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A small-molecule compound targeting CCR5 and CXCR3 prevents the development of asthma

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ABSTRACT: Asthma is associated with increased number of T cells in the lung. CC chemokine receptor 5 (CCR5) and CXC chemokine receptor 3 (CXCR3) have been reported to play important roles in lung T cell homing pathway, and may be potential targets for asthma therapy.

The aim of this study was to investigate a role of CCR5 and CXCR3 in allergen-induced acute asthma and to determine whether a novel small-molecule compound, TAK-779, targeting CCR5 and CXCR3 can attenuate allergic airway responses.

We sensitized mice with ovalbumin (OVA). We measured mRNA expression of chemokine receptors in the lung after the challenge with either aerosolized PBS or OVA. We also treated OVA-sensitized mice with TAK-779. We measured respiratory function, performed bronchoalveolar lavage, and obtained blood and lung.

OVA challenge increased CCR3, CCR5 and CXCR3 expression in the lung. Treatment with TAK-779 significantly attenuated altered respiratory function and pulmonary allergic inflammation. The beneficial effect was associated with reduced expression of CCR5 and CXCR3 in the lung.

These data demonstrate that blockade of CCR5 and CXCR3 using TAK-779, a synthetic non-peptide compound, can prevent the development of asthma features in a mouse model. Thus, CCR5 and CXCR3 may be potential targets for asthma therapy.

Key words:, asthma, CCR5, CXCR3, chemokine, cytokine

Asthma is characterized by a large influx of CD4+ T cells and eosinophils in the bronchial lamina propria [1, 2]. The fact that lung eosinophilia and bronchial hyperreactivity do not occur in the absence of T cells supports a critical role of T cells in asthma [3, 4]. Therefore, preventing the recruitment of T cells to sites of airway inflammation may be an attractive therapeutic strategy for asthma. Lymphocyte recruitment is controlled by adhesion molecules and chemoattractant signals expressed by high endothelial venules (HEVs) and postcapillary venular endothelium. Chemokines are chemotactic cytokines that regulate not only the migration of leukocytes but also their activation and differentiation by binding to specific cell surface receptors on target cells [5, 6]. Chemokines play important roles in a variety of inflammatory disease conditions, such as asthma, inflammatory bowel disease, and infectious diseases [5]. In the field of asthma, several chemokines and chemokine receptors, such as eotaxin, regulated on activation normal T cell expressed and secreted (RANTES), macrophage-inflammatory protein (MIP-1α), monocyte chemoattractant protein-1, -5 (MCP-1, MCP-5), CC chemokine receptor 1, 3, and 5 (CCR1, CCR3, CCR5) have been suggested to be critical in the development of allergic airway inflammatory response in animal models [3, 7-9]. Furthermore, CCR5 and CXCR3 have been reported to be expressed on human lung T cells [10]. These observations indicate that CCR5 and CXCR3 have important roles in T cell homing to the lung.

The objective of the present study was to test the hypothesis that blockade of CCR5 and CXCR3 could prevent the development of asthma mainly by inhibiting the recruitment of T cells to the lung. To this end, we utilized TAK-779, a novel CCR5 and CXCR3 antagonist. TAK-779 has been developed as anti-HIV-1 agent, since CCR5 acts as a major co-receptor for fusion and entry of macrophage-tropic HIV-1 into the host cells [11, 12]. TAK-779 is a synthetic small-molecule non-peptide compound that inhibits the ligands, RANTES, MIP-1α or MIP-1β, from binding to CCR5 and blocks chemokine-induced chemotaxis in vitro [11, 13]. CCR5 is expressed on activated T cells (Th1), macrophages, dendritic cells (DCs), and NK cells [5]. Furthermore, it has been recently demonstrated that TAK-779 also has an ability to block the binding of CXCR3 and IP-10, one of CXCR3's ligands, and also inhibited cell adhesion or chemotaxis induced by its ligands in vitro [13]. CXCR3 is exclusively expressed on activated T cell (Th1), NK cells, and B cells [5, 14, 15]. Therefore,

TAK-779 has the ability to control physiological and pathological responses that is mediated by both CCR5 and CXCR3. Recently, Akashi et al reported that TAK-779 treatment inhibited the recruitment of alloreactive cells into the grafts, which resulted in the prolongation of graft survival using transplant mouse models in which CCR5 and CXCR3 have important roles [16]. Our data indicate that CCR5 and CXCR3 have important roles in the development of allergen-induced asthma. The significant effect of TAK-779 on prevention of physiologic and pathologic features of asthma in a mouse model supports future targeting of CCR5 and CXCR3 in humans.

MATERIALS AND METHODS

Animals

Female BALB/c mice 8 weeks old were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in the animal facility of Nara Medical University that was maintained at 22-24□ with a 12-h dark/light cycle, fed a commercial pelleted mouse food and given water ad libitum under specific pathogen-free conditions according to standard guidelines for the care and use of animals [17]. All experiments were conducted under protocols approved by our institutional review board.

Allergen sensitization and exposure

The mice were sensitized by initial i.p. injection of 0.4 ml phosphate buffered saline (PBS) containing ovalbumin (OVA) (20 μg,□grade 3, Sigma-Aldrich, St. Louis, MO□) and alum (2 mg). After 1 week, these mice were further sensitized with the same reagent. On day 10 after the second sensitization (day 18), the mice were exposed to aerosols of allergen (1% □wt/vol□OVA in PBS, pH 7.4) for 10 min on 3 consecutive days (days 18-20). The aerosol exposure was performed in a chamber using a PARI nebulizer (PARI Japan, Osaka, Japan). As a control group, the mice were sensitized with OVA and exposed to aerosols of PBS.

Administration of TAK-779

A small-molecule, nonpeptide compound, TAK-779 (*N,N*-dimethyl-*N*-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocyclohepten-8-yl]carbonyl]amino]benzyl]tetra-hydro-2*H*-pyran-4-aminium chloride; molecular weight = 531.13) was kindly provided by Takeda Chemical Industries, Ltd. (Osaka, Japan). The chemical structure has been previously shown [11]. It was diluted with sterile water to make a final concentration of 0.5 mg/ml. TAK-779 (250μg/day) was subcutaneously administrated on 3 consecutive days from day 18 to day 20. Mice administrated TAK-779 appeared healthy, showed regular weight gain and activity levels similar to control mice, and had no ulceration at the injection sites.

Pulmonary function testing

Twenty-four hours after the last challenge, the responsiveness of mice to increasing concentrations of aerosolized methacholine was measured using whole body plethysmography (Buxco, Wilmington, NC) as previously described [18, 19]. The main indicator of airflow obstruction, enhanced pause (Penh), which shows strong correlation with the airway resistance examined by standard evaluation methods (see discussion), was calculated from the box waveform [20]. Aerosolized saline or methacholine in increasing concentrations (6, 12, 25, 50 mg/ml) was nebulized to the mice for 1 min, and Penh measurements were taken for 5 min after each dose. Penh values for the 5 min were averaged and used to compare results across treatment groups and individual mice.

Pathological analysis

Twenty-four hours after physiologic testing, the animals were euthanized with sodium pentobarbital (Nakalai Tesque Inc. Kyoto, Japan). Serum was collected and stored at –20□. Bronchoalveolar lavage (BAL) was performed, and BAL fluid (BALF) cells and differentials were counted as previously described [18, 19]. After lavage, some of the lungs were instilled with 10% buffered formalin, removed, and fixed in the same solution. After paraffin embedding, sections for microscopy were stained with hematoxylin and eosin (H&E). An index of pathologic changes in coded H&E slides was delivered by scoring the inflammatory cell infiltrates around airways and vessels for greatest severity (0, normal; 1, < 3 cell diameter thick; 2, 4-10 cells thick; 3, > 10 cells thick) and overall extent (0, normal; 1, < 25% of sample; 2, 25-75%; 3, >75%). The index was calculated by multiplying severity by extent, with a maximum possible score of 9. Metaplastic goblet cells were identified by their abundant cytoplasm filled with mucin and by their flattened nuclei.

Assay of serum IgE

Anti-OVA-specific IgE Ab was measured by ELISA as previously described [21]. For a positive control standard, we used a monoclonal anti-OVA IgE (2C6, Serotec, Kidlington-Oxford, United Kingdom) [18].

Extraction of total RNAs and quantitative real-time PCR analysis

Forty-eight hours after the last challenge, total RNA was isolated from whole lung by using guanidine isothiocyanate methods and was transcribed to cDNA using Omniscript Reverse Transcriptase (QIAGEN, Hilden, Germany) and amplified with oligo dT primers (Amersham Biosciences Corp., Piscataway, NJ). Quantitative real-time PCR analysis was performed by using ABI Prism 7700 sequence detector system (PE Applied Biosystems, Foster City, CA). Primer/probe sets were purchased from PE Applied Biosystems. PCR was carried out with the TaqMan Universal PCR Master Mix (PE Applied Biosystems) using 1 μl of cDNA in a 20 μl final reaction volume. The PCR thermal cycle conditions were as follows: initial step at 95□ for 10 minutes, followed by 40 cycles of 95□ for 15 seconds and 60□ for 1 minute. The expression levels of each mRNA were divided by levels of mRNA of the housekeeping gene β2-microglobulin.

Statistical analysis

Data are presented as mean±SEM. ANOVA analysis of differences among group means was performed using Fisher's protected least significant difference test and the Statview software program (Abacus Concepts, Berkeley, CA). Statistical significance was accepted when $P < 0.05$.

RESULTS

Increased CCR3, CCR5 and CXCR3 expression in the airways after allergen challenge

First, we examined several potent chemokine receptors including CCR5 and CCR3 in this model. Quantitative real-time PCR analysis showed the increased mRNA expression of CCR3, CCR5, and CXCR3 in the lung of OVA-sensitized and exposed mice compared with OVA-sensitized and PBS-exposed mice. (fig. 1; The results are representative of 4 independent experiments.).

Effects of TAK-779 treatment

Next we evaluated the protective effect of TAK-779 administration on key features of this mouse model of asthma. OVA-sensitized and exposed mice showed increased total cells, lymphocytes, and eosinophils on BALF compared with control mice sensitized with OVA and exposed to PBS (fig. 2a). TAK-779 treatment significantly decreased the number of total cells, lymphocytes, and eosinophils on BALF (fig. 2a). Results of semi-quantitative scoring of histological changes further support the qualitative changes presented in figure 2b. While control mice showed robust pathologic changes of allergic pulmonary inflammation (AI) (eosinophil and mononuclear cell infiltration around airways and vessels and goblet cell hyperplasia), TAK-779 treatment significantly diminished the pathologic changes of AI (fig. 2c and 2d). Altered respiratory function was assessed as an increased Penh in response to increasing doses of methacholine. OVA-sensitized and exposed mice showed altered respiratory function (fig. 3), manifest as increased Penh values compared to those seen in control mice sensitized with OVA and challenged with aerosols of PBS. In contrast, OVA-sensitized and exposed mice treated with TAK-779 showed minimal changes in respiratory function, and were comparable to the control group (fig. 3). Serum OVA-specific IgE was increased in OVA-sensitized and exposed mice. TAK-779 treatment did not reduce serum OVA-specific IgE production (fig. 4).

TAK-779 downregulates the expression of chemokine receptors and Th1 cytokines

To further characterize the underlying mechanisms responsible for the marked decrease in allergic response in lungs of TAK-779-treated mice, we analyzed local expression of chemokines, chemokine receptors and cytokines by quantitative real-time PCR. TAK-779 treatment significantly downregulated the expression of CCR5 and CXCR3 that were upregulated in OVA-sensitized and exposed mice compared with mice sensitized with OVA and exposed to aerosols of PBS (fig. 5). In contrast, the expression of CCR3 that was also upregulated in OVA-sensitized and exposed mice was not downregulated by TAK-779 treatment (fig. 5). Moreover, the expression of both Th1 cytokines (IFN-γ and TNF-α) and Th2 cytokine (IL-4 and IL-13) were higher in OVA-sensitized and exposed mice compared with mice sensitized with OVA and exposed to aerosols of PBS (fig. 6). Th1 cytokines (IFN-γ and TNF-α) were significantly downregulated by TAK-779 treatment (fig. 6). In contrast, the expressions of Th2 cytokines (IL-4 and IL-13) were not downregulated by TAK-779 treatment (fig. 6). However, the ligands for CCR5 and CXCR3, RANTES, MIP-1α, IP-10, and Mig were not upregulated in our asthma model (data not shown).

DISCUSSION

The incidence and prevalence of asthma have markedly risen in industrialized countries [22]. Although some success has been achieved with the use of anti-inflammatory drugs, there remain many therapeutic challenges, including chronic airway remodeling changes and steroid-resistant severe asthma. To overcome these problems, novel targets and approaches are needed to suppress both allergic pulmonary inflammation (AI) and airway hyperresponsiveness (AHR), two defining characteristics of asthma. Most current therapies are designed to suppress the inflammation that is caused mainly by inflammatory cells already recruited to the airways. In contrast, inhibiting the initial recruitment of T cells to the airways represents a novel approach, especially since these cells play a central role in the development of asthma.

In this study, we have examined whether blockade of CCR5 and CXCR3 had a beneficial effect on development of asthma features. The context for these studies includes data suggesting that CCR5 and CXCR3 may have an important role in T cell homing to the lung. Campbell et al reported that human lung T cells expressed CCR5 and CXCR3, and only low levels of CCR4 and $\alpha_4\beta_7$. This profile is distinct from that of gut- and skin-homing T cells although both CCR5 and CXCR3 are not specific for the lung [23-26]. These data implied that CCR5 and CXCR3 could be a potent therapeutic target of asthma for inhibiting the recruitment of T cells to the airways.

There were two major findings in this study. First, we found that the expression of the Th1-linked chemokine receptor (CR), CCR5 and CXCR3 were significantly upregulated in the lungs of OVA-sensitized and exposed mice, Th2-mediated asthma model. The expression of the Th2-linked CR, the major CR expressed on eosinophils, CCR3 was also upregulated in this model. Th1 cells appear to preferentially express CCR5 and CXCR3 whereas Th2 cells preferentially express CCR4 and, to a lesser extent, CCR3 [27]. The chemokine system in vivo is extremely redundant because of the large number of different chemokines, the overlap in chemokine function and the pleiotrophy of chemokine-receptor interaction. One of the challenges in this field is to identify which receptors are playing major roles in specific inflammatory conditions. Our findings suggest that the Th2-linked CRs are not the only

potential targets for asthma therapy. The Th1-linked CRs, especially CCR5 and CXCR3 may also be valid targets [28].

Indeed, the second major finding of our study was that TAK-779, that has an ability to antagonize CCR5 and CXCR3, significantly prevented the pathophysiological features of asthma. This was demonstrated by attenuation of altered respiratory function, and decreased AI. Furthermore, TAK-779 treatment significantly down-regulated the expression of CCR5 and CXCR3 in the lung of OVA-induced asthma mice. We cannot to date satisfactorily explain which immune cells were inhibited the recruitment into lungs by TAK-779. However, the hypothesis that TAK-779 has an effect to inhibit the recruitment of immune cells including Th1 cells into the lungs is borne out by the observation of downregulated expression of CCR5 and CXCR3 that are expressed on Th1 cells, and Th1 cytokines (IFN-γ and TNF-α) in the lungs by TAK-779 treatment. It has now become increasingly clear that Th1 cells have a proinflammatory role in Th2-governed disorder, asthma [29-31]. Furthermore, some data has demonstrated that IFN-γ contributes to the development of AHR and AI in experimental asthma [32, 33]. Thus, the beneficial effect of TAK-779 on asthma development may depend, at least in part, on the prevention of infiltration of Th1 cells into the lung. However, we speculate that the prevention of infiltration of other immune cells might contribute to the dramatic inhibition of physiologic (altered respiratory function), and pathologic (BAL and histopathology) measures of asthma severity in our model.

Some limitations of the study merit discussion. We used noninvasive plethysmography to measure Penh as an index of altered respiratory function, a technique that has been the subject of considerable controversy [34]. However, the best, and arguably most useful, correlation of Penh with more invasive measures of airway function is found in BALB/c mice after OVA sensitization and aerosol challenge protocols, as used in our studies. Indeed, Adler et al. reported that Penh correlates well with lung resistance (RL) in the OVA-allergy model in BALB/c mouse [35]. Since direct testing of airway hyperresponsiveness was not performed, we have described the changes in Penh as altered respiratory function. The semi-quantitative analysis of tissue inflammation has limitations since it is not a rigorous morphometric technique. Nevertheless, it complements (and is consistent with) the more

quantitative analysis of AI by lavage. Moreover, the combined analyses of airway function and inflammation responses are internally consistent.

Chvatchko et al reported that blockade of CCR5 using amino-terminally modified RANTES/CC chemokine ligand 5 analogues led to decreased AI, but did not affect AHR in a mouse model of OVA-induced asthma [9]. In contrast, in our experiment, blockade of CCR5 using TAK-779 significantly inhibited altered respiratory function as well as AI. The basis for this is not clear, but may reflect the use of a new synthetic small-molecule non-peptide compound to block CCR5. Amino-terminally modified RANTES/CC chemokine ligand5 analogues have ability to antagonize both CCR1 and CCR5. On the other hand, TAK-779 has been reported to have ability to antagonize both CCR5 and CXCR3, and to control a physiological and pathological response that is mediated by both CCR5 and CXCR3 [13]. Therefore, blockade of both CCR5 and CXCR3 may be more effective to prevent the development of asthma responses than targeting only CCR5, or CCR5 and CCR1. Future studies are needed to address this question by use of mice deficient for either receptor, and by use of neutralizing mAbs or specific receptor small molecule antagonists.

In conclusion, we have demonstrated for the first time that the blockade of CCR5 and CXCR3 by the administration of a novel small-molecule compound, TAK-779 can prevent the development of key features of asthma in a mouse model (both altered respiratory function and AI). Our data indicate that targeting CCR5 and CXCR3 may have a therapeutic potential in clinical asthma. Thus, further studies of the therapeutic potential of TAK-779, other small molecule chemokine receptors antagonists and blocking antibodies targeting CCR5 and CXCR3 for both acute and chronic aspects of asthma are warranted.

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Figure legends

FIGURE 1. CCR3, CCR5, and CXCR3 mRNA expression measured by quantitative real-time PCR analysis. Lungs were collected from PBS and ovalbumin (OVA) group. Results are normalized to β2-microglobulin expression and are expressed as means ± SEM obtained from three to five mice in each group. *: p<0.0005.

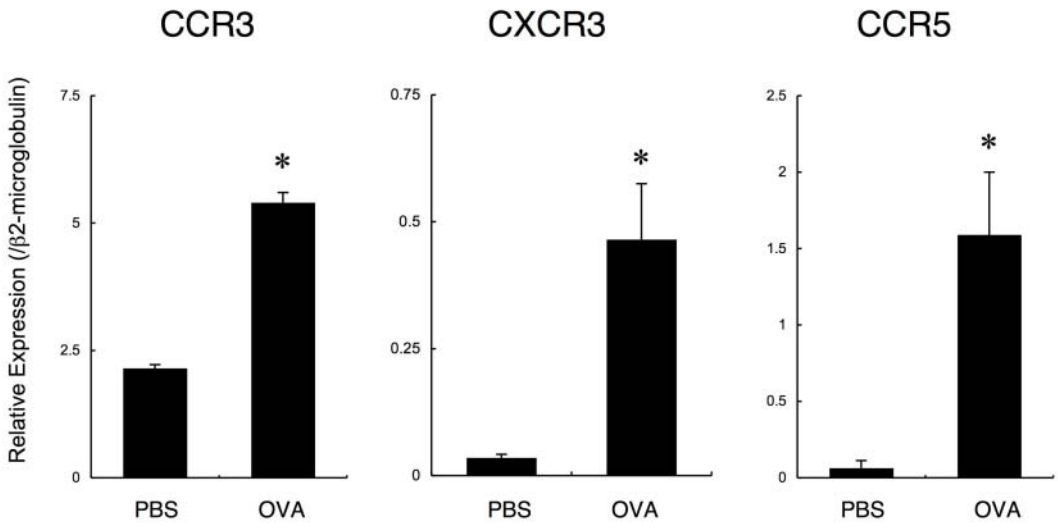


FIGURE 2. Effect of TAK-779 on allergic pulmonary inflammation. (a) BAL cell findings. n = 10 for each group. *: p<0.01 versus other groups; #: p<0.05 versus PBS group. (b) Semiquantative analysis of histopathologic changes, n = 5 per group. *: p<0.005. Representative lung histology findings from OVA (c) and OVA with TAK-779 treatment groups (d). Original magnifications, x200.

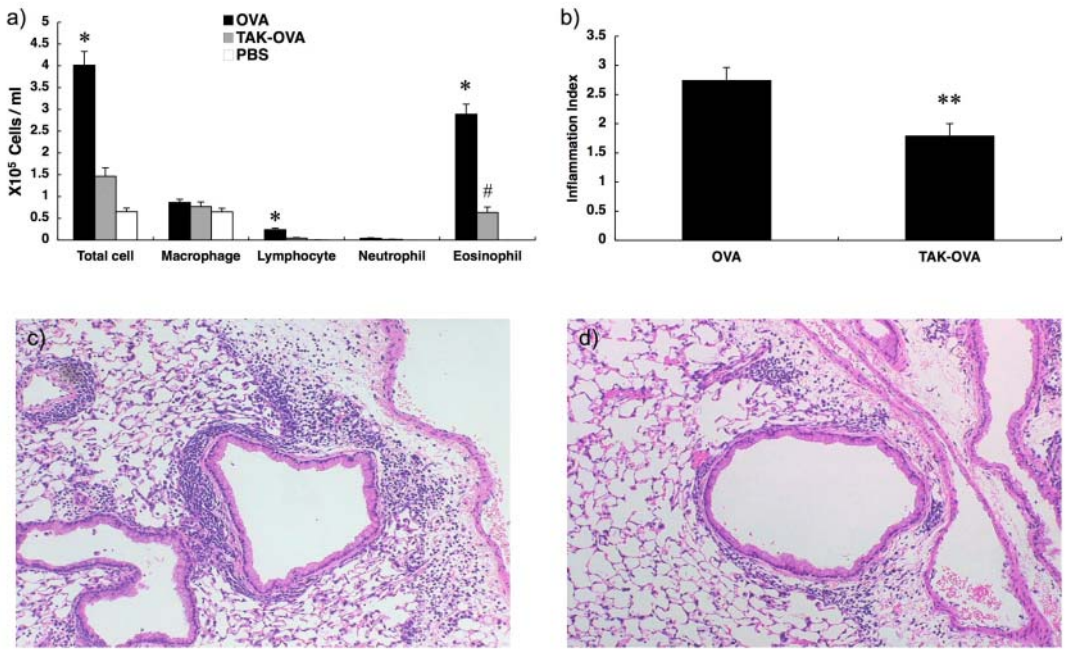


FIGURE 3. Effect of TAK-779 on respiratory function to inhaled methacholine, measured by whole-body plethysmography. n = 5 for each group. *: $p<0.05$ versus other groups; $^{\#}$: $p<0.01$ versus PBS group.

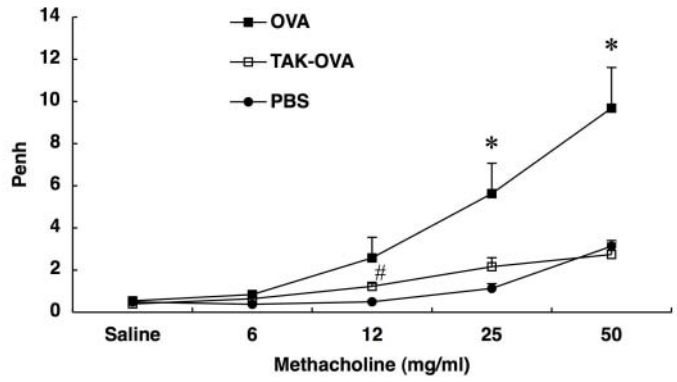


FIGURE 4. Effect of TAK-779 on the production of serum OVA-specific IgE. n = 5 for each group. *: $p<0.0001$ versus other groups.

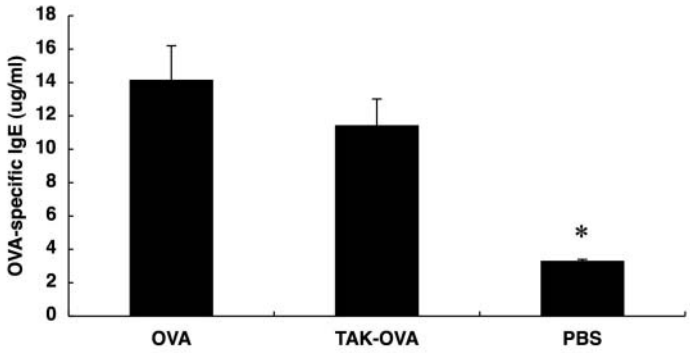


FIGURE 5. Effect of TAK-779 on mRNA expression of CCR3, CCR5, and CXCR3 in the lung measured by quantitative real-time PCR analysis. Lungs were collected from PBS, OVA, and TAK-779-treated OVA group. Results are normalized to β2-microglobulin expression and are expressed as means ± SEM obtained from three to five mice in each group. *: $p<0.05$ versus OVA group.

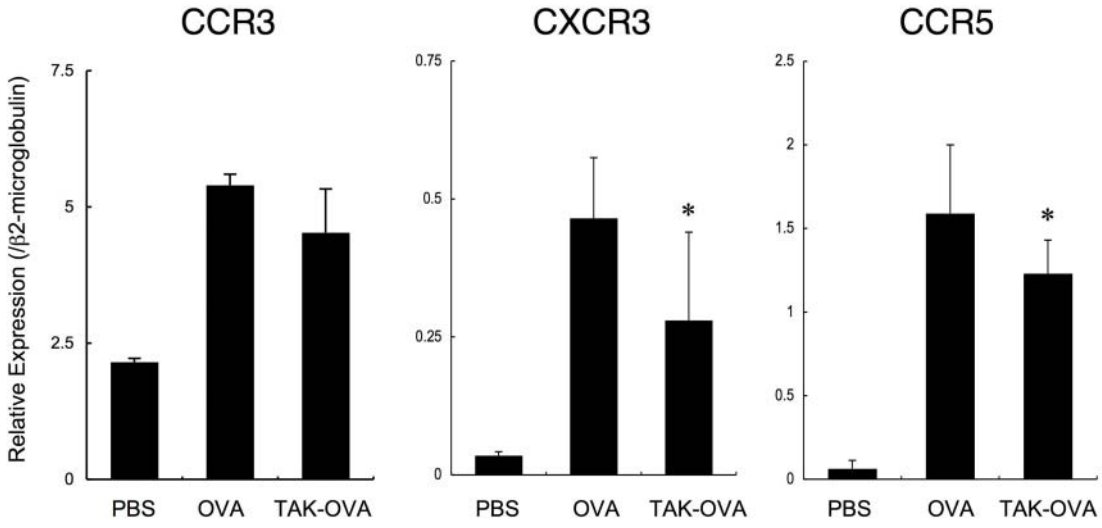
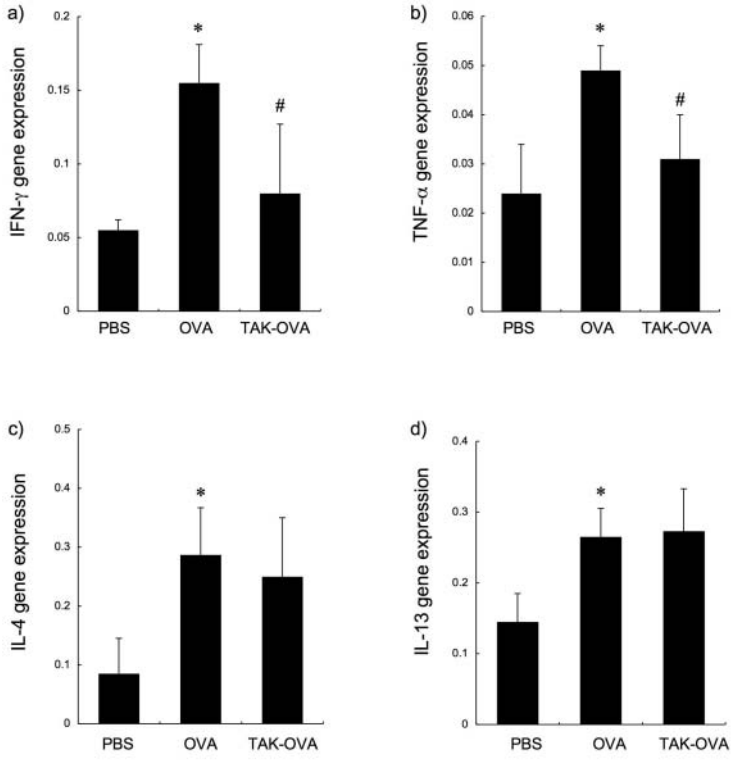


FIGURE 6. Effect of TAK-779 on mRNA expression of cytokines in the lung measured by quantitative real-time PCR analysis. Lungs were collected from PBS, OVA, and TAK-779-treated OVA group. Results are normalized to β2-microglobulin expression and are expressed as means ± SEM obtained from three to five mice in each group. *: $p<0.01$ versus PBS groups; $^{\#}$: $p<0.05$ versus OVA group.


a)
IFN-γ gene expression
0.2
0.15
0.1
0.05
0
*
#
PBS
OVA
TAK-OVA
b)
TNF-α gene expression
0.06
0.05
0.04
0.03
0.02
0.01
0
*
#
PBS
OVA
TAK-OVA
c)
IL-4 gene expression
0.5
0.4
0.3
0.2
0.1
0
*
PBS
OVA
TAK-OVA
d)
IL-13 gene expression
0.4
0.3
0.2
0.1
0
*
PBS
OVA
TAK-OVA