutrophils and macrophages were counted in BALF using cytospins subjected to Wright-Giemsa staining (Wuhan Sanjiang Space Guge Biotech Co., Ltd.) at room temperature. Lung tissue samples were embedded in paraffin, cut into 5 mm sections using a microtome (Leica RM2016; Leica Biosystems, Wetzlar, Germany) and stained with hematoxylin and eosin (both purchased from Wuhan Sanjiang Space Guge Biotech Co., Ltd.). Airway inflammation was assessed using a light microscopy (BX53; Olympus Corporation, Tokyo, Japan).

Preparation of lung homogenate. The lung tissue suspensions were obtained as previously described (21). Briefly, the right lung was dissected prior to being rapidly frozen in liquid nitrogen and stored at -80˚C. Following thawing, the lung tissue sample was homogenized in PBS and centrifuged at 800 x g for 15 min at 4˚C to remove the sediments, and the supernatant was subsequently used for measurement of IL-17 concentration.

Measurement of cytokines. The levels of IL-4, IL-5 and IL-13 in the BALF, as well as those of IL-17 in the lung homogenates were determined by IL-4 (cat. no. 88-7044), IL-5 (cat. no. 88-7054), IL-13 (cat. no. 88-7137) and IL-17 (cat. no. 88-7371) ELISA kits (eBioscience, Inc., San Diego, CA, USA), according to the manufacturer's protocol. Briefly, samples were added to 96-well microtiter plates precoated with monoclonal antibody to mouse IL-4, IL-5 and IL-13 or IL-17 and incubated for 2 h at room temperature. Subsequently, 96-well plates were washed, and Biotin-Conjugate and Streptavidin-HRP were added. After washing 6 times with PBS and 0.25% Tween-20 (Wuhan Sanjiang Space Guge Biotech Co., Ltd.), 100 μl TMB Substrate Solution was added to each well. The plate was incubated for ~10 min in the dark and then 100 μl of Stop Solution was added into each well. Finally, absorbance was determined at 450 nm using a microplate ELISA reader (ELx800; Bio-Rad Laboratories, CA, USA). IL-4, IL-5 and IL-13 or IL-17 concentrations were calculated from a standard curve.

Flow cytometric analysis. The mononuclear cells in the lung tissue samples were obtained as reported previously (22). The cells collected from the lung tissue samples were stimulated with phorbol myristate acetate (50 ng/ml; Sigma-Aldrich) and

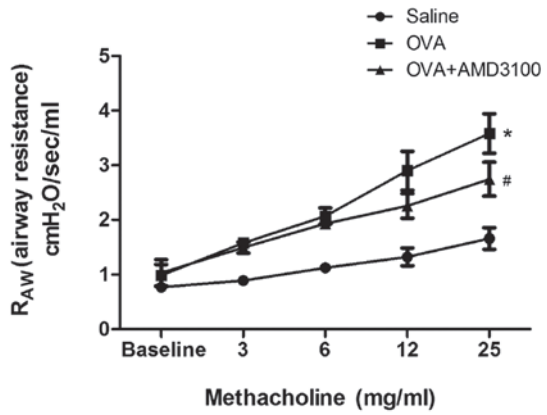

Figure 1. AMD3100 treatment reduces OVA-induced airway hyperresponsiveness. Saline, normal control mice treated with saline only; OVA, OVA-sensitized/challenged mice; OVA + ADM3100, ADM3100-treated and OVA-sensitized/challenged mice. *P<0.05, vs. the saline group and #P<0.05, vs. the OVA group. All data are presented as means ± standard error of the mean for n=4-5 mice/group. OVA, ovalbumin.

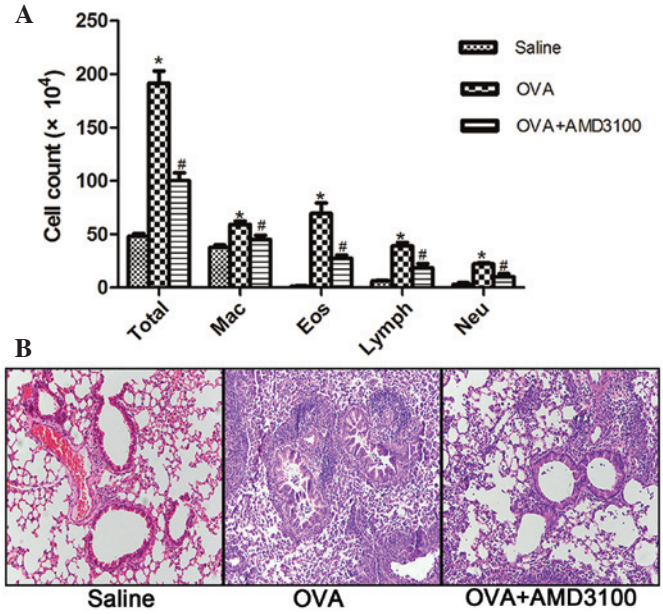

Figure 2. ADM3100 administration attenuates airway inflammation following OVA challenge. BALF and lung tissue samples were collected 24 h following the last challenge with OVA. (A) Differential cell counts for macrophages, eosinophils, lymphocytes and neutrophils were calculated from cytospin preparations. (B) Lung histopathology was assessed with hematoxylin and eosin (original magnification, x200). *P<0.05, vs. the saline group and #P<0.05, vs. the OVA group. All data are presented as means ± standard error of the mean for n=5-6 mice/group. OVA, ovalbumin; Mac, macrophages; Eos, eosinophils; Lymph, lymphocytes; Neu, neutrophils.

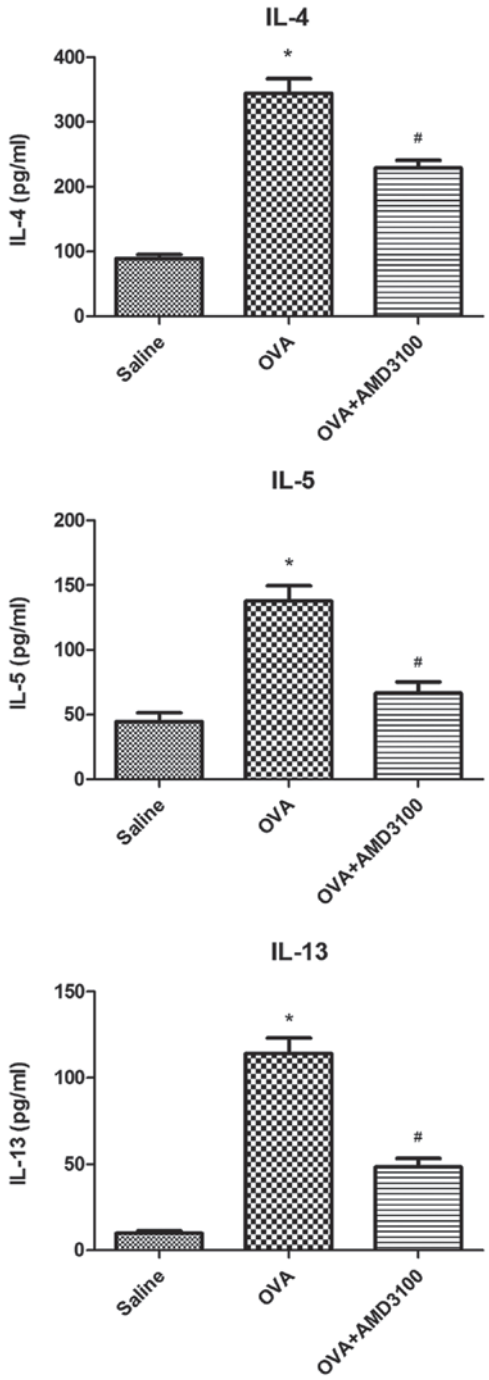

Figure 3. AMD3100 treatment decreases IL-4, IL-5 and IL-13 concentrations in the BALF. IL-4, IL-5 and IL-13 concentrations in the BALF were assessed 24 h following the last challenge with OVA. IL-4, IL-5 and IL-13 production significantly increased in the BALF following OVA sensitization and challenge, and this increase was significantly reduced by treatment with AMD3100. *P<0.01, vs. the saline group and #P<0.05, vs. the OVA group. Results are presented as the means ± standard error of the mean for n=5-6 mice/group. IL, interleukin; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid.

ionomycin (1,000 ng/ml; Sigma-Aldrich) for 5 h at 37˚C in an atmosphere containing 5% CO_2, and bovine serum albumin (50 ng/ml; Sigma-Aldrich) was then added to block the flow of cytokines from the cytoplasm. Following stimulation, the cells were stained with phycoerythrin (PE)-cyanine 5-conjugated anti-mouse CD3 antibody (1:20; cat. no. 17A2; Biolegend, Inc., San Diego, CA, USA) and fluorescein isothiocyanate-conjugated anti-mouse CD8 antibody (1:50; cat. no. 53-6.7; Biolegend, Inc.) for 30 min at 4˚C in the dark. Following cell-surface staining, the cells were washed twice with washing buffer (Biolegend, Inc.), fixed and permeabilised with Fix-Perm solution (BD Biosciences, San Jose, CA, USA) for intracellular staining with PE-conjugated anti-mouse IL-17 antibody (1:100; cat. no. TC11-18H10.1; eBioscience) for 30 min at 4˚C in the dark. The stained cells were used for flow cytometric analysis (BD LSR II; BD Biosciences) in order to determine the number of Th17 and Tc17 lymphocytes. $CD8^+$ T lymphocytes were

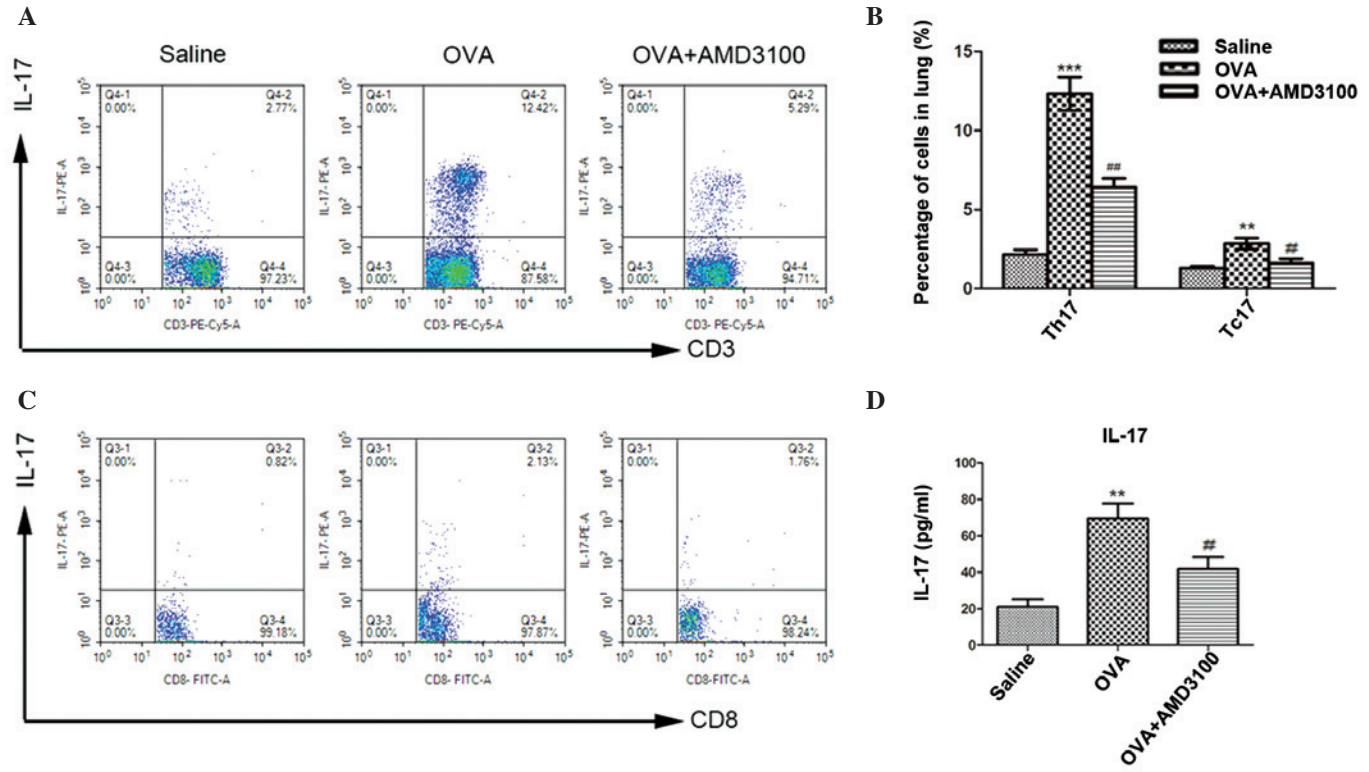

Figure 4. AMD3100 treatment attenuates the development of Th17 and Tc17 cells and decreases the concentration of IL-17 in the lung. (A and C) Representative plots and (B) combined data of Th17 and Tc17 numbers in the lung. IL-17 concentration in the lung homogenate assessed 24 h following the last challenge with OVA. (D) Marked increases in IL-17 production in the lung homogenate following OVA sensitization and challenge were significantly suppressed by treatment with AMD3100 as compared with the saline groups. ***P<0.001, vs. the saline group; **P<0.01, vs. the saline group; $^{\#\#}$P<0.05, vs. the OVA group; $^{\#}$P<0.05, vs. the OVA group. All data are presented as means ± standard error of the mean of n=4-5 mice/group. Th17, T-helper 17 cell; Tc17, T-cytotoxic 17 cell; IL, interleukin; OVA, ovalbumin.

specifically gated, the $CD3^+CD8^-IL17^+$ T lymphocytes were counted as Th17 cells, and the $CD3^+CD8^+IL\text{-}17^+$ T lymphocytes were counted as Tc17 cells.

Statistical analysis. All data were presented as means ± standard error of the mean. Data analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical differences were assessed by one-way analysis of variance. P<0.05 was considered to indicate a statistically significant result.

Results

Treatment with AMD3100 reduced AHR in experimental asthma. To determine the effects of AMD3100 on AHR, airway resistance was measured 24 h following the last challenge with OVA. In OVA-challenged mice, airway resistance was increased in a dose-dependent manner following exposure to methacholine. However, AMD3100 treatment inhibited the increased airway reactivity to methacholine induced by OVA (Fig. 1).

Administration of AMD3100 decreases airway inflammation. ADM3100 administration significantly reduced total cell counts and eosinophil counts in the BALF following OVA sensitization and challenge (Fig. 2A). These changes can also be observed by histological examination of the lung tissue samples from the treated animals; after treatment with ADM3100, inflammatory cell infiltration was inhibited around the airway (Fig. 2B).

Administration of AMD3100 reduces the production of Th2 cytokines. As shown in Fig. 3, the levels of IL-4, IL-5 and IL-13 were significantly increased in OVA-challenged mice, compared with the controls. Administration of AMD3100 significantly reduce these increased levels of IL-4, IL-5 and IL-13 in the BALF of OVA-challenged mice.

AMD3100 suppresses the development of Th17 and Tc17 cells in the lung and IL-17 production in lung homogenates. To investigate the effect of administration of AMD3100 on the development of Th17 and Tc17 cells, $CD3^+CD8^-$ and $CD3^+CD8^+$ T cells were isolated from the lung and gated for expression of IL-17 to compare saline, OVA, and OVA + AMD3100 treatment groups. OVA sensitization and challenge markedly increased Th17 and Tc17 cells in the lung compared with the saline-sensitized and challenged mice. However, this marked increase in Th17 and Tc17 cells was attenuated by AMD3100 administration, which resulted in a significant decrease in the percentage of Th17 and Tc17 cells compared with OVA-challenged mice (Fig. 4A-C). Furthermore, as shown in Fig. 4D, the levels of IL-17 in the lung homogenate were significantly increased in OVA-challenged mice compared with negative controls. Administration of AMD3100 significantly decreased the levels of IL-17 in the lung homogenate of OVA-challenged mice (P<0.01).

Discussion

Previous reports have demonstrated that inhibition of CXCL12/CXCR4 signaling attenuated allergic lung inflammation and AHR (5,6,23). However, the exact underlying mechanisms have yet to be fully understood. The present study investigated whether inhibition of CXCL12/CXCR4 signaling was able to attenuate OVA-induced airway inflammation and AHR by decreasing Th17 and Tc17 pro-inflammatory response. Using a murine model of asthma, the results demonstrated that administration of AMD3100 attenuated allergic airway inflammation along and significantly suppressed Th17 and Tc17 cell infiltration into the lung tissues, as well as decreased IL-17 levels in the lung. These results provide an improved understanding of the mechanisms underlying CXCL12/CXCR4 signaling in the pathogenesis of asthma.

AMD3100 is a soluble CXCR4 inhibitor, which inhibits binding of CXCL12 to CXCR4 and subsequent signal transduction (24). AMD3100 was shown to attenuate allergic pulmonary inflammation and AHR (5). In the present study, the results demonstrated that treatment with AMD3100 prior to allergen challenge significantly decreased airway inflammatory cell accumulation and AHR. The results of the present study were concordant with those of a previous study that demonstrated that specific inhibition of CXCR4 with AMD3100 reduced the number of pathological parameters associated with asthmatic-type inflammation (5). Based on both the results of the present and previous study (5), AMD3100 may effectively inhibit experimental allergic asthma in mice, however, the possible mechanisms underlying this process have yet to be fully understood.

Th17 cell is a type of $CD4^+$ T cell subset, and has been defined by its secreted product, IL-17 (25). In addition to Th17 cells, there are $CD8^+$ T cells named Tc17 cells (26), which also produce IL-17. Th17 cells and Tc17 cells share many similar characteristics, including production of IL-17 (27). Accumulating data suggests that Th17 or Tc17 cells may have an important role in the development of various allergic diseases that have classically been considered to be Th1- or Th2-mediated disorders (28-30). Our recent study also demonstrated an increased proportion of Th17 and Tc17 cells in the peripheral blood of patients with asthma compared with healthy controls, as well as in the spleen cells and lung tissues of asthmatic mice, which suggested that a functional disequilibrium between Th17 and Tc17 cell subsets may contribute to the allergic inflammatory process in asthma (18). Both Th17 and Tc17 cells likely contribute to the immune response in asthma since both have the ability to produce IL-17.

To determined whether the suppression of allergic airway responses induced by AMD3100 is associated with the presence of Th17 and Tc17 cells, the present study investigated the expression levels of Th17 and Tc17 cells in a murine allergic asthma model. The results demonstrated that treatment with AMD3100 attenuated allergic airway inflammation and significantly suppressed Th17 and Tc17 cell recruitment in mice. To the best of our knowledge, this is the first study to report an association between CXCL12 signaling and Th17/Tc17 cells. This investigation provides novel insight into the mechanisms underlying the involvement of the CXCL12/CXCR4 signaling pathway in asthmatic responses during the course of disease development.

In conclusion, the data of the present investigation suggested that inhibition of CXCL12/CXCR4 signaling could suppress the *in vivo* development of Th17 and Tc17 cells. These findings also provide further support for an anti-inflammatory role of AMD3100 as a CXCR4 inhibitor in the treatment of asthma. However, further studies are required in order to explore the precise mechanisms underlying these processes.

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