

ORIGINAL ARTICLE

Aerosol Inhalation-mediated Delivery of an Adeno-associated Virus 5-expressed Antagonistic Interleukin-4 Mutant Ameliorates Experimental Murine Asthma

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Background. T helper 2 (Th2) lymphocytes and associated interleukin (IL) 4 and IL-13 play crucial roles in asthma pathogenesis. In this study, we explored an adeno-associated virus 5 (AAV5) based gene therapy by delivering truncated IL-4 protein to antagonize IL-4 receptor α chain and interrupt asthmatic signal pathway.

Results. A recombinant adeno-associated virus 5 (AAV5) vector harboring a truncated mouse IL-4 gene (AAV5-mIL-4 Δ C22) was prepared. Western blotting showed that the IL-4 mutant protein lacking the C-terminal 22 amino acids was expressed well in AAV5-mIL-4 Δ C22 infected 16HBE and BEAS-2B cells. AAV5-driven green fluorescent protein (AAV5-GFP) served as a control. The biodistribution of vector DNA after AAV5 vector aerosol inhalation was examined by PCR and the result showed that foreign DNA was detectable in the lungs but not in other organs including gonads. The aerosol inhalation-mediated delivery of AAV5-expressed antagonistic IL-4 mutant protein improved the lung function of ovalbumin-induced asthma mice.

Conclusions. The inhalation of aerosolized AAV5-mIL-4 Δ C22 significantly improved the lung function and modulated the immune cell infiltration and associated cytokine expression in the bronchoalveolar lavage fluid (BALF) of ovalbumin-induced asthma mice. © 2019 IMSS. Published by Elsevier Inc.

Key Words: Adeno-associated virus, Gene therapy, Asthma, Antagonist, Truncated interleukin-4 mutant.

Introduction

Asthma is a heterogeneous disease that is usually characterized by recurrent airway hyperresponsiveness and irreversible airway obstruction (1,2). It has been well documented that CD4⁺ T helper 2 (Th2) cells and associated type 2 cytokine (such as IL-4, IL-5 and IL-13) play crucial roles in asthma pathogenesis. IL-4 and IL-13 are key factors for Th2 cell differentiation and activation, eosinophil accumulation and IgE synthesis, as well as mucus production, bronchial fibrosis and airway hyperresponsiveness in asthma. IL-4 and IL-13 share some structural similarities

and they bind the IL-4R α /IL-13R α 1 receptor complex which then activates transcriptional signaling (3). IL-4 binds specifically to the IL-4R α chain, which forms either the IL-4 receptor complex (IL-4/IL-4R α /IL-2 γ C) or the IL-13 receptor complex (IL-4/IL-4R α /IL-13R α 1). The formation of the receptor complex initiates signal transduction via the activation of STAT6, resulting in the transcription of genes that are critical for IL-4 induced effects in the allergic response (3–6). The roles of IL-4 and IL-13 in the pathophysiology of asthma make them very attractive antiasthma targets and the selective neutralization of IL-4 and IL-13 results in the alleviation of asthma symptoms in mouse models (3,7–13). The C-terminal region of IL-4 is critical for triggering gene transcription. Previous reports have shown that human IL-4 mutations or deletion in the C-terminal region has the same capacity for binding to the IL-4R α chain as wildtype IL-4, but has no signaling activity. As

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an IL-4R α chain antagonist, IL-4 mutant protein is effective in preventing both IL-4- and IL-13-induced signal transduction and alleviating asthma symptoms (4,14–17).

In recent years, gene therapy has achieved remarkable success in human and animal models of various diseases (18–20). Asthma is a refractory lung disease that requires high-frequency and long-term medication, which is a heavy burden on patients (21). Gene therapy may provide an economically viable long-term treatment strategy. Of the many viral gene therapy vectors, adeno-associated virus (AAV)-based vectors hold great promise for efficiently targeting the airway epithelium *in vivo* (22). As AAV vectors have shown broad tissue tropism, debate remains over the best-suited AAV serotypes for pulmonary application (23). The infection of target cells with AAV is mediated by various cell-surface associated glycans depending on

the AAV serotype. AAV2 was the first and is currently the most extensively studied serotype in airway diseases but the outcome of an *in vivo* trial was disappointing (24,25). AAV1, AAV5, and AAV6 have been tested in inhaled gene therapy and appear to be more efficient in transducing airway epithelial cells than AAV2 (26,27). Effective transduction of AAV5 into target cells in the airway is considered due to its inherent tropism via interaction with α 2,3 N-linked or O-linked sialic acid receptors present on the apical surface of the airway epithelium (28–32).

In this study, we developed a recombinant AAV5 vector containing a truncated IL-4 gene and used aerosol inhalation-mediated delivery of recombinant vectors into asthma mice to effectively ameliorate the development of asthma.

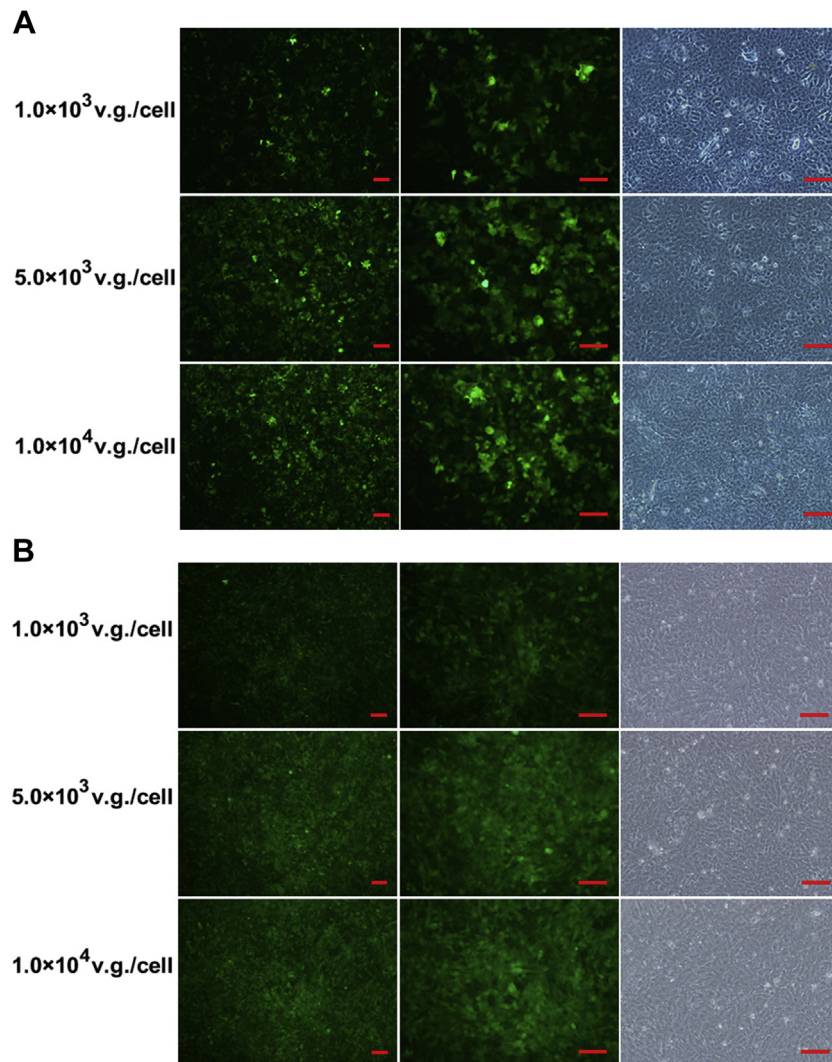


Figure 1. GFP expression in AAV5-GFP transfected 16HBE and BEAS-2B cells. Green fluorescence was detected using a confocal laser scanning microscope 72 h after infection. GFP was detectable in 16HBE (A) and BEAS-2B cells (B); the middle panels are increased magnification images, and increased numbers of GFP-positive cells were observable in cells transfected with an increased dose of AAV5-GFP. Scale bar: 100 μ m.

Results

AAV5 Mediated Gene Expression in 16HBE and BEAS-2B

16HBE and BEAS-2B cells were infected with AAV5-GFP at titers of 1.0×10^3 vector genomes (v.g.)/cell, 5.0×10^3 v.g./cell, and 1.0×10^4 v.g./cell, respectively. Green fluorescence was detected using confocal laser scanning microscope 72 h after infection. The green fluorescence was observed and an increased number of GFP-positive cells were observed after infection with an higher dose of recombinant AAV5-GFP, suggesting that AAV5 can effectively induce the expression of heterogeneous proteins in lung cell lines (Figure 1A, and 1B).

Then we infected 16HBE and BEAS-2B cells with AAV5-mIL-4 Δ C22 (Figure 2A, and 2B) at titers of 2.5×10^3 v.g./cell and 1.0×10^4 v.g./cell, with 5.0×10^3 v.g./cell AAV5-GFP as a control. Western blot analysis was performed to verify the expression of mutant mouse IL-4. A significant immunoreactive band at the appropriate molecular weight was observed in the cell lysates of 16HBE and BEAS-2B cells infected with AAV5-mIL-4 Δ C22 at a titer of 1.0×10^4 v.g./cell, whereas the low titer infection (2.5×10^3 v.g./mL) did not create a distinct immunoreactive band. No band was observed in the control AAV5-GFP infected cell lysate either (Figure 2C). An ELISA assay demonstrated that 1.0×10^4 v.g./cell AAV5-mIL-4 Δ C22 infected cells expressed abundant quantities of mutant IL-4 protein, and the expression sharply decreased in cells infected with a

titer of 2.5×10^3 v.g./mL. Consistent with western blot, the mutant IL-4 protein expression was undetectable in AAV5-GFP infected cells (Figure 2D).

Biodistribution of the Vector DNA

PCR analysis was performed to determine the bio-distribution of vector DNA after aerosol inhalation-mediated delivery of AAV5-GFP. Total DNA was extracted from the targeted lung and distant organs (heart, liver, brain, kidney, lymph nodes and gonads) two weeks and four weeks after inhalation-mediated delivery. PCR was performed with GFP-specific primers. A 160 bp GFP-specific product was detected only in the AAV5-GFP-treated lungs two weeks after inhalation delivery, and no obvious PCR product was identified in the other tested organs (Figure 3).

The Ameliorated Effect of AAV5-mIL-4 Δ C22 on Mice Asthma

After sensitized with 20 μ g ovalbumin (OVA) emulsified in a liquid aluminum suspension and challenged with 100 μ g OVA, BALB/c mice developed pronounced airway hyperactivity (AHR) when inhaling escalating concentrations of the bronchoconstrictor methacholine (Figure 4A). There were no airway allergy signs in the control mice. To test the effect of AAV5-mIL-4 Δ C22 on asthma, recombinant AAV5 was inhaled following the final sensitization (at 14 d). Compared with normal control mice, asthma mice that inhaled AAV5-GFP or PBS showed markedly

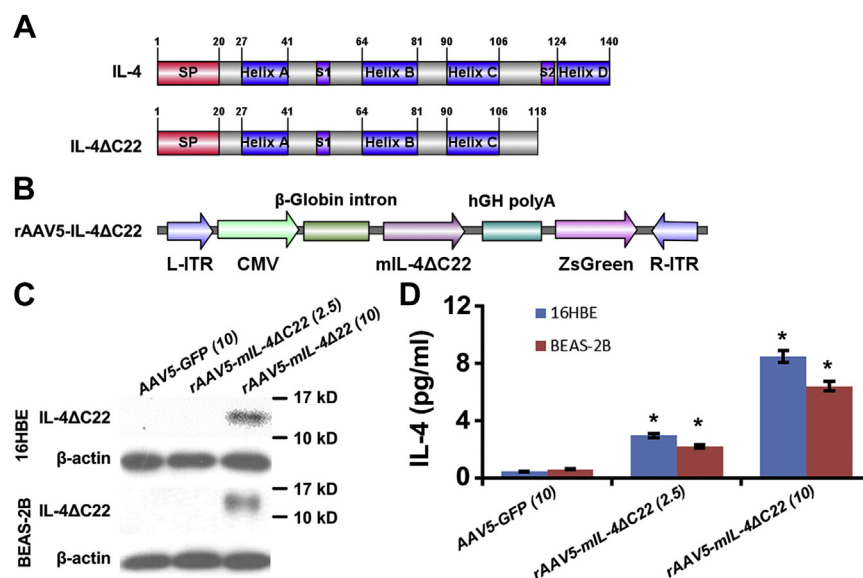


Figure 2. The expression of the truncated mIL-4 mutant in AAV5-mIL-4 Δ C22-transfected cells. (A) Full-length and truncated mouse IL-4. SP: signal peptide; Helix: α -Helix; S: β -sheet. (B) pSNAV-mIL-4 Δ C22 expression cassette. L-ITR: Left inverse terminal repeat; CMV: immediate-early CMV promoter; hGH polyA: human growth hormone polyA signal; ZsGreen: Reef coral *Zoanthus* sp. green fluorescent protein; R-ITR: Right inverse terminal repeat. (C) Western blotting analysis showing an obvious mIL-4 Δ C22-specific band in AAV5-mIL-4 Δ C22 transfected 16HBE and BEAS-2B cells. (D) ELISA assay showing the truncated mIL-4 mutant expression in high dose (10 v.g.) transfected cells was markedly higher than that in cells transfected with a low dose (2.5 v.g.). The truncated mIL-4 mutant protein expression in 16HBE cells was slightly higher than in BEAS-2B cells. *indicates statistically significant ($p < 0.05$) difference existing between different groups.

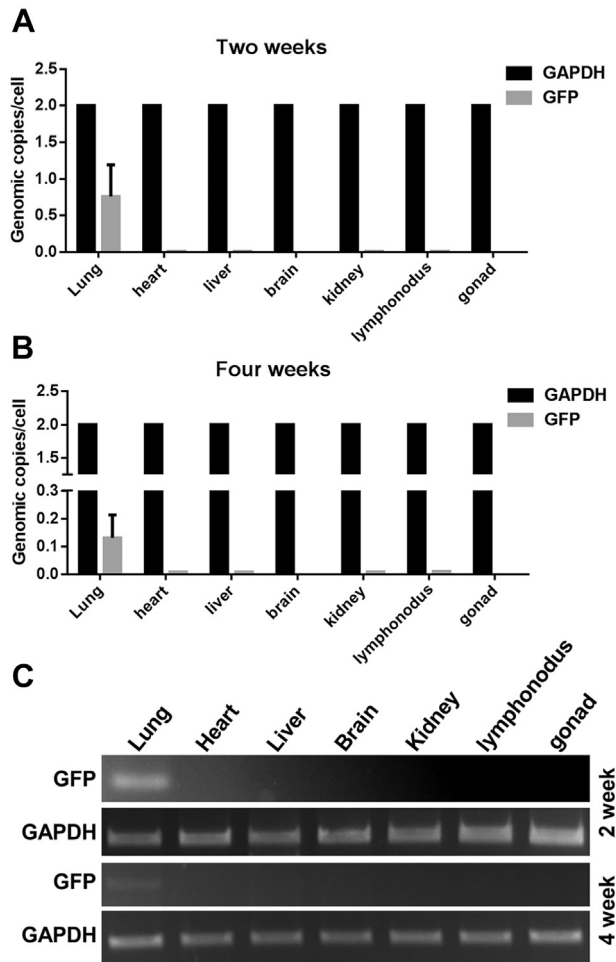


Figure 3. Biodistribution of GFP DNA in mice after AAV5.GFP aerosol inhalation-mediated delivery analyzed by qPCR and agarose electrophoresis. (A and B) qPCR analysis of AAV5-GFP biodistribution at two weeks (A) and four weeks (B) after aerosol inhalation-mediated delivery. (C) Agarose gel electrophoresis of qPCR products.

increased respiratory system resistance (Rrs) and airway resistance (Raw). The increases in Rrs and Raw were significantly reduced in asthma mice that received AAV5-mIL-4 Δ C22 by inhalation. Pulmonary function tests showed that inspiratory capacity (IC) and pulmonary tissue elasticity (H) were dramatically reduced in asthma mice treated with PBS or AAV5-GFP, and this reduction was significantly restored in AAV5-mIL-4 Δ C22 treated asthma mice (Figure 4).

The Effect of AAV5.mIL-4 Δ C22 Delivery on Immune Cell Infiltration in the BALF of Asthma Mice

To determine whether there is an effect of AAV5-mIL-4 Δ C22 on the immune component in the mouse airway, BALF was collected, and the immune cells and inflammatory cytokines were measured. Immune cell counting showed a significant increase in neutrophil ($p < 0.0001$)

and eosinophil ($p < 0.0001$) numbers in asthma mice compared with normal control mice. The increase in eosinophil numbers was much more significant than the increase in neutrophil numbers. A marked decrease in macrophage numbers ($p < 0.0001$) was also detected in asthma mice. Asthma mice showed somewhat fewer lymphocytes than normal control mice, but the difference was not significant ($p > 0.05$). AAV5-GFP treatment had no significant impact on immune cell numbers in the BALF of asthma mice. In asthma mice receiving AAV5-mIL-4 Δ C22, the extent of the increase in neutrophil and eosinophil numbers, as well as the decrease in the macrophage number, was significantly reduced compared with that in asthma mice treated with AAV5-GFP or PBS (Table 1).

The Effect of AAV5-mIL-4 Δ C22 Delivery on Airway Cytokine Expression in Asthma Mice

Cytokines were measured to evaluate the impact of AAV5-mIL-4 Δ C22 inhalation on the local airway immune function. BALF was collected, and the concentrations of IL-4, IL-5, IL-13 and IFN- γ were measured. The results showed a significant increase in cytokine levels in asthma mice treated with AAV5-GFP or PBS. In AAV5-mIL-4 Δ C22-treated asthma mice, the elevation of IL-4, IL-5, IL-13 and IFN- γ levels was significantly reduced (Figure 5).

Materials and Methods

Animals

Female BALB/c mice were obtained from China Academy of Military Medical Sciences (Beijing, China) and were housed under standard (specific pathogen-free) conditions with OVA-free diets. All experimental procedures were reviewed and approved by the Beijing University of Chinese Medicine Animal Care and Use Committee and were performed in accordance with the institutional guidelines for the Care and Use of Laboratory Animals.

Plasmids and Vector Preparation

The coding sequence of wild-type mouse interleukin 4 was downloaded from the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>, GenBank accession number NM_021283). Wild-type mouse IL-4 cDNA was obtained by reverse transcription PCR and inserted into the T-vector. Δ C22 mouse IL-4 cDNA (mIL-4 Δ C22) was obtained by PCR amplification with a 5' oligonucleotide containing a *KpnI* site and a 3' oligonucleotide incorporating a stop codon at position 119 and a *SalI* site (forward, 5' gggGGTACCatgggtctcaacccccagcta 3' and reverse, 5' tctGTCGACtactcattcatggtgcagcttatcg 3'). PCR fragments were digested with *KpnI* and *SalI*, subcloned into the AAV5-CMV backbone and verified by DNA sequencing

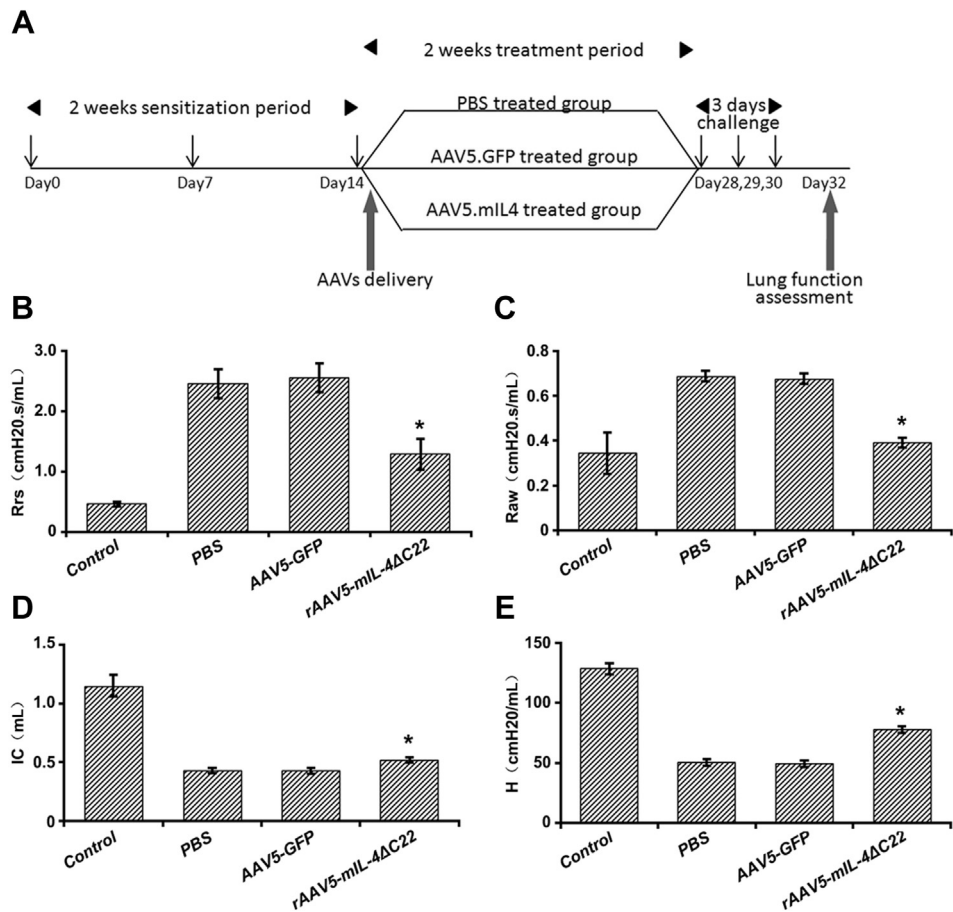


Figure 4. The lung function analysis of asthma mice. (A) Schematic diagram of this study. Asthma was induced in mice, and normal mice served as non-asthma controls. For asthma mice, AAV5-mIL-4ΔC22 was delivered by aerosol, and PBS and AAV5-GFP were used as control treatments. (B–E) Pulmonary function analysis showing that respiratory system resistance (Rrs, B) and airway resistance (Raw, C) were significantly reduced in AAV5-mIL-4ΔC22-treated asthma mice. The inspiratory capacity (IC, D) and pulmonary tissue elasticity (H, E) were markedly improved in asthma mice treated with AAV5-mIL-4ΔC22 via aerosol inhalation. *indicates statistically significant ($p < 0.05$) difference existing between different groups.

on an Applied Biosystems Model 3730 DNA Sequencing System (Invitrogen Biotechnology Co., Shanghai, China). The sequence-verified mIL-4ΔC22 was driven by a CMV intermediate-early promoter followed by BGH poly A. The expression cassette was flanked by AAV5 inverted terminal repeats (ITRs). The large-scale production and purification of vectors were performed by Vector Gene Technology Company Ltd. Vectors were prepared using the three plasmid cotransfection method. For purification, vectors were freeze-thawed, and supernatants were collected following centrifugation. The supernatants were subjected to PEG precipitation followed by benzonase

treatment, chloroform extraction, and ion-exchange chromatography. The products were then ultrafiltrated and microfiltrated. The AAV5-GFP vector served as a control. The titers of the vector batches were 5×10^{11} v.g./mL for AAV5-mIL-4ΔC22 and 1×10^{12} v.g./mL for AAV5-GFP.

Cell Culture and Transfection

Human lung epithelial cell lines BEAS-2B (CRL9609) and 16HBE were kindly provided by Dr. Xiaoxi Lv, from the Institute of Materia Medica, Chinese Academy of Medical

Table 1. The effect of AAV5-mIL-4ΔC22 delivery on immune cells in BALF from asthma mice

	Macrophage (%)	Lymphocyte (%)	Neutrophil (%)	Eosinophil (%)
Control	89.2 ± 6.2	10.7 ± 2.3	0.1 ± 0.1	0.0 ± 0.0
Asthma	36.3 ± 5.7	7.8 ± 1.3	18.5 ± 5.5	37.4 ± 6.7
AAV5-GFP	38.1 ± 5.5	8.2 ± 1.7	17.5 ± 5.9	36.2 ± 7.1
rAAV5-mIL-4ΔC22	58.3 ± 5.9*	8.5 ± 1.2	11.2 ± 4.5*	22.0 ± 3.3*

$n = 5$, * $p < 0.05$.

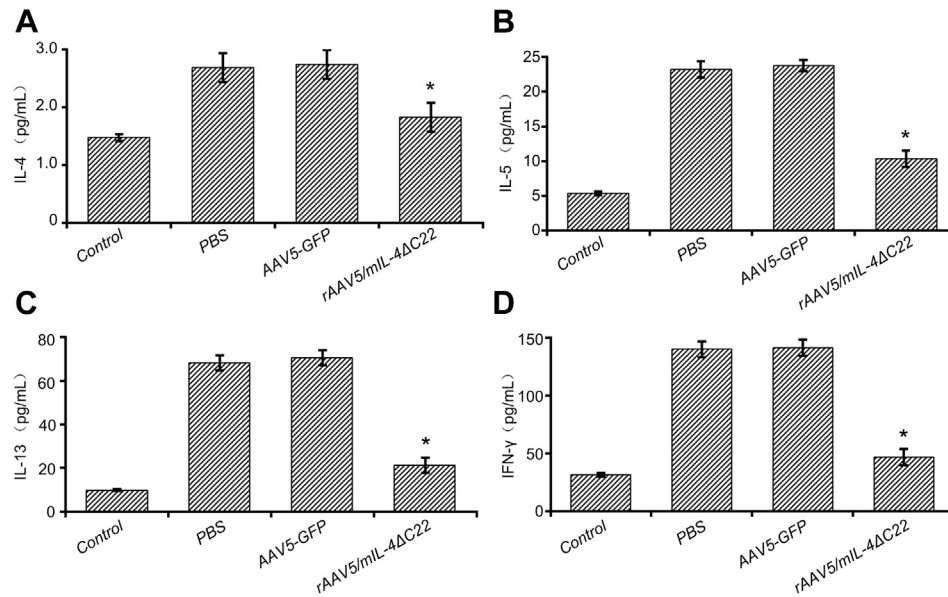


Figure 5. The BALF cytokine levels in asthmic mice. Asthma was induced in mice, and normal mice served as nonasthma controls. For asthmic mice, AAV5-mIL-4ΔC22 was delivered via aerosol, and PBS and AAV5-GFP were used as control treatments. BALF was collected, and associated cytokine levels were detected by ELISA. (A–D) The results showed that the levels of the immune cytokines IL-4, IL-5, IL-13 and IFN-γ were increased in asthmic mice and that this increase was reduced in AAV5-mIL-4ΔC22-treated asthmic mice. *indicates statistically significant ($p < 0.05$) difference existing between the AAV5-mIL-4ΔC22-treated group and the Control group.

Sciences and Peking Union Medical College. Cells were cultured in DMEM (Gibco) supplemented with 10% heat-inactivated FBS (PAN Biotech) and 1% penicillin-streptomycin (Invitrogen). A total of 1.0×10^6 cells were seeded in six-well plates and incubated at 37°C in a 5% CO_2 atmosphere; 24 h later, the cells were transfected with AAV5-GFP or AAV5-mIL-4ΔC22. For AAV5-GFP, 1.0×10^3 v.g./cell, 5.0×10^3 v.g./cell or 1.0×10^4 v.g./cell viral particles were added to each well, respectively. The green fluorescence was observed using fluorescence microscope 72 h after transfection. For AAV5-mIL-4ΔC22, 2.5×10^3 v.g./cell or 1.0×10^4 v.g./cell viral particles were added to each well; 72 h later cells were harvested and cell lysates were analyzed by Western blot to detect the intracellular expression levels of mIL-4ΔC22 with a rabbit polyclonal antibody to IL-4 (Abcam, ab9728). The extracellular expression levels of mIL-4ΔC22 in the cell culture medium were measured with a mouse IL-4 ELISA kit (Abcam, ab100710) according to the manufacturer's instructions.

Murine Model of Allergic Asthma and the Administration of rAAV Vectors

Eight-week-old female BALB/c mice (6–8 per group) were sensitized by i.p. injection of 20 μg OVA (Sigma, Grave V) emulsified in 150 μL of liquid aluminum suspension in a total volume of 200 μL on 0, 7 and 14 d. Mice were challenged via the airway with 100 μg OVA (0.2% in saline) for 3 d (28–30 d). Control mouse groups received saline sensitization and challenges. The AAV5-treated mouse groups received AAV5-GFP or AAV5-mIL-4ΔC22 via airway

delivery following the last sensitization (14 d). Forty-eight hours after the last challenge, the lung function was assessed. A total of 2.0×10^{11} v.g. viral particles diluted in 10 mL saline was used for aerosol delivery. Mice were placed in plastic cages that were sealed with tape (33,34), which is an unrestrained, whole-body aerosol exposure system. rAAV vectors were aerosolized using a nebulizer at flow rate of 10 liters/min using air. The nebulizer has been shown to produce fine particle aerosols in the range of approximately 2.5 μm diameter for effective lung deposition. The nebulization of 10 mL rAAV vector solution took approximately 30 min.

Vector DNA Biodistribution

Quantitative PCR (qPCR) analysis was performed to evaluate the biodistribution of rAAV5 vector DNA. Mice were sacrificed at 2 weeks and 4 weeks after inhalation-mediated viral delivery and total DNA was extracted from AAV5-GFP targeted lung and distant organs using a Qiagen DNeasy kit. Primers for GFP (forward, 5' CGCACCATCTTCTTCAAG3'; reverse, 5' TGTGGCTGTTGTAGTTGTA 3') and GAPDH (forward, 5' TGAC GTGCCGCCTGGAGAAA 3'; reverse, 5' AGTGTAGCCCAAGATGCCCTTCAG 3') were used to amplify 160 bp and 98 bp fragments respectively. The qPCR was performed on an ABI 7500 real-time PCR machine using a SYBR Premix Ex Taq II kit (Takara) in a 20 μL reaction volume. The genomic copy number of GFP (N_{GFP}) can be roughly calculated with following formula: $N_{\text{GFP}} = N_{\text{GAPDH}} / 2^{(\text{Ct}_{\text{GFP}} - \text{Ct}_{\text{GAPDH}})}$. All samples were analyzed in triplicate.

Lung Function Assessment and Bronchoalveolar Lavage

Mice were challenged for 5 min with aerosolized PBS or increasing concentrations of methacholine (Sigma) dissolved in PBS and anesthetized by tribromoethanol. The airway function was analyzed using a pulmonary function instrument (Flexivent pulmonary system). Inspiratory capacity (IC) was recorded under the TLC mode. Respiratory system resistance (Rrs) and main airway resistance (Raw) were recorded in SnapShot mode. Pulmonary tissue elasticity (H) was recorded under Primewave mode.

Immediately after lung function assessment, the lungs were lavaged via the tracheal tube with PBS (twice, 0.8 mL, 37°C). The BALF was centrifuged at a low speed, and the enriched cell suspensions were dripped on a slide, dried naturally and then stained with Leukostat. At least 100 nucleated cells were counted and classified. The proportions of macrophages, lymphocytes, neutrophils and eosinophils were recorded. Cell counting and classification was performed by two independent standard hematological procedures. The undiluted supernatants were collected for cytokine immunoassay using ELISA kits according to the manufacturer's instructions with detection limits of 0.819 pg/mL for IL-4 (Abcam), 1 pg/mL for IL-5 (Thermo), 2.8 pg/mL for IL-13 (Invitrogen) and 5.3 pg/mL for IFN- γ (Invitrogen).

Statistical Analysis

Data are expressed as the mean $\pm$ standard deviation (SD). Groups were compared with one-way analysis of variance, and $p < 0.05$ was considered to be significantly different. Analyses were performed using software (SPSS version 13.0; SPSS, Chicago, IL). All experiments were repeated at least twice.

Discussion

Allergic asthma is a heterogeneous disease characterized by inflammation of the airways that causes increase airway reactivity and structural changes in the bronchial walls (35–37), clinically characterized by recurrent episodes of wheezing, shortness of breath, chest tightness, persistent cough and increased sputum (38). The clinical manifestation of asthma has been confirmed in murine models in which Th2-type immune responses are induced. A good understanding of the disease pathophysiology is necessary to facilitate the exploration of nucleotide/gene therapy-based treatment strategies aimed at reducing airway inflammation and altering pathologic allergic reactions (39,40). In this study, we investigated the effect of an AAV5-based airway gene therapy designed to produce a truncated IL-4 protein, an antagonist of the IL-4 receptor α chain, on the development of asthma in mice. We prepared the recombinant AAV5 vector harboring the truncated IL-4 gene (AAV5-

mIL-4 Δ C22) and demonstrated that mouse IL-4 mutant protein was secreted in AAV5-mIL-4 Δ C22 transfected HBE and BEAS cells *in vitro*. The vector DNA was detected in the targeted airway tissues but not detectable in other distant organs following aerosol inhalation. In OVA-induced asthma mice, lung function was improved after recombinant AAV5 vector aerosol inhalation. These studies extend the potential utility of rAAV-mediated gene therapy for the treatment of allergic asthma.

A functional and effective therapeutic gene is vital in gene therapy studies. A previous report showed that blockade of IL-4 signaling alone cannot block AHR in sensitized mice and that the blockade of IL-13 signaling is also required (41). IL-4 and IL-13 share some structural similarities and interact with an overlapping and complex receptor signaling system (42). IL-4 receptor α chain (IL-4R α) functions within these receptor complexes: the IL-4R α /common- γ chain/IL-4 signaling complex (called the type I receptor), and both the IL-4R α /IL-13R α 1/IL-4 and the IL-4R α /IL-13R α 1/IL-13 signaling complexes (both called the type II receptor). The Th2 cytokines IL-4 and IL-13 exert their biological effects on nonhematopoietic airway cells through this type II receptor and then activate the downstream pathway and induce eosinophil accumulation and IgE synthesis (43). Previous studies have shown that human IL-4 with mutations in Arg121, Tyr124 or Ser125C sites can bind to the IL-4R α normally but lack signal transduction activity. These mutants, considered IL-4R α antagonists, can competitively inhibit normal IL-4- and IL-13-mediated signal transduction (44,45). Mouse IL-4 with a Q116 mutation or Y119 deletion also bind to the IL-4R α normally but lack the ability to trigger signal transduction, thus interrupting IL-4-induced cell proliferation, differentiation and IgE production (16,17,46). Signal transduction requires amino acid sites, as mentioned above, located in the C-terminal domain of the IL-4 peptide chain. Researchers deleted 22 amino acids in the C-terminus of mouse IL-4 and found that this mutant inhibited IL-4/IL-13-induced STAT6 phosphorylation and IgE production *in vitro* and inhibited lung eosinophil aggregation, AHR and IgE production in antigen-induced AHR mice (16,17). The C-terminal domain is the essential domain for signal transduction; either mutation or deletion of the IL-4 C-terminal domain inhibits signal transduction, but the ability to specifically bind to the cell-surface receptor with a high affinity is retained. IL-4 C-terminal domain mutants act as antagonists of the IL-4R α -chain and can disturb the IL-4- and IL-13-mediated downstream signaling pathways, thus relieving the increased airway reactivity and IgE secretion (3). Based on these previous findings, we designed experiments and successfully constructed a truncated mouse IL-4 gene encoding a truncated IL-4 mutant protein lacking 22 amino acids at the C-terminus (AAV5-mIL-4 Δ C22), which served as an antagonist of the IL-4R α -chain with the aim of simultaneously targeting the IL-4

and IL-13 pathways. Our results showed restored lung function and downregulation of the expression of Type 2 cytokines such as IL-4, IL-5 and IL-13 in asthma mice treated with AAV5-mIL-4 Δ C22 inhalation. IL-4 and IL-13 signaling cascades activate tyrosine kinases of the Janus family (Jak-1, Jak-3 and Tyk-2), leading to recruitment, phosphorylation and dimerization of Stat-6, which translocates to the nucleus and regulates transcription of IL-4/IL-13-responsive genes involved in Th2 differentiation and activation (47). Activated Th2 cells are known as the main source of IL-4, IL-5, and IL-13 in asthma (48), and blockage of IL-4 and IL-13 signaling pathways with antagonistic IL-4 mutant protein might interfering with the positive feedback of IL-4 and IL-13 production by Th2 and downregulate the expression levels of IL-4, IL-5 and IL-13.

In viral vector-mediated gene therapy, the biological distribution of viral vector DNA is an important issue. In this study, aerosol inhalation was adopted to deliver recombinant AAV5 vector into the tested mice, and the dissemination in airway tissue and other distant organs, including the gonads, was subsequently investigated to examine whether the AAV5 vector spread to other organs. The results showed that the vector DNA was detectable only in the airway tissue and not in other tested organs, which might be attributable to the integrity of the blood-air barrier. Furthermore, an applicable gene therapy strategy should include a feasible gene delivery system in association with the effective production of therapeutic molecules in targeted tissue without, at least, obvious systemic spreading. Our study showed that the AAV5-delivered reporter gene GFP was well expressed in the bronchiolar epithelium-derived cell line *in vitro*, confirming that the bronchiolar epithelium can be successfully transfected by AAV5 and that foreign genes can be expressed, and suggesting that therapeutic molecules might be produced predominantly by bronchiolar epithelial cells in the airway after aerosol delivery. This deduction is supported by numerous earlier reports in rats. In fact, AAV5 is considered a particularly good candidate for gene therapy in the respiratory tract (32), and a previous report found that recombinant AAV5-mediated gene transfer was approximately 50 fold more efficient than that of AAV2 and was more efficient than AAV2 in transferring transgenes to the murine airway *in vivo* (30). In our subsequent experiments, the analysis with a small-animal ventilator showed a significant improvement in lung function in asthma mice treated with rAAV5 vector by aerosol-mediated delivery, which strengthens the hypothesis that a therapeutic molecule is produced locally following rAAV5 vector aerosol inhalation.

This gene therapy approach of dual targeting of IL-4/IL-13 may hold hope for patients suffering from asthma in the future. However, many questions remain unresolved. The turnover of target cells may dilute the transgene, and a re-inhalation strategy would be required. The therapeutic effects in higher animal asthma models need to be

investigated. A more effective AAV5 vector using a tissue-specific promoter modulating the therapeutic gene expression would be more practical than the current vector.

Conflict of Interest

The authors have no conflicts of interest to declare.

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