

MECHANISMS

The Effects of Antisense Interleukin-4 Gene Transferred by Recombinant Adeno-Associated Virus Vector on the Airway Remodeling in Allergic Rats

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Background. Th2-derived cytokines, including interleukin-4 (IL-4), are considered to play an important role in the development of airway remodeling of asthma. **Objectives.** Our previous study has demonstrated that a recombinant adeno-associated virus containing antisense against IL-4 gene (rAAV-asIL4) vector could significantly suppress the expression of IL-4 protein and airway inflammation in the rat models of allergic asthma. In this study, we applied the rAAV-asIL4 vector to allergic rats to investigate the effects of anti-IL4 therapy on airway remodeling in allergic asthma. **Methods.** rAAV-asIL4 was used to infect the ovalbumin (OVA)-sensitized and challenged rats by tail-vein injection. IL-4 protein in bronchoalveolar lavage fluid (BALF) was detected by enzyme-linked immunosorbent assay. The number of eosinophils in BALF was counted. Transforming growth factor-beta1 (TGF-beta1) and TGF-beta2-positive cells in the peribronchial space were detected by immunohistochemical staining, and collagen deposition beneath the basement membrane was detected by Sirius red stain. The lung tissues were collected for histologic analysis of total bronchial wall area (W_{At}) and airway smooth muscle area (W_{Am}). **Results.** rAAV-asIL4 significantly decreased IL-4 protein in BALF of OVA-sensitized and challenged rats. The number of eosinophils in BALF, the TGF-beta1 and TGF-beta2-positive cells in the peribronchial space were also suppressed. Moreover, the rAAV-asIL4 treatment inhibited the area of Sirius red staining in airways and the increase in W_{At} and W_{Am} . **Conclusion.** These results suggest that rAAV-asIL4 may attenuate the airway remodeling process relevant to the inhibition of airway inflammation. This study provides elementary evidence for the potential utility of rAAV-asIL4 as an approach to gene therapy for asthmatic airway remodeling.

Keywords airway remodeling, asthma, gene therapy, interleukin-4, recombinant adeno-associated virus

INTRODUCTION

Asthma is a chronic disease characterized by reversible airway obstruction, airway inflammation, and airway hyperresponsiveness (AHR). Although current standard therapies (including corticosteroids and β_2 -receptor agonists) effectively provide symptomatic control for the majority of asthma patients, some patients with severe or difficult asthma still do not respond well to these therapies (1, 2). Accumulating evidence (3–7) suggests that continuous airway inflammation often leads to airway remodeling, and then leads to drug resistance and persistent AHR. Thus, it is necessary to find some new therapeutic agents to attenuate airway inflammation for the treatment of asthmatic airway remodeling.

Th2 cells, one sort of CD4⁺ T lymphocytes, are currently considered to play an important role in the chronic airway inflammation of asthma. IL-4 is implicated in multiple pathologies of asthma, especially airway inflammation and hyperactivity, and anti-IL4 therapy could inhibit those pathological changes (8–16). Our

previous research results also support that view (12). But whether anti-IL4 therapy could inhibit airway remodeling has not been elucidated. Leigh et al. (17) reported that the wild-type mice developed airway remodeling after chronic allergen exposure, whereas IL-4 deficient (–/–) mice were protected from developing airway remodeling. Therefore, it is worthwhile to explore the hypothesis that anti-IL4 therapy might be beneficial in treatment of chronic asthmatic airway remodeling.

Although the therapies, such as anti-IL4 monoclonal antibody (14–16) and IL-4 antisense oligodeoxynucleotide, were beneficial and feasible (8), there are still some problems such as immunity reaction and less-persistent effect. As a promising vector for long-term gene therapy, recombinant adeno-associated virus-2 (AAV-2) offers attractive advantages such as safety, no inflammatory reaction, and the ability to infect dividing and non-dividing cells (18–21). In our previous study, a recombinant AAV-2 vector transferring antisense oligonucleotide against IL-4 (rAAV-asIL4) was constructed and found to attenuate airway inflammation by inhibiting IgE and inflammatory cell infiltration (12).

Airway remodeling is another characteristic of asthma. In this study, whether rAAV-asIL4 could attenuate airway remodeling in OVA-sensitized and challenged rats will be investigated for the first time.

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MATERIALS AND METHODS

Materials

rAAV-asIL4 and rAAV-EGFP (enhanced green fluorescence protein) were constructed as reported previously by us (12).

Briefly, the psub201 plasmid (provided by Dr. Richard Jude Samulski, University of North Carolina, NC, USA; 19–21) was reconstructed by replacing Rep and Cap regions by the cytomegalovirus immediate early (CMV IE) promoter, a multiple clone site (MCS) and polyA. The IL-4 cDNA was inserted into the MCS region in an antisense orientation. The resulting plasmid was termed pasIL4 (plasmid containing antisense IL-4 cDNA) and was verified by sequencing at Shanghai Bioengineering Co. Ltd (Shanghai, China). The EGFP gene was inserted into the MCS region to construct pRGFP plasmid. pasIL4, AAV packaging plasmid pXX2, and Ad help plasmid pXX6-80 (provided by Dr. Richard Jude Samulski) were cotransfected into 293T cells (ATCC, Manassas, VA, USA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After 48 hours, the cells were harvested and submitted to single-step gravity-flow column purification to yield rAAV-asIL4 (12, 22). rAAV-EGFP was also harvested by the method of three plasmids cotransfection and single-step gravity-flow column purification. The titers of rAAV-asIL4 and rAAV-EGFP were measured by Southern blot analyses using a dig-labeled probe. Then rAAV-asIL4 and rAAV-EGFP were stored at -80°C until use.

Rat IL-4 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Jingmei Biotech Corporation (Shenzhen, China). Rabbit polyclonal antibody against rat transforming growth factor-beta1 (TGF-beta1) and goat polyclonal antibody against rat TGF-beta2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Sirius red and ovalbumin (OVA) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Heat-killed *Bacillus pertussis* was purchased from Beijing Institute of Biological Products (Beijing, China).

Asthma Models and rAAV Administration

All animal experiments were carried out according to the guidelines set by the Chinese Council on Animal Care and approved by the Tongji Medical College Committee[0] on Animal Experimentation. The research protocol was reviewed and approved by the Tongji Medical College Committee[0]. Forty pathogen-free adult male Sprague-Dawley rats (aged 10 weeks and weighing 180–210 g at the start of the study) were obtained from the animal supply center at Tongji Medical College (Wuhan, Hubei Province, China). The animals were randomly divided into four groups: wild-type (Wt) group ($n = 10$), OVA-sensitized and challenged group (asthma group, $n = 10$), rAAV-asIL4 treatment group (treatment group, $n = 10$), and rAAV-EGFP negative control group (EGFP group, $n = 10$). The allergic rat model was established by the method as described previously (23, 24). Rats from the

asthma group, treatment group, and EGFP group were all sensitized with subcutaneous injections of 1 ml 0.2% (w/v) OVA (Grade III, Sigma, St. Louis, MO, USA) complex with 10% (w/v) aluminum hydroxide in their footpad, neck, back, groin, and abdomen on day 1. In addition, each animal received an intramuscular injection of 2×10^{10} heat-killed *B. pertussis* cells. Two weeks after sensitization, each rat inhaled 1% (w/v) OVA 30 minutes every other day for 4 weeks to challenge their airways. Animals in the Wt group received a subcutaneous injection of 1 ml natrii chloride saline and were exposed to aerosolized natrii chloride saline.

Rats in the treatment and EGFP groups were pretreated with 1 ml [5×10^{12} vector genome/ml (v.g./ml)] rAAV-asIL4 and rAAV-EGFP, respectively, 1 day before sensitization (day 0) and 1 day before challenge (day 13) through tail-vein injection (12, 24). Rats in the Wt and asthma groups were injected with natrii chloride saline instead of viral vectors.

Twenty-four hours after the last exposure to OVA or natrii chloride saline, each rat was anesthetized with an intraperitoneal injection of 60 mg/kg pentobarbital. The bronchoalveolar lavage fluid (BALF) and lung tissues from each rat were then collected for analysis.

Infection Efficiency of rAAV Vectors Determined by Fluorescence Microscopy

The left upper lung tissues from the EGFP group were harvested for cryosections. Two slides from the sequential section of each sample were used to check the infection efficiency of rAAV-EGFP vectors under the fluorescence microscope. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. The infection efficiency of rAAV-EGFP vectors was calculated by comparing the number of cells showing green fluorescence with the number of cells showing blue fluorescence.

IL-4 Protein in Bronchoalveolar Lavage

All rat lungs ($n = 10$) were lavaged three times with 3 ml 0.9% natrii chloride saline. BALF samples were collected and centrifuged for supernatant. IL-4 protein in BALF was measured using ELISA according to the manufacturer's recommendations. Each BALF sample was detected three times and mean value was used for analysis.

Eosinophil Count in Bronchoalveolar Lavage

BALF samples were collected and centrifuged to pellet cells. Eosinophil count was performed on cell smears stained with Wright-Giemsa in duplicate using the standard morphologic criterion of enumeration of at least 300 cells using an immersion objective microscope (25, 26).

Immunohistochemical Staining

The lung tissues from different groups were equivalently inflated with an intratracheal injection of 4% paraformaldehyde and embedded in paraffin.

Sections were incubated overnight at 4°C with the rabbit polyclonal antibody against rat TGF-beta1 at a dilution of 1:100. As a negative control, the primary

antibody was substituted with normal rabbit serum. Then, the sections were incubated with goat anti-rabbit biotin-labeled secondary antibodies (Zhongshan Goldenbridge Biotechnology Co. Ltd. Beijing, China). Immunoreactivity was visualized with diaminobenzidine (DAB). Number of TGF-beta1-positive cells were counted in the airway wall using a light microscope. As previously described (27), results were expressed as the number of positive cells per bronchiole. At least 10 bronchioles were counted in each slide.

Sections ($n = 10$) from each group were immunostained with goat polyclonal antibodies specific for rat TGF-beta2 at a dilution of 1:100. Normal goat serum was used as a negative control. Then, sections were incubated with rabbit anti-goat biotin-labeled secondary antibodies (Zhongshan Goldenbridge Biotechnology Co. Ltd). Immunoreactivity was visualized with DAB. Numbers of TGF-beta2-positive cells were counted in the airway wall using a light microscope. Results were expressed as the number of positive cells per square millimeter of bronchial area (28).

Sirius Red Staining

Sirius red staining was used to evaluate the deposition of total collagen in the airway wall (29). All lung sections ($n = 10$) were deparaffinized and incubated with 0.1% Sirius red in saturated picric acid for 90 minutes. The sections were observed under polarized light (Nikon (Nikon Eclipse E800, Tokyo, Japan)). Collagen was identified by birefringence. The area of total collagen deposition in the airway wall was measured with a computerized image analysis system (Image-Pro Plus, version 4.5, for Windows TM, Media Cybernetics, Bethesda, Maryland, USA). The perimeter of the epithelial basement membrane (P_{bm}) was also measured. The value of total collagen content was expressed as the area of total collagen deposition per millimeter P_{bm} (28).

Morphometry Analysis

All sections of rat lungs ($n = 10$) from each group were stained with hematoxylin-and-eosin (H&E). Then the H&E-stained tissue sections were examined using light microscopy and morphometric measurements were performed on sections at 200 $\times$ magnifications under a Nikon microscope (Nikon).

For each group, three tissue sections of each animal were analyzed. Only large airways cut in cross-sections (defined by a ratio of minimal internal diameter to maximal internal diameter less than 0.5) were examined. Airways with a length of basement membrane (P_{bm}) > 2 mm were defined as being large. Thirty large airways per experimental group were analyzed. The following parameters were measured: the perimeter of epithelial basement membrane (P_{bm}) and the outer adventitial perimeter (P_o), the areas defined by these parameters (A_{bm} and A_o), and the area of airway smooth muscle (W_{Am}). The total airway wall area ($W_{At} = A_o - A_{bm}$) was calculated based on the measured values. The total

airway wall area (W_{At}) and airway smooth muscle area (W_{Am}) were measured. The mean values of W_{At} and W_{Am} were normalized to the square of P_{bm} as previously described (28).

Statistical Analysis

All data were expressed as mean $\pm$ standard deviation and analyzed by one-way ANOVA using SPSS 12.0. A p value less than .05 was considered statistically significant.

RESULTS

rAAV-asIL4-Contained Antisense IL-4 cDNA

The rat IL-4 cDNA sequence (according to the sequence offered by PubMed NM_201270) was inserted into the plasmid in an antisense orientation to construct the plasmid pasIL4. By sequencing, we confirmed the sequence as shown in the pasIL4.

ATGGGTCTCA	GCCCCACCT	TGCTGTCACC
CTGTTCTGCT	TTCTCATATG	TACCGGGAAC
GGTATCCACG	GATGTAACGA	CAGCCCTCTG
AGAGAGATCA	TCAACACTTT	GAACCAGGTC
AACACCACGG	AGAACGAGCT	CATCTGCAGG
GCTTCACGGG	TGCTTCGCAA	ATTTTACTTC
CCACGTGATG	TACCTCCGTG	CTTGAAGAAC
AAGTCTGGGG	TTCTCGGTGA	ACTGAGGAAA
CTCTGTAGAG	GTGTCAGCGG	TCTGAACTCA
CTGAGAAGCT	GCACCGTGAA	TGAGTCCACG
CTCACAACAC	TGAAAGACTT	CCTGGAAAGC
CTAAAAAGCA	TCCTACGAGG	GAAATACTTG
ACAGAAAAAG	GGACTCCATG	CACCGAGATG
TTTGTACCAG	ACGTCCTTAC	GGCAACAAGG
CAGTCCTGCA	CTTCCATGTC	CTAA.

Infection Efficiency of rAAV-asIL4 and rAAV-EGFP

As shown in Figure 1, among the total lung cells whose nuclei were stained blue by DAPI, a number were infected by rAAV-EGFP and expressed green fluorescence protein in the cytoplasm. rAAV-EGFP also transfected both inflammatory cells and structural cells in the lung tissues.

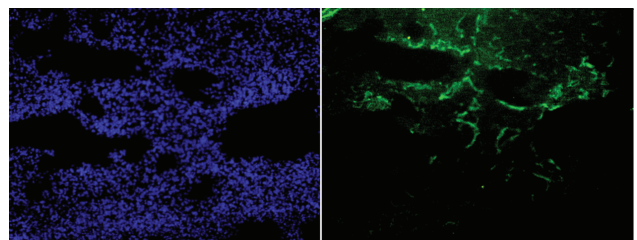


FIGURE 1.—EGFP and DAPI were observed using fluorescence microscopy (200 $\times$). The lung tissues from rats in EGFP group, which were sensitized and challenged by OVA and treated by rAAV-EGFP, were made for serial sections. Frozen pathologic sections were made and observed under a fluorescence microscopy to detect EGFP. In left figure, the cell nuclei were stained by DAPI stain, and showed blue fluorescence, whereas in right figure, the cells in lung tissues, which were infected by rAAV-EGFP, showed green fluorescence. rAAV-EGFP could infect both inflammatory cells and structural cells in the lung tissues.

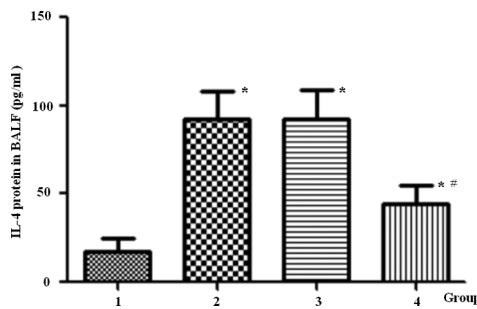


FIGURE 2.—Levels of IL-4 protein in BALF from different groups. Values represent mean $\pm$ standard deviation. Bar 1 refer to wild-type group ($n = 10$); bar 2 to asthma group ($n = 10$); bar 3 to EGFP group ($n = 10$); and bar 4 to treatment group ($n = 10$). A significant difference compared with wild-type group was shown as * $p < .01$ and that compared with asthma group or EGFP group was shown as # $p < .01$.

Effect of rAAV-asIL4 on IL-4 Protein in BALF of OVA-Induced Allergic Rat

As shown in Figure 2, OVA sensitization and challenge markedly increased the levels of IL-4 protein in BALF of the asthma group, when compared with those in Wt group ($p < .01$). A similar increase in IL-4 protein levels was also observed in the EGFP group. In the group treated with rAAV-asIL4, the IL-4 protein level was reduced by about 52% in BALF when compared with the asthma group ($p < .01$), although the protein levels were still higher than those in normal rats ($p < .01$ compared between treatment group and Wt group).

Effect of rAAV-asIL4 on the Eosinophils Count in BALF

Eosinophils in the treatment group were decreased in both the blood and the BALF ($p < .05$, compared between treatment group and asthma group). In contrast, eosinophils in the asthma and EGFP groups were significantly increased (Figure 3, $p < .05$, as compared with Wt group).

Effect of rAAV-asIL4 on TGF-Beta1- and TGF-Beta2-Positive Expressing Cells in the Airways of Allergic Rats

In allergic rats, TGF-beta1 staining protein was found in the bronchial epithelium, macrophages, smooth muscle, PMN, and sub-epithelial ECM. TGF-beta1-positive cells increased in allergic rats compared with those in Wt

group ($p < .05$, Figure 4). Treatment with rAAV-asIL4 reduced the number of TGF-beta1-positive cells in the airways ($p < .05$), but the number of TGF-beta1-positive cells was still higher than Wt rats ($p < .05$). Treatment with rAAV-EGFP had no effect on the number of TGF-beta1-positive cells compared with those in the asthma group.

Staining for TGF-beta2 was found mainly in the inflammatory cells. TGF-beta2-positive cells, which were rarely observed in the airways of Wt rats, strikingly increased in allergic rats ($p < .05$, Figure 5). Treatment with rAAV-asIL4 remarkably reduced the number of TGF-beta2-positive cells in the airways ($p < .05$), although they were still higher than in Wt rats ($p < .05$). In contrast, treatment with rAAV-EGFP had no effect on the number of TGF-beta2-positive cells ($p > .05$).

Effect of rAAV-asIL4 on the Area of Sirius Red Staining in Airways

The collagen in the airway wall was stained using Sirius red. By detecting the area of Sirius red staining, the content of collagen in the airways was analyzed. As compared with Wt rats, OVA sensitization and challenge significantly increased the area of Sirius red staining in airways ($p < .05$, Figure 6). The area of Sirius red staining reduced in the treatment group ($p < .05$), although was still higher than the Wt rats ($p < .05$). Nevertheless, treatment with rAAV-EGFP had no effect on the area of Sirius red staining in airways ($p > .05$).

Effect of rAAV-asIL4 on Airways Morphometry

As shown in Figure 7, OVA sensitization and challenge remarkably increased the total airway wall area (W_{At}) and airway smooth muscle area (W_{Am}) ($p < .05$, Figure 7) when compared with normal rats. Treatment with rAAV-asIL4 reduced the W_{At} and W_{Am} ($p < .05$), although was still higher than control ($p < .05$). Treatment with rAAV-EGFP had almost no effect on the W_{At} and W_{Am} ($p > .05$).

DISCUSSION

This study is the first that we know of to use an IL-4 antisense gene employed by a rAAV vector in the study of asthmatic airway remodeling. Results show that

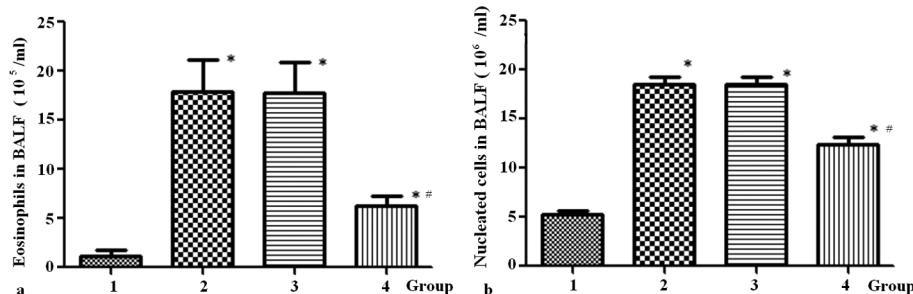


FIGURE 3.—The number of eosinophils (a) and nucleated cells (b) in BALF in the rats of different groups. Values represent mean $\pm$ standard deviation. Bar 1 refer to wild-type group ($n = 10$); bar 2 to asthma group ($n = 10$); bar 3 to EGFP group ($n = 10$); and bar 4 to treatment group ($n = 10$). Significant differences compared with wild-type group shown as * $p < .05$; and compared with allergic group as # $p < .05$.

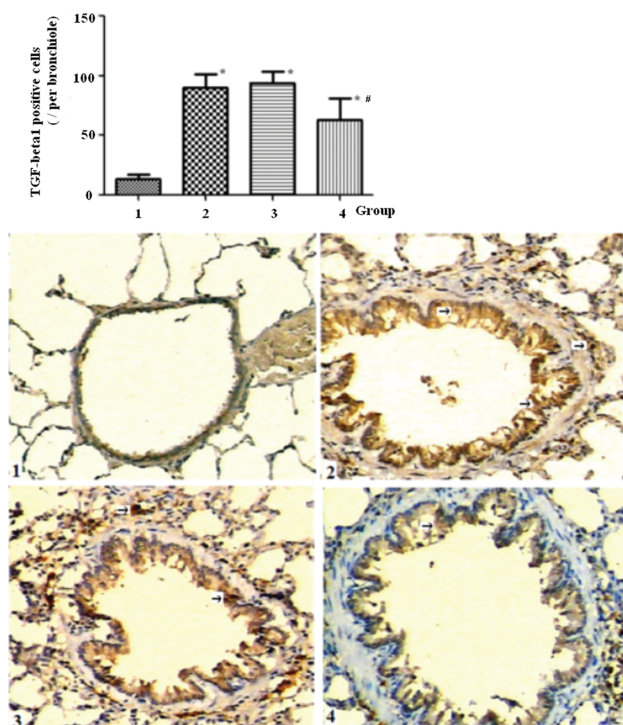


FIGURE 4.—The number of TGF-beta1-positive cells in the airways in the rats of different groups. Values represent mean $\pm$ standard deviation. Bar 1 and Slide 1 refer to wild-type group ($n = 10$); bar 2 and Slide 2 to asthma group ($n = 10$); bar 3 and Slide 3 to EGFP group ($n = 10$); and bar 4 and Slide 4 to treatment group ($n = 10$). Significant differences compared with wild-type group shown as $*p < .05$; and compared with asthma group as $#p < .05$. Some TGF-beta1-positive cells were shown by arrowhead in slides.

rAAV-asIL4 attenuated airway remodeling in allergic rats, including collagen deposition beneath the basement membrane, and the area of the total airway wall and airway smooth muscle. These changes were accompanied by a reduction of IL-4 protein, the eosinophils counts in the BALF, and the number of TGF-beta1 and TGF-beta2-positive cells in airway walls. These data demonstrate the role of IL-4 in airway remodeling and suggest its link with airway inflammation.

IL-4 plays a critical role in the induction of a local Th2 response (30) and the development of airway inflammation in the lung (31). In this study, we observed rats for longer periods compared with our previous study and found that IL-4 protein expression was reduced by 52% after 4 weeks along with a significant reduction in eosinophils in the BALF. These results were similar to those of other studies testing anti-IL4 therapies (8–10). This suggests that the effect of rAAV-asIL4 on airway remodeling may be inhibition of IL-4 protein expression and airway inflammation.

The TGF-betas are a family of dimeric polypeptide growth factors consisting of three isoforms: TGF-beta1, 2, and 3. TGF-betas contribute to the development of peribronchiolar fibrosis (32). TGF-beta1 and TGF-beta2 were found independently to contribute to the increase of eosinophils, lymphocytes, and sub-epithelial deposition of collagen (33). In our study, rAAV-asIL4 treatment

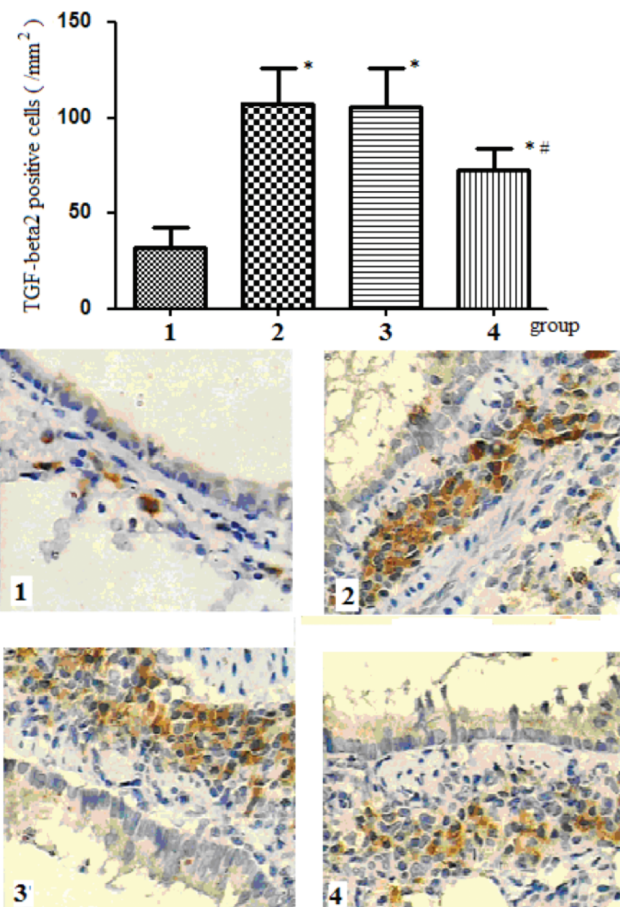


FIGURE 5.—The number of TGF-beta2-positive cells in the airways in the rats of different groups (200 $\times$). Values represent mean $\pm$ standard deviation. Bar 1 and Slide 1 refer to wild-type group ($n = 10$); bar 2 and Slide 2 to asthma group ($n = 10$); bar 3 and Slide 3 to EGFP group ($n = 10$); and bar 4 and Slide 4 to treatment group ($n = 10$). Significant differences compared with wild-type group shown as $*p < .05$; and compared with asthma group as $#p < .05$.

led to a reduction in the number of TGF-beta1-positive cells. These cells, which include bronchial epithelium, smooth muscle, and PMN, may all contribute to airway remodeling. Meanwhile, a reduction in the number of TGF-beta2-positive cells was also observed, mainly the inflammatory cells in the airways, which also contribute to airway remodeling. According to our results, we propose that the suppression of IL-4 and inflammatory cells resulting from rAAV-asIL4 treatment might lead to a reduction in the number of TGF-beta1- and TGF-beta2-positive cells.

The peribronchial fibrosis is a critical feature of airway remodeling. According to previous studies, IL-4 could directly or indirectly induce the activation of fibroblasts and the deposition of collagen (34–39). TGF-beta1 and TGF-beta2 could also activate the underlying fibroblasts or myofibroblasts to induce the deposition of collagen and upregulation of smooth muscle cells, which contribute to tissue remodeling (40–43). In this study, the collagen in the airway wall reduced after rAAV-asIL4 treatment. Those changes might be caused by the decrease of IL-4, TGF-beta1, and TGF-beta2. Treatment with rAAV-asIL4

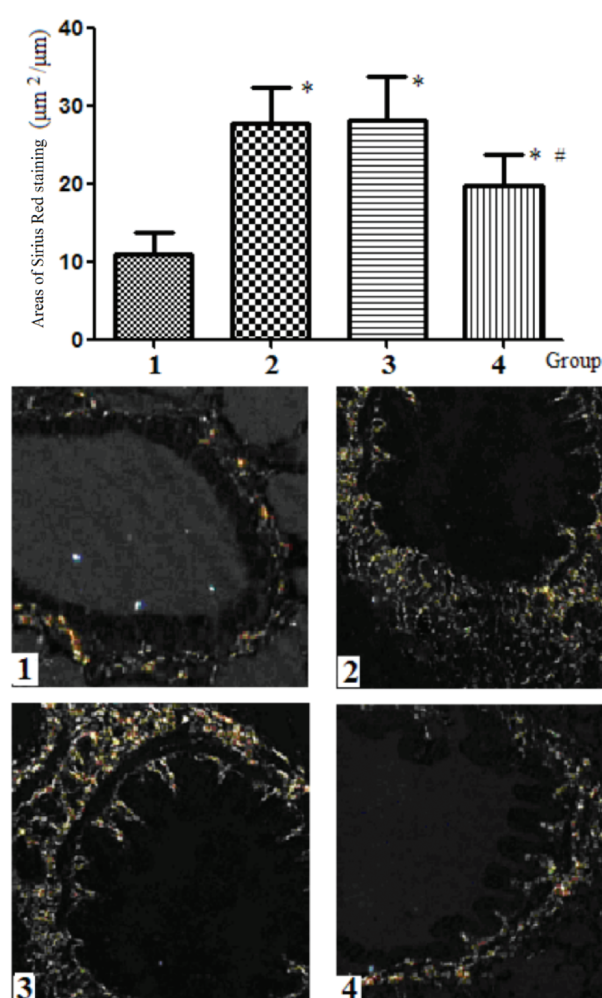


FIGURE 6.—Areas of Sirius red staining in the airways in the rats of different groups (200 $\times$). Values represent mean $\pm$ standard deviation. Bar 1 and Slide 1 refer to wild-type group ($n = 10$); bar 2 and Slide 2 to asthma group ($n = 10$); bar 3 and Slide 3 to EGFP group ($n = 10$); and bar 4 and Slide 4 to treatment group ($n = 10$). Significant differences compared with wild-type group shown as $*p < .05$; and compared with asthma group as $\#p < .05$.

did not completely prevent the peribronchial fibrosis as compared with controls, which might be partly attributed to the involvement of other mediators in the process of peribronchial fibrosis, such as IL-5 and IL-13 (44, 45).

The thickness of reticular basement membrane, in parallel with collagen, is directly correlated with airway remodeling. In this study, the deposition of collagens, the thickness of reticular basement membrane, W_{At} , and W_{Am} were reduced by rAAV-asIL4 in the OVA-sensitized and challenged rats. These may be partly because of the reduction of IL-4 protein, TGF- β 1, TGF- β 2, and peribronchial fibrosis.

In this experiment, the degree of the decrease of the collagen in the airway wall and airway pathological changes by rAAV-asIL4 treatment was less than the report of Komai (46). In their study, the allergic model used IL-4 gene knockout mice. We speculated that the difference may be because of the incomplete IL-4 inhibition of rAAV-asIL4 compared with IL-4 gene knockout methods.

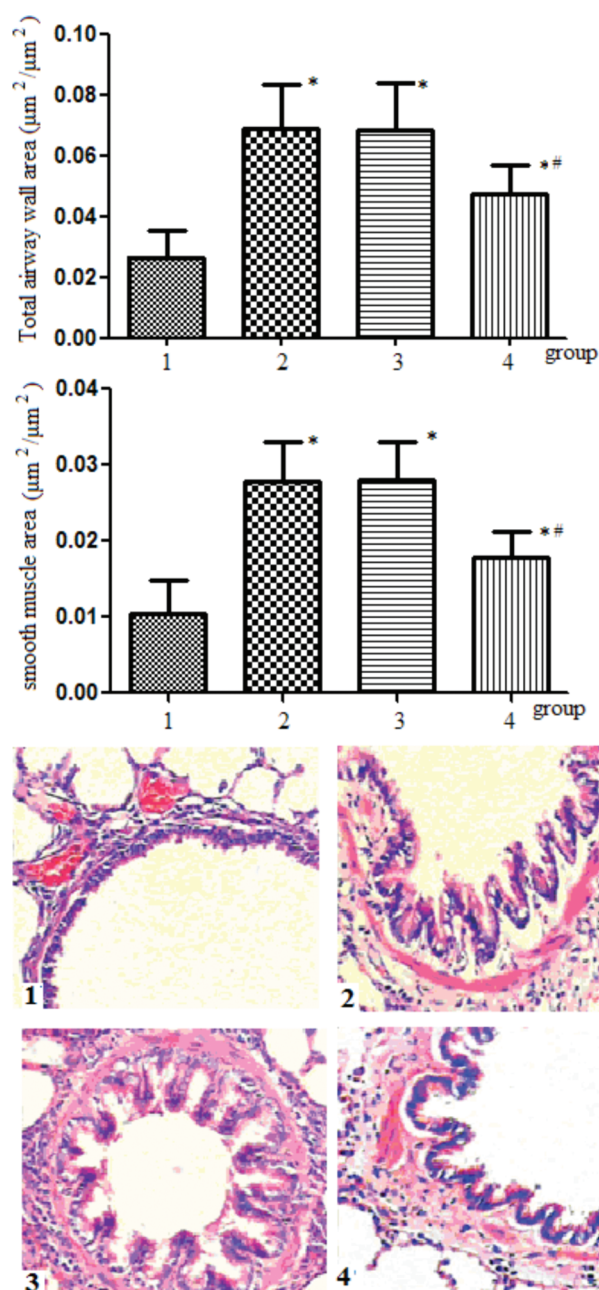


FIGURE 7.—Total airway wall area (WAt) and smooth muscle area (WAm) in the rats of different groups (200 $\times$). Values represent mean $\pm$ standard deviation. Bar 1 and Slide 1 refer to wild-type group ($n = 10$); bar 2 and Slide 2 to asthma group ($n = 10$); bar 3 and Slide 3 to EGFP group ($n = 10$); bar 4 and Slide 4 to treatment group ($n = 10$). Significant differences compared with wild-type group shown as $*p < .05$; and compared with asthma group shown as $\#p < .05$.

In summary, this study shows that the delivery of antisense IL-4 gene mediated by rAAV had the potential of inhibiting airway remodeling in allergic rats. An AAV vector has previously been used to deliver an IL-4 inhibitor to the lung, but the IL-4 antisense gene employed in this work is novel. These results suggest that rAAV-asIL4-based gene therapy may be valuable in the treatment of allergic asthma.

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REFERENCES

- Jenkins HA, Cool C, Szeffler SJ, Covar R, Brugman S, Gelfand EW, Spahn JD. Histopathology of severe childhood asthma: a case series. *Chest* 2003; 124(1):32–41.
- Heaney LG, Robinson DS. Severe asthma treatment: need for characterising patients. *Lancet* 2005; 365(9463):974–976.
- Broide DH. Immunologic and inflammatory mechanisms that drive asthma progression to remodeling. *J Allergy Clin Immunol* 2008; 121(3):560–570.
- Doherty T, Broide D. Cytokines and growth factors in airway remodeling in asthma. *Curr Opin Immunol* 2007; 19(6):676–680.
- Miller M, Cho JY, McElwain K, McElwain S, Shim JY, Manni M, Baek JS, Broide DH. Corticosteroids prevent myofibroblast accumulation and airway remodeling in mice. *Am J Physiol Lung Cell Mol Physiol* 2006; 290(1):L162–L169.
- Sagani S, Payne DN, Zhu J, Wang Z, Nicholson AG, Bush A, Jeffery PK. Early detection of airway wall remodelling and eosinophilic inflammation in preschool wheezers. *Am J Respir Crit Care Med* 2007; 176(9):858–864.
- Phipps S, Benyahia F, Ou TT, Barkans J, Robinson DS, Kay AB. Acute allergen-induced airway remodeling in atopic asthma. *Am J Respir Cell Mol Biol* 2004; 31(6):626–632.
- Molet S, Rumos-Barbon D, Martin JG, Hamid Q. Adoptively transferred late allergic response is inhibited by IL-4, but not IL-5, antisense oligonucleotide. *J Allergy Clin Immunol* 1999; 104(1):205–214.
- Nishikubo K, Murata Y, Tamaki S, Sugama K, Imanaka-Yoshida K, Yuda N, Kai M, Takamura S, Sebald W, Adachi Y, Yasutomi Y. A single administration of interleukin-4 antagonistic mutant DNA inhibits allergic airway inflammation in a mouse model of asthma. *Gene Ther* 2003; 10(26):2119–2125.
- Zavorotinskaya T, Tomkinson A, Murphy JE. Treatment of experimental asthma by long-term gene therapy directed against IL-4 and IL-13. *Mol Ther* 2003; 7(2):155–162.
- Ahn JH, Kim CH, Kim YH, Kim SJ, Lee SY, Kim YK, Kim KH, Moon HS, Song JS, Park SH, Kwon SS. Inflammatory and remodeling events in asthma with chronic exposure to house dust mites: a murine model. *J Korean Med Sci* 2007; 22(6):1026–1033.
- Cao Y, Xiong WN, Xu YJ, Zhang ZX, Gao BA, Du CL, Lu JY, Ye T. Recombinant adeno-associated virus-mediated inhibiting of interleukin-4 expression in rat model of asthma. *Chin Med J (Engl)* 2006; 119(3):223–225.
- Ma Y, Hayglass KT, Becker AB, Halayko AJ, Basu S, Simons FE, Peng Z. Novel cytokine peptide-based vaccines: an interleukin-4 vaccine suppresses airway allergic responses in mice. *Allergy* 2007; 62(6):675–682.
- Steinke JW. Anti-interleukin-4 therapy. *Immunol Allergy Clin North Am* 2004; 24(4):599–614.
- Hart TK, Blackburn MN, Brigham-Burke M, Dede K, Al-Mahdi N, Zia-Amirhosseini P, Cook RM. Preclinical efficacy and safety of pascolizumab (SB 240683): a humanized anti-interleukin-4 antibody with therapeutic potential in asthma. *Clin Exp Immunol* 2002; 130(1):93–100.
- Steinke JW, Borish L. Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. *Respir Res* 2001; 2(2):66–70.
- Leigh R, Ellis R, Wattie JN, Hirota JA, Matthaei KI, Foster PS, O'Byrne PM, Inman MD. Type 2 cytokines in the pathogenesis of sustained airway dysfunction and airway remodeling in mice. *Am J Respir Crit Care Med* 2004; 169(7):860–867.
- Hildinger M, Auricchio A. Advances in AAV-mediated gene transfer for the treatment of inherited disorders. *Eur J Hum Genet* 2004; 12(4):263–271.
- Rabinowitz JE, Rolling F, Li C, Conrath H, Xiao W, Xiao X, Samulski RJ. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol* 2002; 76(2):791–801.
- Wu Z, Asokan A, Samulski RJ. Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Mol Ther* 2006; 14(3):316–327.
- Xiao X, Li J, Samulski RJ. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* 1998; 72(3):2224–2232.
- Auricchio A, Hildinger M, O'Connor E, Gao GP, Wilson JM. Isolation of highly infectious and pure adeno-associated virus type 2 vectors with a single-step gravity-flow column. *Hum Gene Ther* 2001; 12:71–76.
- Tang HF, Song YH, Chen JC, Chen JQ, Wang P. Upregulation of phosphodiesterase-4 in the lung of allergic rats. *Am J Respir Crit Care Med* 2005; 171:823–828.
- Zeng D, Cao Y, Song Q, Cao C, Liu X, Xu Y, Xiong W. Effects of antisense interleukin-5 gene transferred by recombinant adeno-associated virus to allergic rats. *Respirology* 2010; 15:132–140.
- Renzi PM, Olivenstein R, Martin JG. Inflammatory Cell populations in the airways and parenchyma after antigen challenge in the rat. *Am Rev Respir Dis* 1993; 147:967–974.
- Palmans E, Kips JC, Pauwels RA. Prolonged allergen exposure induces structural airway changes in sensitized rats. *Am J Respir Crit Care Med* 2000; 161:627–635.
- Song DJ, Min MG, Miller M, Cho JY, Yum HY, Broide DH. Toll-like receptor-9 agonist inhibits airway inflammation, remodeling and hyper-reactivity in mice exposed to chronic environmental tobacco smoke and allergen. *Int Arch Allergy Immunol* 2010; 151:285–296.
- Vanacker NJ, Palmans E, Kips JC, Pauwels RA. Fluticasone inhibits but does not reverse allergen-induced structural airway changes. *Am J Respir Crit Care Med* 2001; 163(3 Pt 1):674–679.
- Cohn L, Herrick C, Niu N, Homer R, Bottomly K. IL-4 promotes airway eosinophilia by suppressing IFN-gamma production: defining a novel role for IFN-gamma in the regulation of allergic airway inflammation. *J Immunol* 2001; 166(4):2760–2767.
- Coyle AJ, Le Gros G, Bertrand C, Tsuyuki S, Heusser CH, Kopf M, Anderson GP. Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am J Respir Cell Mol Biol* 1995; 13(1):54–59.
- Brusselle GG, Kips JC, Tavernier JH, van der Heyden JG, Cuvelier CA, Pauwels RA, Bluethmann H. Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin Exp Allergy* 1994; 24(1):73–80.
- Vignola AM, Chanez P, Chiappara G, Merendino A, Pace E, Rizzo A, la Rocca AM, Bellia V, Bonsignore G, Bousquet J. Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 1997; 156(2 Pt 1):591–599.
- Bottoms SE, Howell JE, Reinhardt AK, Evans IC, McNulty RJ. TGF-beta isoform specific regulation of airway inflammation and remodelling in a murine model of asthma. *PLoS One* 2010; 5:e9674.
- Doucet C, Brouty-Boyé D, Pottin-Clémenceau C, Canonica GW, Jasmin C, Azzarone B. Interleukin (IL) 4 and IL-13 act on human lung fibroblasts. Implication in asthma. *J Clin Invest* 1998; 101(10):2129–2239.
- Hashimoto S, Gon Y, Takeshita I, Maruoka S, Horie T. IL-4 and IL-13 induce myofibroblastic phenotype of human lung fibroblasts through c-Jun NH2-terminal kinase-dependent pathway. *J Allergy Clin Immunol* 2001; 107(6):1001–1008.
- Liu X, Kohyama T, Wang H, Zhu YK, Wen FQ, Kim HJ, Romberger DJ, Rennard SI. Th2 cytokine regulation of type I collagen gel contraction mediated by human lung mesenchymal cells. *Am J Physiol Lung Cell Mol Physiol* 2002; 282(5):L1049–L1056.
- Saito A, Okazaki H, Sugawara I, Yamamoto K, Takizawa H. Potential action of IL-4 and IL-13 as fibrogenic factors on lung fibroblasts in vitro. *Int Arch Allergy Immunol* 2003; 132(2):168–176.
- Plante S, Semlali A, Joubert P, Bissonnette E, Laviolette M, Hamid Q, Chakir J. Mast cells regulate procollagen I (alpha 1) production by bronchial fibroblasts derived from subjects with

- asthma through IL-4/IL-13 ratio. *J Allergy Clin Immunol* 2006; 117(6):1321–1327.
39. Shao DD, Suresh R, Vakil V, Gomer RH, Pilling D. Pivotal Advance: Th-1 cytokines inhibit, and Th-2 cytokines promote fibrocyte differentiation. *J Leukoc Biol* 2008; 83(6):1323–1333.
 40. Zhang S, Smartt H, Holgate ST, Roche WR. Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an in vitro co-culture model of airway remodeling in asthma. *Lab Invest* 1999; 79(4):395–405.
 41. Wicks J, Haitchi HM, Holgate ST, Davies DE, Powell RM. Enhanced upregulation of smooth muscle related transcripts by TGF beta2 in asthmatic (myo) fibroblasts. *Thorax* 2006; 61(4):313–319.
 42. Serpero L, Petecchia L, Sabatini F, Giuliani M, Silvestri M, Di Blasi P, Rossi GA. The effect of transforming growth factor (TGF)-beta1 and (TGF)-beta2 on nasal polyp fibroblast activities involved upper airway remodeling: modulation by fluticasone propionate. *Immunol Lett* 2006; 105(1):61–67.
 43. Gamulescu MA, Chen Y, He S, Spee C, Jin M, Ryan SJ, Hinton DR. Transforming growth factor beta2-induced myofibroblastic differentiation of human retinal pigment epithelial cells: regulation by extracellular matrix proteins and hepatocyte growth factor. *Exp Eye Res* 2006; 83(1):212–222.
 44. Nath P, Leung SY, Williams AS, Noble A, Xie S, McKenzie AN, Chung KF. Complete inhibition of allergic airway inflammation and remodeling in quadruple IL-4/5/9/13 $-/-$ mice. *Clin Exp Allergy* 2007; 37:1427–1435.
 45. Borowski A, Kuepper M, Horn U, et al. Interleukin-13 acts as an apoptotic effector on lung epithelial cells and induces pro-fibrotic gene expression in lung fibroblasts. *Clin Exp Allergy* 2008; 38: 619–628.
 46. Komai M, Tanaka H, Masuda T, Nagao K, Ishizaki M, Sawada M, Nagai H. Role of Th2 responses in the development of allergen-induced airway remodelling in a murine model of allergic asthma. *Br J Pharmacol* 2003; 138(5):912–920.