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ORIGINAL ARTICLE

Therapeutic Effect of Intratracheal Administration of Murine IL-4 Receptor Antagonist on Asthmatic Airway Inflammation

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Objective. It is well known that IL-4 and IL-13 play critical roles in the pathogenesis of asthma. In this study, by overexpressing murine IL-4 receptor antagonist (mIL-4RA), a competitive antagonist for both IL-4 and IL-13, we investigated the therapeutic effects of mIL-4RA on mouse asthmatic airway inflammation. **Material and methods.** BALB/c mice were randomly divided into four groups: healthy control mice; ovalbumin (OVA) sensitized/challenged mice; OVA sensitized/challenged mice intratracheally administered with mIL-4RA plasmid (mIL-4RA group); and OVA sensitized/challenged mice intratracheally administered with control plasmid (control plasmid group). The airway inflammation was determined by histopathological examinations. Cytokines were measured by enzyme-linked immunosorbent assay (ELISA). Flow cytometry was used to analyze CD4 and CD8 T-lymphocyte subsets. **Results.** Compared to the control plasmid-treated mice, intratracheal administration of mIL-4RA expressing plasmid on the sensitization phase protected the mice from the subsequent induction of asthmatic airway inflammation. The eosinophilic infiltration in bronchoalveolar lavage fluid (BALF) was significantly reduced compared to that of the control ($p < 0.01$). Interestingly, intratracheal administration of mIL-4RA regulated the Th1/Th2 cytokine imbalance in local airway with increased IL-13 levels and decreased IFN- γ levels compared to the control plasmid group. However, although we did see the decreased level of IL-4 and IL-13 in serum, the serum level of IFN- γ is not changed in the mIL-4RA group, suggesting that mIL-4RA could not correct the imbalance of Th1/Th2 cytokines in serum. In addition, intratracheal administration of mIL-4RA had no effect on the ratio of CD4/CD8 T-lymphocyte subsets in the peripheral blood, lung, or spleen. **Conclusions.** This study demonstrated that intratracheal administration of mIL-4RA attenuated the asthmatic inflammation and regulated the Th1/Th2 cytokine imbalance in local airway with minimal systemic effects. This method may serve as a potential therapeutic option for treating asthma.

Keywords interleukin 4 asthma, IL-4, receptor antagonist

INTRODUCTION

The clinical manifestations of allergic asthma are believed to result from a dysregulated T helper 2 lymphocyte (Th2)-biased response to antigen (1). Interleukin (IL)-4 and IL-13 are two closely related Th2 cytokines. They share many biological activities such as, induction of IgE isotype switching, differentiation of Th2 lymphocytes, up-regulation of MHC class II expression (2), promoting eosinophil transmigration, increasing mucus secretion, and airway hyperresponsiveness (AHR) (3–5). IL-4 exerts its biological effects by binding to the IL-4R alpha-chain that complexes with the common gamma-chain. Formation of the receptor heterodimer is critical for many IL-4-induced effects associated with allergic responses (6). The IL-4R alpha-chain also forms the functional signaling heterodimer with IL-13R alpha chain (IL-13R alpha 1/IL-4R alpha). This explains the similar biological functions between IL-4 and IL-13 (7, 8). Mutagenesis studies have identified the critical regions for the ligation of IL-4 with the IL-4R alpha-chain. Mutation of amino acids glutamine 116 and tyrosine 119 to aspartic acid in murine IL-4 produces a mutant

protein (Q116D/Y119D), which binds to the IL-4R alpha-chain with high affinity but fails to induce signal transduction (8, 9). Another mutant study represents a murine IL-4 truncated protein (C118 deletion), in which the terminal amino acids from position 119 onward are deleted. Researches on this murine IL-4R antagonist (IL-4RA) showed that it inhibited the development of allergic airway eosinophilia, AHR, and serum level of OVA-IgE (10). However, this IL-4R antagonist protein has some barriers in clinical trials because of its short half-life. A high concentration of plasma IL-4RA must be maintained for a long period for them to have suppressive effects. Therefore, we hypothesized that delivering of this therapeutic protein by gene transfer may improve the efficacy of asthma treatment (1, 11). In the present study, by intratracheally administering the mIL-4RA expressing plasmid we evaluated the therapeutic effects of this mIL-4RA on mouse asthmatic inflammation.

MATERIAL AND METHODS

Animals

Female BALB/c mice, 6 to 8 weeks of age, weighing 18 ± 0.5 g, were purchased from Experimental Animal Center of Chinese Academy of Sciences, Shanghai, China. Mice were housed in microisolator cages in a specific pathogen-free (SPF) condition with 12-hour light-dark cycles. All mice were maintained on OVA-free diets.

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Allergen Sensitization and Airway Challenge

The allergen mixture of OVA (Grade V, Sigma, USA) and aluminum potassium sulfate (alum, Sigma) for sensitization was previously described by Oh et al. (12). Briefly, mice were sensitized by intraperitoneal (i.p.) and subcutaneous (s.c.) injection of 100 μ g OVA complexed with alum in a total volume of 200 μ L on days 0 and 14. Mice were challenged 30 minutes via the airways with a nebulized 5% OVA (Grade III, Sigma, USA) daily for 10 days (days 21 to 30). Healthy control mice were sensitized and challenged with normal saline (NS). The mice were sacrificed 24 hours after the last challenge.

mIL-4RA Expression Vector Construction

The mIL-4 RA (C118 deletion) cDNA was amplified by polymerase chain reaction (PCR). The primers used for mIL-4RA were: (forward: 5'-CCGCTCGAGGCCACCATGCATATCCACGGATGCGACA-3', reverse: 5'-CGGAATTCTTC AATCCATTTGCATGATGCTC-3'). The 5' oligonucleotide of the primer contains a *Xho*I site, and the 3' oligonucleotide is incorporated with a stop codon at position 119 and an *Eco*RI site. The PCR conditions used in this study were: 94°C for 5 minutes, followed by 94°C (20 seconds), 55°C (20 seconds), 72°C (15 seconds) for 30 cycles, and 72°C for 3 minutes. PCR fragments were digested by *Xho*I and *Eco*RI (TaKaRa, Japan) and were subcloned into plasmid pIRES2-EGFP (BD, USA). The recombinant pIRES2-EGFP/IL-4RA construct was confirmed by *Xho*I and *Eco*RI double digestion and DNA sequencing. Purified Plasmid was extracted with EndoFree Plasmid Maxi Kit (Qiagen, China).

In Vivo Gene Delivery to Airway

The approach of intratracheal administration was performed according to the method described by Champion et al. (13). Briefly, mice were anesthetized with a mixture of ketamine/xylazine and were placed in a supine position on a surgical table. Using sterile techniques, the trachea was approached via a midline neck incision and was isolated through blunt dissection. A 22-gauge blunt needle was inserted into the trachea and instilled vector DNA or NS. Complexes of 10 μ g vector DNA + 2 μ L in vivo-jetPEI (Polyplus-Transfection Inc., New York, USA) + 40 μ L 5% (w/v) glucose were directly instilled into the tracheal tube on the day of first sensitization. Immediately before instillation, a forced exhalation was achieved by circumferential compression of the thorax, and the compression was released after intratracheal instillation. This approach results in a forceful inspiration that will facilitate dispersion of vector to distal air spaces.

Bronchoalveolar Lavage Fluid (BALF) Collection

Mice were placed in a supine position, and the trachea was isolated by blunt dissection. Lungs were lavaged with pre-chilled phosphate buffered solution (PBS) 0.5 mL for twice. BALF was collected and was centrifuged at 500 g for 5 minutes to recover cells. After centrifugation, the supernatant was collected and was stored at -20°C for cytokine analysis. Cell pellets were resuspended in PBS and the total cell number was determined by counting with a hemocytometer. Differential cell counts were performed by counting at least 200 cells, stained with Wright-Giemsa and differentiated by standard hematological procedures.

Measurement of Cytokine

Cytokine levels in the BALF or serum were measured by standard enzyme-linked immunosorbent assay (ELISA). Procedures were performed according to the recommended protocol of the manufacturer. The limit of detection for IL-4 (Bender MedSystems, Austria), was 1.98 pg/mL, for IFN- γ (Bender MedSystems, Austria) was 5.28 pg/mL, and for IL-13 (BioSource International, USA) was <2 pg/mL.

FACS Analysis

At sacrifice, spleen and lung tissues were harvested and smashed into pieces. Mononuclear cells were purified by passing the smashed tissue through a 15- μ m stainless steel mesh and the following density gradient centrifugation. Spleen mononuclear cells, lung mononuclear cells, or peripheral blood mononuclear cells (PBMC) were incubated with FITC-conjugated anti-CD3, PE-conjugated anti-CD4, anti-CD8, or isotype controls at 4°C for overnight. All the antibodies mentioned above were purchased from Becton, Dickinson & Co. (USA). After washing, cells were examined (10,000 gated events were analyzed) by flow cytometry. Results are expressed as the percentage of cells with an individual surface marker.

Histopathology and Immunohistochemistry

For histopathological examinations, lung tissues were fixed with 4% formalin, embedded in paraffin, and cut into 5- μ m-thick sections. Sections were deparaffinized and rehydrated with xylene and ethanol series. For histopathological examinations, sections were stained by hematoxylin-eosin (HE). For immunohistochemistry, sections were incubated for 10 minutes with 3% hydrogen peroxide in methanol to remove endogenous peroxidase activity. Goat non-immune serum was used to block the non-specific binding. Sections were incubated overnight at 4°C with rat monoclonal anti-IL-4, rabbit polyclonal anti-IL-13 or rat monoclonal anti-IFN- γ antibodies (Santa Cruz, CA, USA). After being washed three times with PBS, sections were incubated with specific biotinylated-conjugated secondary antibodies (Santa Cruz, CA, USA) for 30 minutes at 37°C followed by incubation with HRP-streptavidin complex (Santa Cruz, CA, USA) for 30 minutes at 37°C. Finally, horseradish peroxidase substrate solution was added and the staining was stopped by rinsing with deionized H₂O repeatedly. All slides were counterstained with hematoxylin. The positive staining was identified as dark-brown either on the cell membrane or in the cytoplasm depending on the individual antibodies.

Cell Culture and Plasmid Transfection

Human epithelial cell line, 9HTE cells, were kindly provided by Dr. Liping Jiang from the Clinical Immunology Research Laboratory of the Children's Hospital of Chongqing Medical University. The cells were cultured in plastic flasks with RPMI 1640 medium (Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; Sijixin, Inc., Beijing, China), 10³ U/mL penicillin (Invitrogen, Shanghai, China), 1 μ g/mL streptomycin (Invitrogen) at 37°C with 5% CO₂. The cells were passaged (divided into daughter cells) every 3 days with 0.25% trypsin (Sigma, St Louis, MO, USA) and 1 mM EDTA (Sigma). For transfection, cells were seeded in 6-well plates at 1 $\times$ 10⁶ cells per well and were allowed

to grow overnight. The cells in each well were transfected with a mixture of 0.4 μg vector DNA and 10 μL Effectene Transfection Reagent (Qiagen, USA) in 600 μL fresh 1640 culture medium. Fourth-eight hours post-transfection, the supernatant from the transfected 9HTE cells was harvested.

Statistical Analysis

The results were expressed as mean $\pm$ deviation. One-way analysis of variance (ANOVA) was used to determine the differences between all groups. Comparisons for all pairs were made using Student-Newman-Keuls (SNK) test. $p < 0.05$ was considered statistically significant.

RESULTS

Production of mIL-4RA Protein by 9HTE Cells

The expression of mIL-4RA protein was confirmed by transfecting 9HTE cells with mIL-4RA expression plasmid. Since the transfected 9HTE cells express both GFP and mIL-4RA, the expression of mIL-4RA can be visually monitored in living 9HTE cells by detecting the GFP expression using an inverted phase fluorescence microscope. The results showed that the transfected 9HTE cells started to express GFP at 6 hours post-transfection and reached to a peak around 48 hours. The supernatant from the transfected 9HTE cells was collected 48 hours post-transfection and was analyzed by ELISA. The level of mIL-4RA in the supernatant was 30.6 ± 8.2 pg/mL for pIRES2-EGFP/IL-4RA and 2.5 ± 1.2 pg/mL for control vector-transfected cells (Figure 1), suggesting a high efficient transfection in 9HTE cells.

Administration of mIL-4RA Inhibited the Development of Pulmonary Inflammation

To analyze the effects of mIL-4RA on pulmonary inflammation, recombinant pIRES2-EGFP/IL-4RA vector was directly instilled into trachea on the day of first sensitization. Pulmonary histopathological examinations showed that the inflammatory cell infiltration in

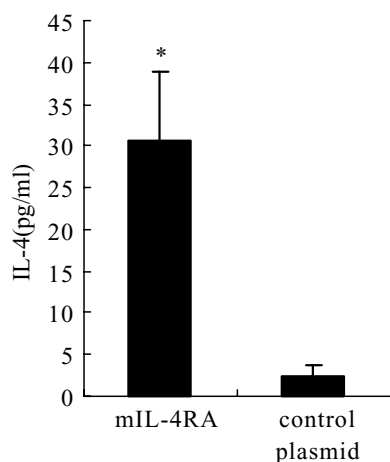


FIGURE 1.—Production of mIL-4RA in the culture supernatant of 9HTE cells. 9HTE cells were transfected with mIL-4RA expression plasmid or control plasmid. Culture supernatant was harvested 48 hours post-transfection and the quantitative analysis was analyzed using ELISA. * $p < 0.01$ versus control plasmid ($n = 5$).

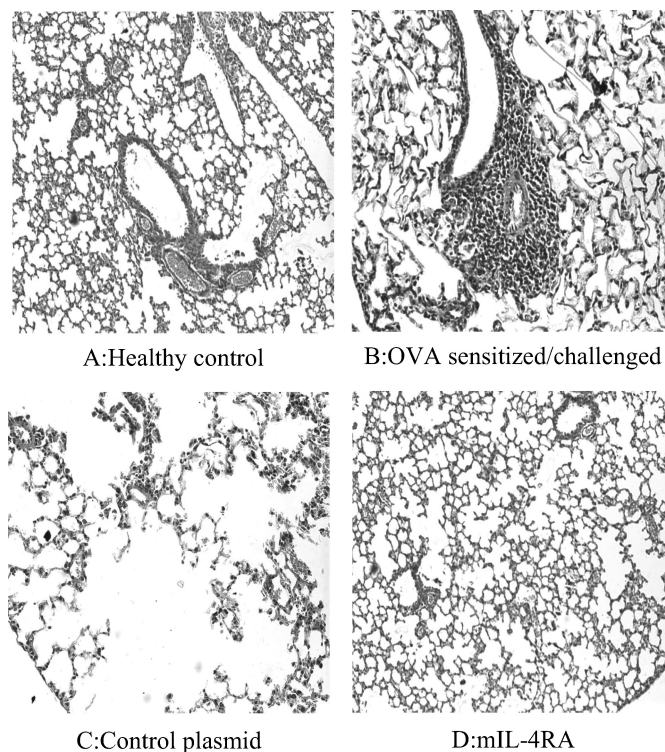


FIGURE 2.—Histopathological examinations of the lungs. Lung sections were stained with HE to detect allergic airway pathologic conditions; (A) healthy control mice (NS sensitized/challenged); (B) OVA sensitized/challenged mice; (C) OVA sensitized/challenged mice + intratracheally administered with control plasmid; (D) OVA sensitized/challenged mice + intratracheally administered with mIL-4RA expression plasmid.

the lung and pulmonary emphysema were greatly attenuated in OVA sensitized/challenged mice administered with pIRES2-EGFP/IL-4RA vector. But the OVA sensitized/challenged mice administered with control vector did not demonstrate any inhibitory effects on the inflammatory cell infiltration and pulmonary emphysema (Figure 2). These results suggest that local administration of mIL-4RA to airway relieved the pulmonary pathological abnormalities.

BALF Total Cell Number and Eosinophils are Inhibited by mIL-4RA Administration

To evaluate the effects of mIL-4RA on airway inflammation and cell infiltration, the number of total cells and the percentage of eosinophils in total leukocytes recovered from BALF were assessed. As demonstrated in Figures 3 and 4, compared to the healthy control mice, the OVA sensitized/challenged mice had drastically increased total cell numbers and a remarkable influx of eosinophils in BALF. After intratracheally administered with mIL-4RA, the total number of cells and percentage of eosinophils were greatly reduced, whereas we did not observe any significant changes in the total cell numbers and eosinophil in the control vector group. These results suggested that mIL-4RA administration inhibited the inflammatory cell transmigration to the local airway, especially the eosinophil infiltration.

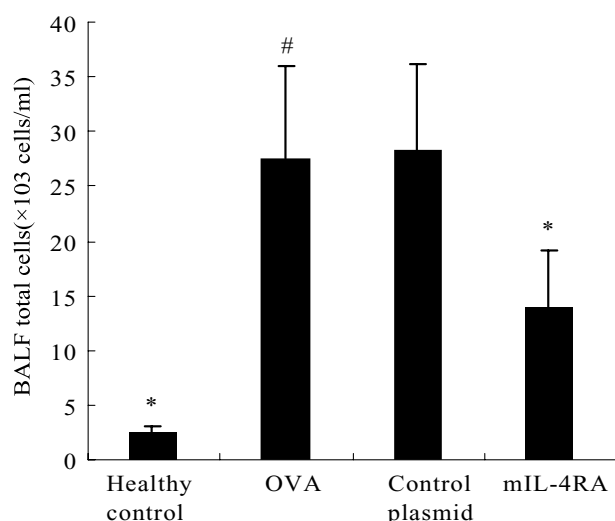


FIGURE 3.—The number of total cells in BALF. BALF was collected 48 hours after the last challenge. Data are calculated as mean $\pm$ SD ($n = 8$ for each group) * $p < 0.05$, # $p > 0.05$ (versus control plasmid).

Regulation of the Th1/Th2 Cytokine Levels in BALF

To test whether mIL-4RA could regulate the Th1/Th2 cytokine imbalance, the levels of Th2 cytokines IL-4, IL-13, and Th1 cytokines IFN- γ in BALF were measured using standard ELISA. As shown in Figure 5, the Th1/Th2 cytokine level was imbalanced in local airway of OVA sensitized/challenged mice. The levels of IL-4 and IL-13 were significantly increased and the level of IFN- γ was significantly reduced compared with the control mice (Figure 5). After administration of mIL-4RA, the level of IL-13 in BALF was dramatically reduced and the level of IFN- γ was increased compared to the control plasmid-treated mice ($p < 0.01$, $n = 8$). However, we did not find a statistical difference in the level of IL-4 between mIL-4RA plasmid- and control vector-treated mice ($p > 0.05$, $n = 8$).

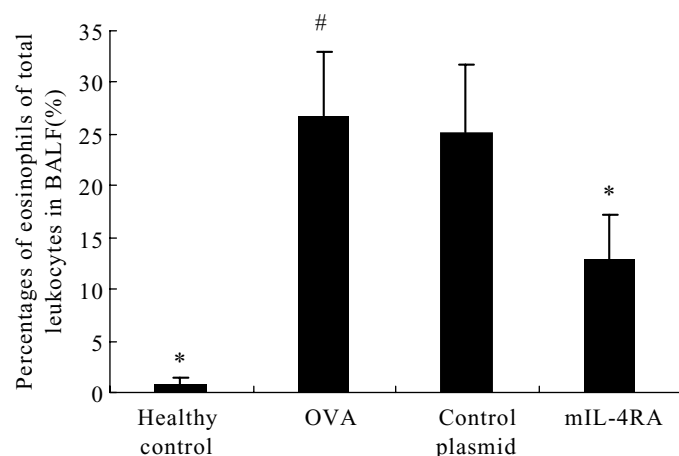


FIGURE 4.—The percentage of eosinophils in total leukocytes in BALF. BALF was collected 48 hours after the last challenge. Data are given as mean $\pm$ SD ($n = 8$ for each group) * $p < 0.01$, # $p > 0.05$ (versus control plasmid).

Levels of Th1/Th2 Cytokines in Serum

The levels of IL-4, IL-13, and IFN- γ in serum were also analyzed using standard ELISA. As demonstrated in Figure 6, the Th1/Th2 cytokine imbalance in serum of OVA sensitized/challenged mice was obvious with increased IL-4 and IL-13 and reduced IFN- γ levels. Compared to control vector-treated mice, the levels of IL-13 and IL-4 were reduced following mIL-4RA plasmid administration ($p < 0.05$, $n = 8$). No statistical difference was found in the level of IFN- γ between control vector-treated mice and mIL-4RA-treated mice ($p > 0.05$, $n = 8$).

Immunohistochemistry Assessment

To further confirm the ELISA results obtained above, we performed immunohistochemistry studies. As shown in Figure 7, we observed a weak staining of IL-13 and an intense staining of IFN- γ in the lung tissues from the mice administered with mIL-4RA compared to the control vector-treated mice. These results were coincidence with the cytokine levels in BALF detected by ELISA. This further indicated that intratracheal administration of mIL-4RA plasmid is able to regulate the Th1/Th2 cytokine imbalance in local airway.

CD4 and CD8 T-Lymphocyte Subsets in PBMC, Lung, and Spleen

To investigate whether administration of mIL-4RA will influence CD4 and CD8 T-lymphocyte subsets, we performed fluorescence-activated cell sorter (FACS) analysis. Our results showed that there were no statistical differences in the CD4/CD8 T-lymphocyte subsets among the mice of OVA sensitized/challenged, OVA sensitized/challenged with control plasmid, OVA sensitized/challenged with mIL-4RA, and healthy control mice either in the PBMC or lung or spleen (data not shown). These results suggested that intratracheal administration with mIL-4RA has no effect on the production of CD4/CD8 T-lymphocyte subsets either in the PBMC, lung, or spleen.

DISCUSSION

The Th2 closely related cytokines, IL-4, and IL-13 are considered to play crucial roles in the development of airway inflammation and AHR, and both are interesting targets for asthma therapies. Tomkinson et al. (10) have developed a mutant protein, mIL-4RA (C118 deletion in mIL-4) and found that it can inhibit the airway eosinophilia and AHR in mouse asthmatic model during allergen challenge. However, as the pharmacokinetic half-life of mIL-4RA protein was only 0.83 hours, resulting in the barriers for its clinical use. In the present study, to achieve higher concentration of mIL-4RA protein in local airway, we intratracheally delivered the mIL-4RA plasmid in the mouse asthmatic model. This kind of administration restricts the expression of mIL-4RA in local airway and lung with minor systemic absorption. Our results showed that there was a significant attenuation of airway inflammation and allergic parameters on mIL-4RA-treated mice. This suggests that a single administration of mIL-4RA plasmid on the first sensitization can effectively prevent both IL-4 and IL-13 induced allergic airway inflammation.

It is known that the Th1/Th2 cytokine imbalances exist in asthma. The CD4⁺ Th2 cytokines are considered to play

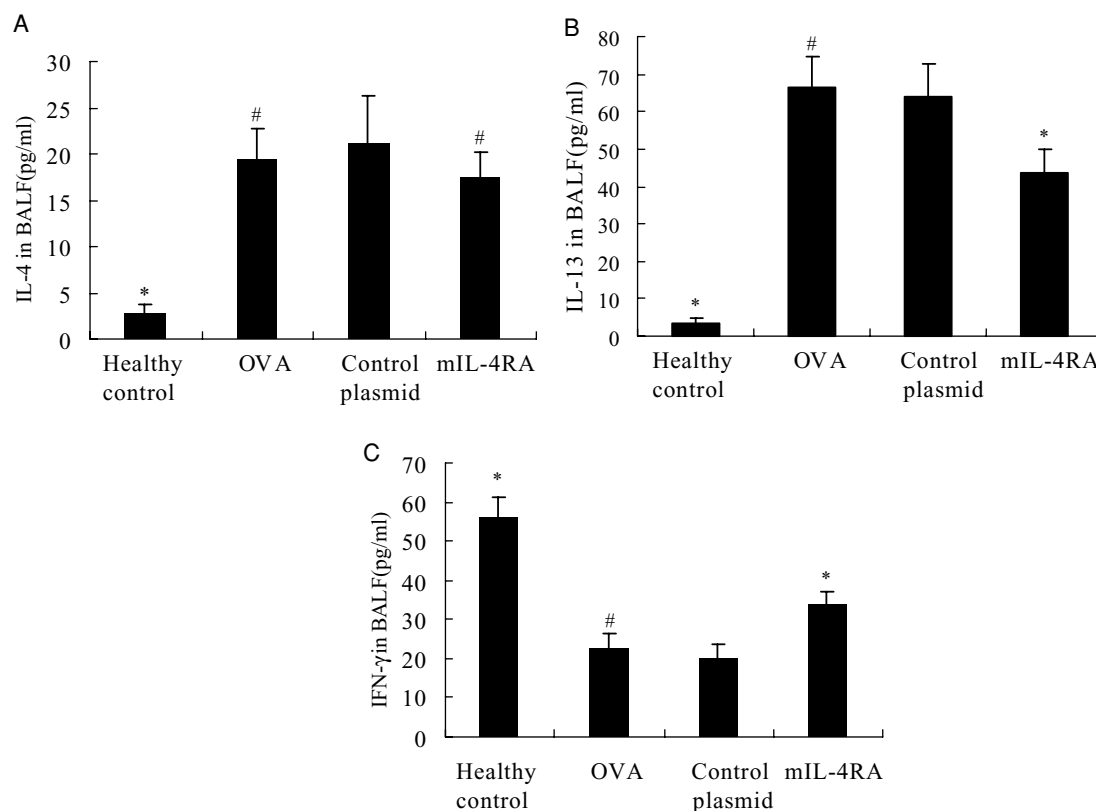


FIGURE 5.—The level of cytokines in BALF. BALF was collected 48 hours after the last challenge; (A) IL-4; (B) IL-13; (C) IFN- γ . Data are shown as mean $\pm$ SD ($n = 8$ for each group) * $p < 0.01$, # $p > 0.05$ (versus control plasmid).

important roles in orchestrating the influx of inflammatory cells into the airways. Therefore, to change the expression levels of Th1 and Th2 cytokines, in other words, to enhance the Th1 immune responses while inhibiting the Th2 responses, will be an ideal way to treat asthma. In this study, we found that following intratracheal administration of mIL-4RA plasmid, the levels of Th2 cytokines IL-13 in BALF were largely reduced and the Th1 cytokine, IFN- γ in BALF were significantly increased, compared to that of the control vector-treated mice. Since IL-4 and IL-4RA could not be distinguished by the rat anti-mIL-4 antibody we used in the ELISA kit, the level of IL-4 in mice treated with mIL-4RA was actually the total level of IL-4 and IL-4RA together. This explains why we did not observe a significant reduction of IL-4 in mIL-4RA-treated mice. However, from the results of IFN- γ and IL-13, we found that intratracheal administration of mIL-4RA plasmid significantly elevated the Th1 cytokines and inhibited the Th2 cytokines in BALF, indicating that intratracheal administration of mIL-4RA plasmid regulates the Th1/Th2 cytokine imbalance in the local airway. Although the mechanisms need to be further investigated, it has been reported (14,15) that both IL-4 and IL-13 modulate macrophage functions and the release of IL-12, which promote the Th1 response.

Regarding to the levels of the Th1/Th2 cytokines in serum, our data showed that intratracheal administration of mIL-4RA significantly inhibited elevation of IL-13 induced by during asthmatic inflammation but has no effect on the level

of IFN- γ . Interestingly, the levels of total IL-4/IL-4RA in serum were not high; however, the level of IL-13 was reduced in mice treated with IL-4RA. These results suggest that although we do not detect high titers of IL-4RA in serum, intratracheal administration of IL-4RA is able to inhibit the production of Th2 cytokines. Meanwhile, although we did not see a great correction of Th1/Th2 imbalance in serum, we did observe obvious alleviation of allergic airway inflammation and pulmonary pathological abnormalities. This suggests that the Th1/Th2 cytokine imbalance in local airway may play more important roles in the pathogenesis of asthma compared to the Th1/Th2 imbalance in serum.

Despite the fact that asthma is a CD4⁺ Th2 cytokines predominant disease, the CD8⁺ T-lymphocytes also play important roles in asthma. However, the roles of CD4 and CD8 T cell in asthma still remain unclear. Lee et al. found that there was an increased ratio of CD4/CD8 in PBMC while a decreased ratio of CD4/CD8 in BALF in the asthmatic patients compared to that of the control subjects (16). The appearance of asthma symptoms for asymptomatic subjects was associated with an increase in airway inflammation, particularly the CD4/CD8 ratio and airway fibrosis (17). Harmanci et al. (18) found that there is no difference in the percentage of CD4⁺, CD8⁺ T lymphocytes, and CD4/CD8 ratios between the asthmatic and control groups. Kanazawa et al. found that the CD4/CD8 ratio was significantly increased following IFN- γ therapy in asthmatic patients who responded to IFN- γ . In addition, the increase in the CD4/CD8 ratio after IFN therapy

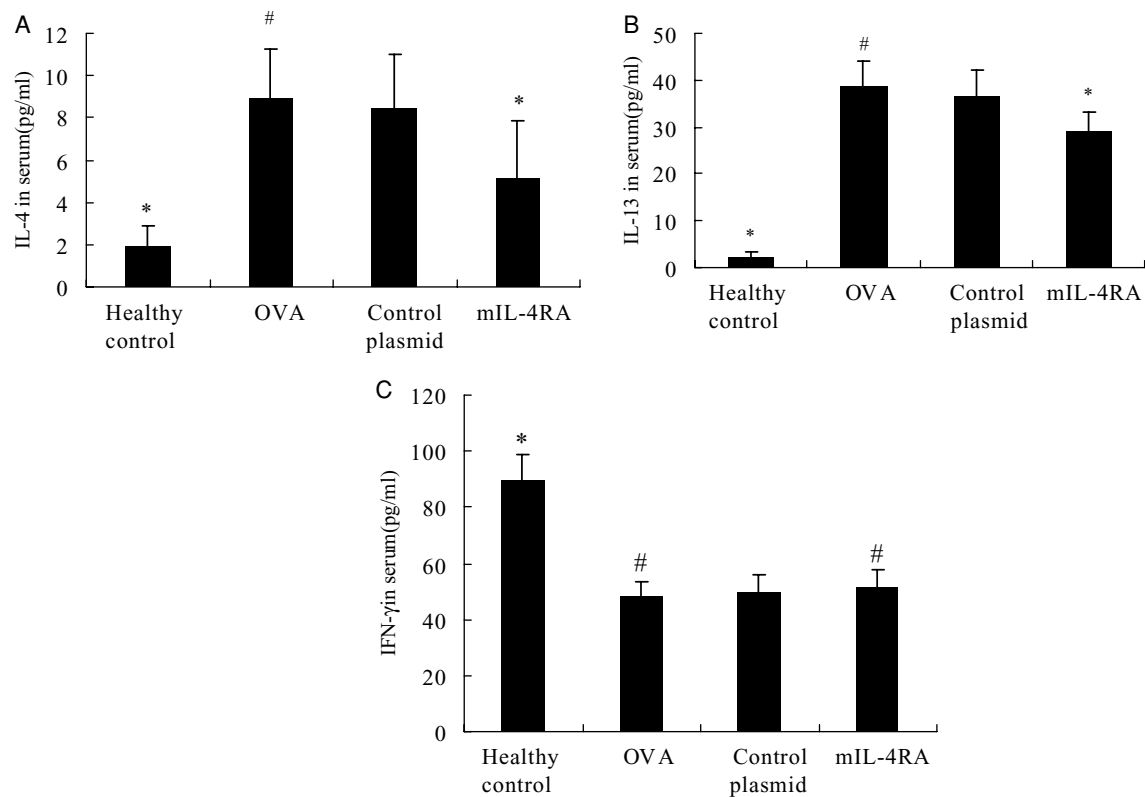


FIGURE 6.—The levels of cytokines in serum. Sera were collected 48 hours after the last challenge; (A) IL-4; (B) IL-13; (C) IFN- γ . Data are given as mean $\pm$ SD ($n = 8$ for each group * $p < 0.05$, # $p > 0.05$ (versus control plasmid))

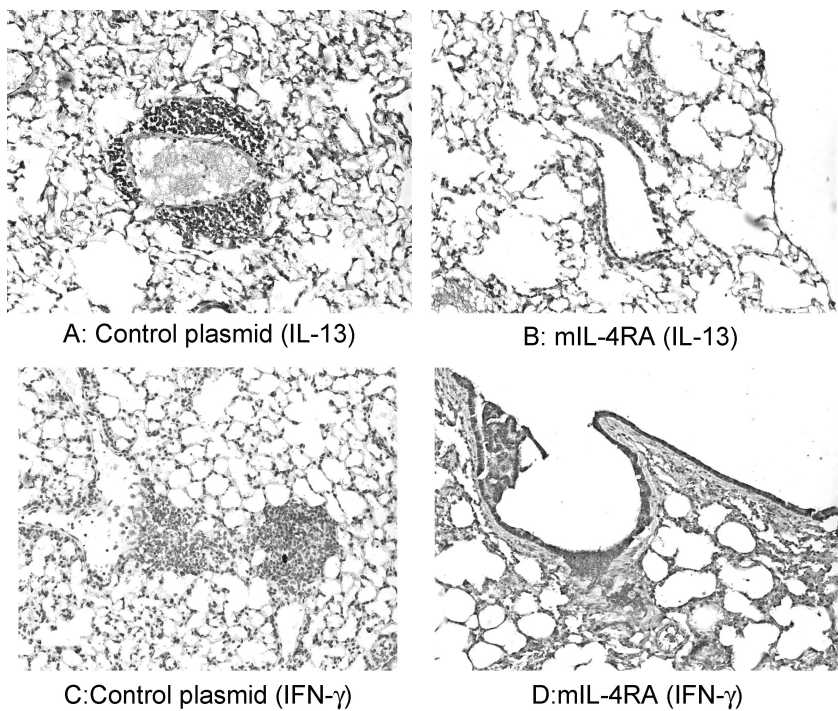


FIGURE 7.—Immunohistochemistry assessment of the expression of IL-13 and IFN- γ in the lung. Compared with the OVA sensitized/challenged mice treated with control plasmid (A and C), weak staining of IL-13 (B) and intense staining of IFN- γ (D) were observed in mice treated with mIL-4RA plasmid. Positive staining was demonstrated as brown.

was significantly correlated with the increase in FEV₁ after salbutamol administration (19). In this study, we did not find any differences in the ratio of CD4/CD8 among the four groups in the PBMC, lung, or spleen. This demonstrated that in our asthmatic model, the ratio of CD4/CD8 T-lymphocyte subsets was not different from the healthy control subjects, and blocking IL-4 and IL-13 by mIL-4RA probably does not influence the production of CD4/CD8 T-lymphocyte subsets. Furthermore, this result suggested that the inhibitory effect of mIL-4RA on our asthmatic model was not through regulating the number of CD4/CD8 T-lymphocyte subsets.

In conclusion, our research demonstrated that a single intratracheal administration of mIL-4RA plasmid on the day of first sensitization effectively prevented both IL-4 and IL-13 induced airway eosinophilia, relieved the pulmonary pathological abnormalities, and regulated the Th1/Th2 cytokine imbalance in the local airway with minor systemic effects. Because mIL-4RA inhibits both IL-4 and IL-13 (the two key mediators in asthma), it may serve as a potential therapeutic option in asthma treatment.

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