

# Antisense Interleukin-5 Reduces Eosinophil Infiltration and Hyperresponsiveness in an Allergic Asthma Model

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**SUMMARY** Interleukin-5 (IL-5) involves in the development of airway inflammation and hyperresponsiveness through activation of eosinophils. Thus, inhibition of IL-5 expression seems to be an attractive approach for asthma therapy. In this study, an antisense IL-5 gene transferred by recombinant adeno-associated virus (asIL-5) was constructed to transfect murine allergic asthma model. Our results showed that asIL-5 efficiently inhibited the IL-5 mRNA expression and significantly attenuated the inflammation in lung tissues. Significant decreasing of eosinophils and inflammatory cells were found in peripheral blood and bronchoalveolar lavage fluid (BALF). In addition, significant inhibition of airway hyperresponsiveness (AHR) was also found in the mice treated with asIL-5. These observations demonstrate that antisense oligonucleotide against IL-5 delivered by adeno-associated virus system is possibly an efficacious therapeutic strategy for allergic asthma and other eosinophil-related disorders.

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Allergic asthma is a disease characterized by elevated levels of allergen-specific immunoglobulin E, chronic airway inflammation, reversible airway obstruction, and airway hyperresponsiveness (AHR) to various broncho-constrictive stimuli.<sup>1-3</sup> Clinical and experimental studies have shown strong correlation between the presence of eosinophils and their products and airway hyper-reactivity.<sup>4</sup> Study results have demonstrated that the airway inflammation is the major contributing factor to pathogenesis and pathobiology of allergic asthma, the levels of airway inflammation highly correlate with the clinical symptoms and degree of airway obstruction and airway hyperresponsiveness (AHR).<sup>5-7</sup> It is now widely recognized that eosinophils are the major cell types that play a very important role in the generation of the asthma inflammation. The levels of eosinophils and their inflammatory products in the lung well

correlate with disease severity.<sup>7</sup>

At present, interleukin (IL-5) is thought to be a major chemokine of eosinophils. IL-5 has the capability of enhancing the differentiation, activation, expansion, mobilization, and *in situ* survival of eosinophils.<sup>8-10</sup> Therefore, blocking or depleting IL-5 from asthmatic patients will result in both allergic inflammation and symptom remission. In fact, a few lines of investigations have shown that passive administration of anti-IL-5 monoclonal antibodies or experiment vaccines against IL-5 not only inhibited

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recruitment of eosinophils from the bone marrow into the targeted organs, but also attenuated allergic inflammation and clinical symptoms.<sup>11-13</sup> In addition, pharmacological modulation of gene expression has been thought to be potential approaches for treatment of allergic diseases. Manipulation of gene expression in mRNA level is more efficient than in protein level because more than 5,000 copies of a protein are produced by one mRNA molecule.<sup>14</sup> Therefore, in this study, we designed an antisense interleukin-5 which was expressed in adeno-associated virus (AAV) system to test its capability of silencing the IL-5 expression in a murine model of allergic asthma.

## MATERIALS AND METHODS

### Construction and preparation of recombinant adeno-associated virus expressing antisense IL-5

The recombinant adeno-associated virus expressing antisense IL-5 (rAAV-asIL-5) was constructed and prepared as previous reported.<sup>15</sup> Briefly, the murine antisense IL-5 gene was firstly cloned into pSNAV plasmid (Vector Gene Technology Co. Ltd., China) and yielded a recombinant plasmid named as pSNAV-asIL-5. Thereafter, BHK-21 cells (ATCC, USA) were transfected with the plasmid pSNAV-asIL-5 by Lipofectamine reagent (Invitrogen, USA). After 24 hours, the cell lines expressing pSNAV-asIL-5 were obtained through G418 selection. These cell lines were plated onto a 15-cm-diameter dish and incubated at 37°C in 5% CO<sub>2</sub>. When it reached 90% of the dish, the cells were infected by helper virus HSV-1, which containing the necessary *rep* gene and *cap* gene for rAAV virus replication and packaging at a multiplicity of infection. After 48 hours, the cells were removed and a single-step gravity-flow column purification method was carried out. The rAAV-asIL-5 vectors were saved at -80°C for animal experiment.

### Animals

Specific pathogen-free male BALB/c mice between 6 and 8 weeks of age were purchased from the Experimental Animal Center of Hainan Province, People's Republic of China. The mice were housed in macrolon cages in a laminar flow cabinet and provided with ovalbumin (OVA)-free food and water *ad*

*libitum*. Animals were handled and treated in accordance with the guidelines of Dutch Committee on Animal Experimentations. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Hainan Provincial Key Laboratory of Tropical Medicine.

### Murine asthma model and therapeutic protocols

To establish the mouse allergic asthma model, mice were sensitized to ovalbumin (OVA, Sigma-Aldrich, USA) by 2 intraperitoneal injections (7 days apart) of 100 µl alum-precipitated antigens composed of 10 µg OVA absorbed onto 2.25 mg alum (ImjectAlum; Pierce Biotechnology, Rockford, USA). Thereafter, the animals were exposed to aerosol challenge containing 1% OVA (1 mg/ml saline) for 60 minutes/day. The aerosol challenge was performed in a Plexiglas exposure chamber coupled to a Pari LC Star nebulizer (particle size 2.5–3.1 µm; PARI Respiratory Equipment, Richmond, USA) driven by compressed air at a flow rate of 6 liters/minute.

To test the therapeutic anti-asthma effects, mice were randomly divided into four groups of 10 mice each. Group 1 (asIL-5): mice were sensitized and challenged with OVA, 0.2 ml (1 × 10<sup>13</sup> vector genomes [v.g.]/ml) rAAV-asIL-5 were administered *via* tail vein injection on the day of sensitization (day 0) and the day of challenge (day 14). Group 2 (AAV): mice were sensitized and challenged with OVA, 0.2 ml (1 × 10<sup>13</sup> v.g./ml) adeno-associated virus were administered *via* tail vein injection on the day of sensitization (day 0) and the day of challenge (day 14). Group 3 (NS): mice were sensitized and challenged with OVA, 0.2 ml normal saline was administered *via* tail vein injection on the day of sensitization (day 0) and the day of challenge (day 14). Group 4 (naive): mice were not sensitized and challenged with OVA, 0.2 ml normal saline was administered *via* tail vein injection on the day of sensitization (day 0) and the day of challenge (day 14).

### Real-time quantitative PCR

The lungs of mice were homogenized with TRIZOL reagent (Invitrogen, Gaithersburg, USA) and the total RNA was extracted according to the manufacturer's instruction. The total RNA was

treated with rDNase I for 30 minutes. cDNA synthesis was performed with random hexamer primers. Quantitative real-time PCR was performed in a Multicolor Real-Time PCR Detection System (Bio-Rad, CA, USA). All reported mRNA levels were normalized to the  $\beta$ -actin mRNA level.

### Lung histology

For histological evaluation of lung tissues, lungs were removed and fixed in 10% phosphate-buffered formalin. The left lobe of each lung was embedded in paraffin, sectioned at 3–5  $\mu$ m, and stained with hematoxylin and eosin. Cellular infiltrates were assessed in five randomly selected fields with a microscope at 200x magnification. Groups were analyzed in a blind fashion as done previously.<sup>16,17</sup>

### Bronchoalveolar lavage

At the end of the experiments, mice were sacrificed by CO<sub>2</sub> asphyxiation, and samples of bronchoalveolar lavage fluid (BALF) and lung tissue were collected as previously described.<sup>16</sup> Briefly, ten lungs per group were perfused through the pulmonary artery and lavaged through the trachea three times with 1 ml of PBS. Total BALF cells were determined using a hemacytometer. BALF cells were then spun onto slides by cytocentrifugation, then fixed and visualized with Hansel stain (Lide Laboratories, Florissant, Mo., USA). Differential cell counts were made on at least 400 cells using standard morphological criteria.<sup>16,17</sup>

### Quantification of eosinophils

Peripheral blood samples, lung tissues and BALF were collected at the end of the experiments. Carbol's chromotrope-hematoxylin stain was used for the identification of eosinophils. Eosinophils in the peripheral blood, BALF, and lung tissues were identified based on morphological characteristics and quantified as previously described.<sup>16,17</sup>

### Measurement of airway hyperresponsiveness

Airway hyperresponsiveness (AHR) was assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole body plethysmograph (model PLY 3211, Buxco Electron-

ics, Troy, USA). Measurements of a dimensionless parameter known as the enhanced pause (Penh) were performed as previously described.<sup>18,19</sup> Briefly, mice were placed in the plethysmograph chamber and exposed to an aerosol of normal saline (NS) (baseline readings) and then to cumulative concentrations of  $\beta$ -methacholine ranging from 3 to 50 mg/ml. The aerosol was generated by an ultrasonic nebulizer and drawn through the chamber for 2 minutes. The inlet was then closed, and Penh readings were taken for 3 minutes and averaged. The values from 10 normal mice (without sensitizing and aerosol challenging with OVA, no vaccine immunization) of the same age were used for the baseline data. Values from the above-mentioned four experimental groups were reported as the percent increase over the baseline values from the normal mice at corresponding time points.

### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation (SD) and analyzed by one-way ANOVA and *q* test using SPSS 12.0. A *p* value less than 0.05 was considered statistically significant.

## RESULTS

### Inhibition of IL-5 mRNA expression in lung tissues

Compared with the non-sensitized mice (naive), the IL-5 mRNA expression in the mice treated with only adeno-associated virus (AAV) or normal saline (NS) had  $5.84 \pm 1.13$  and  $5.12 \pm 0.98$ -fold increase, respectively. However, IL-5 mRNA levels of mice treated with rAAV-asIL-5 (asIL-5) were significantly decreased, about  $1.02 \pm 0.32$ -fold compared with that found in the naive mice. More than 4-fold reduction of IL-5 mRNA was found in mice treated with asIL-5 compared with those treated with AAV or NS (Fig. 1).

### Remission of inflammation in lung tissues

Lung sections from the mice treated with asIL-5 (Fig. 2A) showed almost normal lung histology compared with the naive mice (Fig. 2D), with only marginal perivascular and peribronchiolar lymphocytic infiltrates. In contrast, lung sections from mice treated with AAV (Fig. 2B) or NS (Fig. 2C)

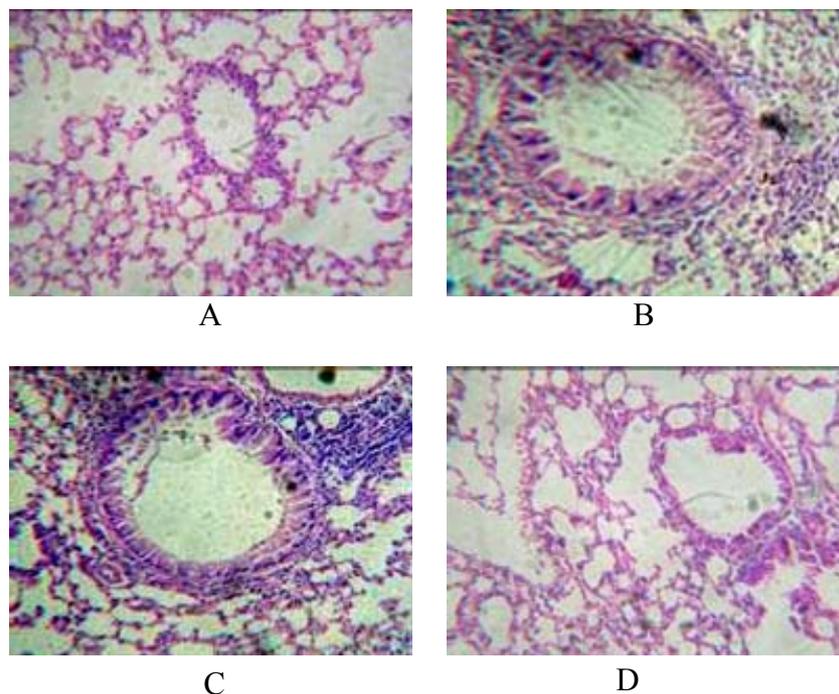
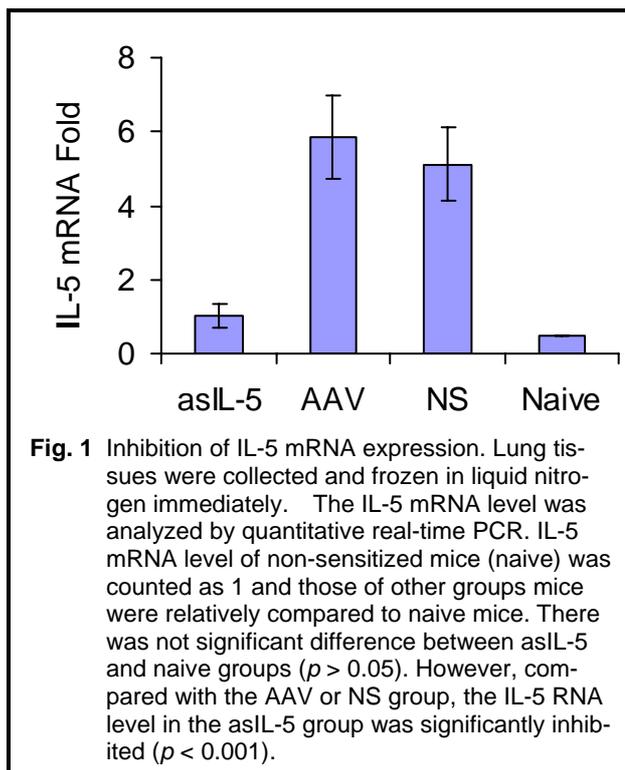
showed significant airway inflammation with peribronchiolar and perivascular infiltrates, consisting of lymphocytes, eosinophils and some neutrophils.

### Decreasing of eosinophil infiltration

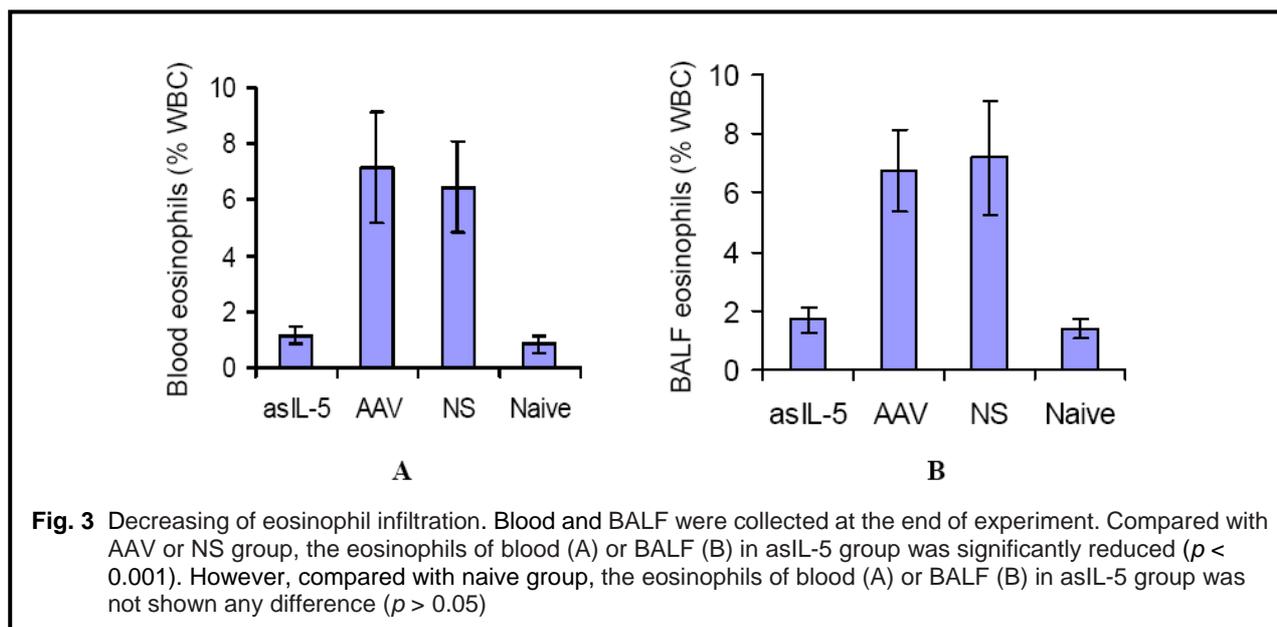
The number of eosinophils in BALF (Fig. 3A) and peripheral blood (Fig. 3B) treated with asIL-5 were found to be comparable to those in the naive mice. None any significant difference was shown between asIL-5-treated mice and naive mice. However, compared with the AAV-treated and NS-treated mice, eosinophils were significantly attenuated in the asIL-5-treated mice ( $p < 0.001$ ).

### Inhibition of AHR development

AHR was measured with a whole-body plethysmograph by challenging with increasing concentrations of methacholine at different time points. Fig. 4 demonstrated the strand of AHR development. The mice treated with AAV or NS developed significant AHR. However, mice treated with asIL-5



**Fig. 2** Remission of inflammation. Lung tissues were fixed in formalin and stained with hematoxylin and eosin at the day of sacrifice. Compared with naive mice (D), lung tissues from mice treated with asIL-5 had only minimal inflammation (A); lung tissues from mice treated with AAV (B) and with NS (C) revealed significant inflammation, significant peribronchiolar mononuclear cell infiltrates consisting of lymphocytes, eosinophils, and some neutrophils. 200x magnification.

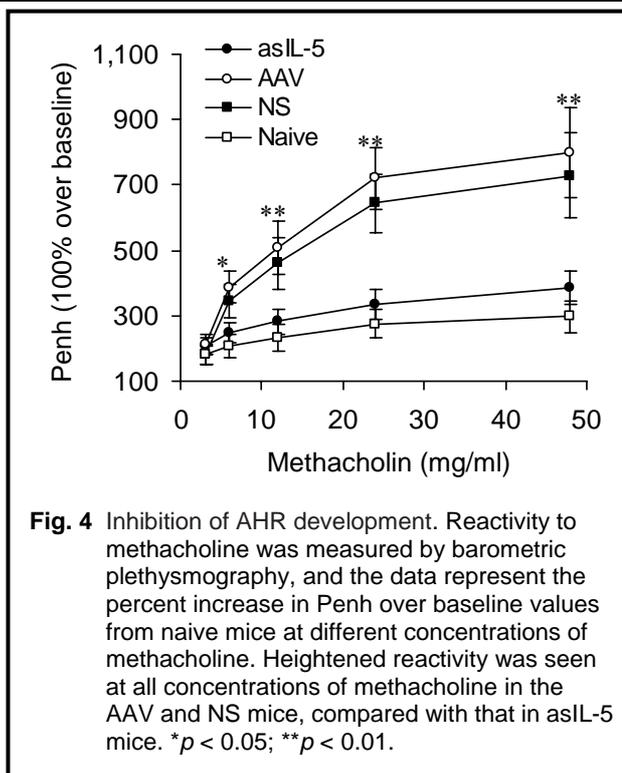


showed dramatically reduced AHR, which were almost comparable to that found in the naive mice (Fig. 4).

## DISCUSSION

The development of the antisense approach started in the late 1970s after the discovery that the expression of a specific gene product could be inhibited using a short complementary DNA sequence.<sup>20</sup> Since then, the antisense strategy has enjoyed exponential gains in interest in the world. In 1998, the first antisense oligonucleotide, Vitravene™ (Fomivirsen), was approved for use against cytomegalovirus-induced retinitis by local injection.<sup>21</sup> Antisense oligonucleotides have been used for a variety of purposes, including target validation, gene function studies and as experimental therapy for different diseases. The antisense oligonucleotides have been used most frequently for cancer-related targets including oncogenes, signaling molecules and mutant tumor suppressor genes.<sup>22-24</sup> Besides, pathogen-associated and other disease-related gene products such as ICAM-1 and TNF- $\alpha$  (for Crohn's disease and Rheumatoid Arthritis, respectively) have also been targeted.<sup>25,26</sup>

To date, there have been few specific antagonists or molecular targets directed against eosinophils. It is thus desirable to have other therapeutic targets. Several studies have demonstrated that the



function of eosinophils is principally under the control of a subset of Th2 cell-derived cytokines including IL-5, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Although each of these cytokines has the capability of regulating the survival, adhesion, priming and activation of terminally differentiated eosinophils, IL-5 alone is capable of promoting the terminal differentiation of bone

marrow-derived eosinophil precursors into mature eosinophils.<sup>27</sup> Furthermore, while IL-3 and GM-CSF can activate a broad spectrum of hematopoietic cell types, the effects of IL-5 are restricted to cells of the eosinophil/basophil lineage.<sup>28,29</sup> Thus, blocking the activity of IL-5 seems the most specific way of targeting the eosinophils, potentially leading to the highest therapeutic benefit with the smallest number of adverse consequences. In addition, several studies have demonstrated that the expression of IL-5 in the lung was inversely correlated with pulmonary dysfunction in asthma patients, and the level of expression was directly correlated with the number of eosinophils detected in asthmatic airways.<sup>30-32</sup> Reducing IL-5 levels also reduced AHR independently of its role in eosinophilia, probably through the effects of IL-5 on airway smooth muscle.<sup>33</sup> At present, although some studies have shown that monoclonal antibody against IL-5 could reach satisfied anti-asthma effects,<sup>34</sup> these therapeutic approaches exist many shortages. Treatment with monoclonal antibody is a kind of passive immunotherapy which needs complex manipulation and a long-term usage of the antibody *in vitro*, representing an expensive economic burden for asthma patients. Therefore, it is highly warranted to develop a novel approach for treatment of asthma.

Gene therapy has shown its attractive future in cancer and other chronic or genetic refractory diseases. Several viral vectors, such as lentivirus, adenovirus, rAAV and vesicular stomatitis virus, have been used for gene therapy.<sup>35</sup> The rAAV vector, which has the capability of either integrating into the genome or remaining in the nucleus as a stable episome, offers several theoretical advantages compared with other vectors, such as the lack of inflammatory response, the ability to infect the dividing and non-dividing cells, especially including T lymphocytes.<sup>36,37</sup> In addition, the AAV is relatively safe in gene transport process, because AAV can not produce viral proteins that stimulate inflammatory reactions, and not replicate inside cells which results in not any presence of a wild-type adenovirus.<sup>38,39</sup> Furthermore, the AAV is effective as a vector as it contains sufficient capacities for the insertion of various exogenous genes.<sup>40</sup> Therefore, it is possible for exogenous gene to produce a sustained and effective expression.

Our current study results demonstrated that asIL-5 was capable of inducing therapeutic anti-asthma efficacies in the murine model of asthma. Treatment with antisense oligonucleotide against IL-5 (asIL-5) significantly decreased expression of IL-5 mRNA and reduced airway inflammation and AHR in the pulmonary compartments of mice sensitized and challenged with OVA. We thus supposed that it may be the reduction of IL-5 expression that reduced tissue infiltration of eosinophils and result in remission of inflammation and AHR. Compared with monoclonal antibodies and other currently used approaches, antisense oligonucleotide delivered by intravenous injection is easier and more economic. Therefore, our findings demonstrated that gene therapy with antisense oligonucleotide against IL-5 could be a potential therapeutic approach to the treatment of allergic asthma and possibly other eosinophil-related disorders.

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