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RNA interference against interleukin-5 attenuates airway inflammation and hyperresponsiveness in an asthma model*

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Abstract: Interleukin-5 (IL-5) accompanies the development of airway inflammation and hyperresponsiveness through the activation of eosinophils. Therefore, interference of IL-5 expression in lung tissue seems to be an accepted approach in asthma therapy. In this study, we designed a small interfering RNA (siRNA) to inhibit the expression of IL-5. The siRNAs against IL-5 were constructed in a lentivirus expressing system, and 1.5×10^6 IFU (inclusion-forming unit) lentiviruses were administered intratracheally to ovalbumin (OVA)-sensitized murine asthmatic models. Our results show that lentivirus-delivered siRNA against IL-5 efficiently inhibited the IL-5 messenger ribonucleic acid (mRNA) expression and significantly attenuated the inflammation in lung tissue. Significant decrease of eosinophils and inflammatory cells were found in peripheral blood, bronchoalveolar lavage fluid (BALF), and lung tissue. In addition, significant inhibition of airway hyperresponsiveness (AHR) was found in the mice treated with siRNA against IL-5. These observations demonstrate that siRNA delivered by means of the lentivirus system is possibly an efficacious therapeutic approach for asthma.

Key words: Interleukin-5 (IL-5), Small interfering RNA (siRNA), Airway hyperresponsiveness (AHR), Allergy, Lentivirus
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INTRODUCTION

Allergic asthma is characterized by elevated levels of allergen-specific immunoglobulin E, chronic airway inflammation, reversible airway obstruction, and airway hyperresponsiveness (AHR) to various broncho-constrictive stimuli (Barthel *et al*., 2008; Sugita *et al*., 2003; Woodruff *et al*., 2001). Clinical and experimental studies have shown strong correlation between the presence of eosinophils and their products and AHR (Humbles *et al*., 2004).

Studies have demonstrated that the airway inflammation is the major contributing factor to pathogenesis and pathobiology of allergic asthma, and the levels of airway inflammation often correlate with the clinical symptoms and degree of airway obstruction and AHR (Busse and Lemanske, 2001; Fixman *et al*., 2007; Walter and Holtzman, 2005). It is now widely recognized that eosinophils are the major cell types that play a pivotal role in the generation of asthma inflammation. The levels of eosinophils and their inflammatory products in the lung well correlate with disease severity (Busse and Lemanske, 2001). At present, interleukin-5 (IL-5) is thought to be a major chemokine of eosinophils, and has the capability of enhancing the differentiation, activation, expansion, mobilization, and in situ survival of eosinophils (Cho *et al*., 2004; Mattes and Foster, 2003; Shen *et al*., 2003). Thus, the 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

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administration of anti-IL-5 monoclonal antibodies or experimental vaccines against IL-5 not only inhibited recruitment of eosinophils from the bone marrow into the targeted organs, but also attenuated allergic inflammation and clinical symptoms (Menzies-Gow *et al*., 2003; Simon *et al*., 2005; Sutton *et al*., 2005).

Recently, pharmacological modulation of gene expression has been thought to be a potential approach for treatment of allergic diseases. Manipulation of gene expression in messenger ribonucleic acid (mRNA) level is more efficient than that in protein level because more than 5000 copies of a protein are produced by one mRNA molecule (Popescu, 2005; Jiang *et al*., 2008). Therefore, in the current study, we designed a small interfering RNA (siRNA) against IL-5 and constructed it in a lentivirus system to test its capability of silencing the IL-5 expression in lung tissue in an ovalbumin (OVA)-induced murine model of allergic asthma.

MATERIALS AND METHODS

Preparation of lentiviruses expressing siRNA against IL-5 (siIL-5)

Before our study, we have tried several siRNA expression cassettes (siRNA-EC), which targeted the coding regions or 5′ untranslated region (5′UTR) of IL-5 mRNA. We found that an siRNA-EC sequence (5′-AAGATATTCGTGTACCGCACG-3′) targeting 5′UTR of IL-5 mRNA has the best interfering effect in an EL4 (a mouse lymphoma cell line, purchased from ATCC, USA)-infected cell system. Plasmids for producing lentivirus were provided by Dr. Hua Li (Tumor Center, Sichuan University, China). The siRNA-ECs were digested with *Eco*RI and *Hin*dIII and ligated into pTY-linker vector for the lentivirus-delivering system. Thereafter, UAS-BLA HEK 293T cells (Invitrogen, USA) were seeded in a 10-cm dish the day before transfection of the plasmids. The lentivirus vector containing siRNA-EC sequence or control sequence (5′-GTCAGAGTGTGCCTTGAC TG-3′) was offered by siRNA expression cassette kit and lacked homology to any murine genes. The siRNA-EC with three other packaging plasmids for virus generation, transfer vector, packaging vector, and envelope vector, was mixed at ratios of 3, 3, 2, and 1, respectively, and co-transfected into 293T cells. After 18~24 h, fresh complete media were changed. Forty-eight hours after transfection, lentivirus particles were produced into the supernatant. Filtrated lentivirus-containing supernatant was concentrated with Microcon YM-100 Centrifugal Filter Unit (Millipore, USA) and stored at −80 °C.

Animals

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 Medical College. Specific pathogen-free male BALB/c mice between 6 and 8 weeks of age were purchased from the Experimental Animal Center of Hainan Province, China. The mice were housed in macrolon cages in a laminar flow cabinet and provided with OVA-free food and water ad libitum.

Mouse asthma model and therapeutic protocols

To establish the mouse allergy asthma model, mice were sensitized to OVA (Sigma-Aldrich, USA) by 2 intraperitoneal injections (7 d apart) of 100 μl alum-precipitated antigens composed of 10 μg OVA absorbed into 2.25 mg alum (ImjectAlum; Pierce Biotechnology, Rockford, USA). Thereafter, the animals were exposed to aerosol challenge containing 1 mg/ml OVA for 60 min/d. 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, USA) driven by compressed air at a flow rate of 6 L/min. On Day 37, 1.5×10^6 IFU (inclusion-forming unit) lentivirus containing siRNA against IL-5 (siIL-5) or 3×10^6 IFU lentivirus containing control siRNA (cIL-5), was administered intratracheally into the anaesthetized animals, and positive control mice were administered normal saline (NS). The naive group was not sensitized with OVA nor administered with anything (Naive). At the end of the experiments, lung tissue was collected and studied.

Quantitative real-time polymerase chain reaction

The lungs of mice were homogenized with Trizol reagent (Invitrogen, Gaithersburg, USA). The total RNA was extracted according to the manufacturer's instructions and treated with recombinant DNase I

(rDNase I) for 30 min. cDNA synthesis was performed with random hexamer primers. Quantitative real-time polymerase chain reaction (PCR) was performed in a Muticolor Real-Time PCR Detection System (Bio-Rad, CA, USA). All reported mRNA levels were normalized to the β-actin mRNA level.

Lung histology

For histological evaluation of lung tissue, the lung tissues were dislodged and then fixed in 10 mg/ml formalin in phosphate-buffered saline (PBS). The left lobe of each lung tissue was embedded in paraffin, sectioned at 3~5 μm, and stained with hematoxylin and eosin (H&E). Inflammatory cell infiltration in each section was assessed in five randomly selected fields with a microscope at 200× magnification. Data were analyzed in a blind fashion as previously described (Hertz *et al*., 2001; Tan *et al*., 2008).

Bronchoalveolar lavage

At the end of the experiments, mice were sacrificed by means of CO_2 asphyxiation, and the samples of bronchoalveolar lavage fluid (BALF) and lung tissues were collected as previously described (Hertz *et al*., 2001). Briefly, 10 lungs per group were perfused through the pulmonary artery and lavaged through the trachea 3 times with 1 ml of PBS. Total number of BALF cells was determined using a hemacytometer. BALF cells were spun onto slides by cytocentrifugation, and then fixed and visualized with Hansel stain (Lide Laboratories, Florissant, Mo., USA). Differential cell counts were determined on at least 400 cells by morphological characteristics (Hertz *et al*., 2001; Tan *et al*., 2008).

Quantification of eosinophils

Peripheral blood samples, lung tissues, and BALF cells were collected at the end of the experiments. Carbol's chromotrope-hematoxylin stain was used for the detection of eosinophils. Eosinophils in the peripheral blood, BALF, and lung tissue were judged based on the morphological characteristics and quantified as previously reported (Hertz *et al*., 2001; Tan *et al*., 2008).

Measurement of AHR

AHR was assessed by β-methacholine-induced airflow obstruction from conscious mice that were placed in a whole body plethysmograph (model PLY 3211, Buxco Electronics, Troy, USA). Measurements of a dimensionless parameter known as the enhanced pause (Penh) were performed as previously described (Hamelmann *et al*., 1997; Hertz e*t al*., 2001). Briefly, mice placed in a plethysmograph chamber were exposed to an aerosol of NS water (as baseline) and then to cumulative concentrations of β-methacholine ranging from 3 to 48 mg/ml. The aerosol was generated by an ultrasonic nebulizer (PARI Respiratory Equipment, USA) and drawn through the chamber for 2 min. Thereafter, the inlet was closed, and Penh readings were collected for 3 min 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 4 experimental groups were reported as the percentage increase over the baseline values from the normal mice at corresponding time points.

Statistical analysis

Data was expressed as mean±standard deviation (*SD*) and analyzed by one-way analysis of variance (ANOVA) and *q* test using SPSS 12.0. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Knock-down of IL-5 mRNA expression in lung tissues

The levels of IL-5 mRNA in the lungs were determined by quantitative real-time PCR analysis. The IL-5 mRNA expression of the OVA-sensitized mice treated with NS had an (8.26±1.63)-fold increase compared with the naive mice. However, the IL-5 mRNA levels of mice treated with siIL-5 interfered significantly by (1.45±0.43)-fold compared with those of the naive mice; the cIL-5 slightly reduced IL-5 mRNA expression; almost 3-fold reduction of IL-5 mRNA were found in the mice treated with siIL-5 compared with those treated with cIL-5 or NS (Fig.1). These results show that siIL-5 effectively knocked down the expression of IL-5 in lung tissue.

Significant remission of inflammation in lung tissues

Lung sections from the mice treated with siIL-5 showed almost normal lung histology compared with the naive mice (Fig.2d), with only marginal perivascular and peribronchiolar lymphocytic infiltrates (Fig.2a). In contrast, lung sections from mice immunized with cIL-5 or NS showed clear airway inflammation with peribronchiolar and perivascular infiltrates, consisting of lymphocytes, eosinophils, and some neutrophils (Figs.2b and 2c). These results suggest that siIL-5 significantly reduced airway inflammation in the murine model of allergic asthma.

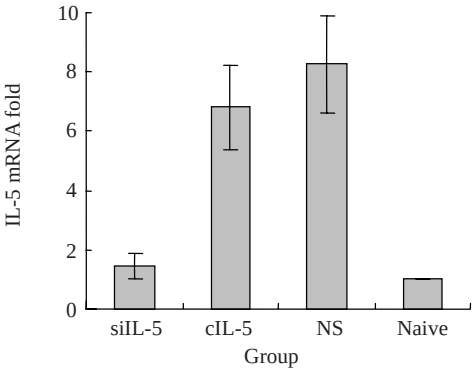

Fig.1 Knock-down of IL-5 mRNA expression

Lung tissue was collected and frozen in liquid nitrogen immediately. The IL-5 mRNA level was analyzed by quantitative real-time PCR. IL-5 mRNA level of non-sensitized mice (Naive) was counted as 1 and those of other groups of mice were relatively compared with the naive mice. There was no significant difference between the siIL-5 and Naive groups (P>0.05). However, compared with the cIL-5 or NS group, the IL-5 RNA level in the siIL-5 group was significantly inhibited (P<0.001). Data are expressed as mean±*SD*

Significant decrease of eosinophils and inflammatory cells

Blood, BALF, and lung tissues were collected at the end of the experiment. The numbers of eosinophils in blood (Fig.3a), BALF (Fig.3b), and lung tissue (Fig.3c) were assessed by applying Carbol's chromotrope-hematoxylin staining. No significant difference in the numbers of eosinophils in blood, BALF, and lung tissue was found between the siIL-5-treated mice and the naive mice. However,

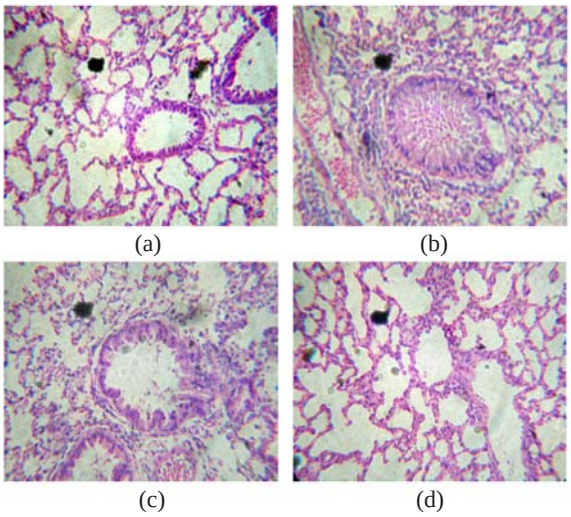

Fig.2 Remission of inflammation

Lung tissue was fixed in formalin and stained with hematoxylin and eosin (H&E) at the day of sacrifice. Compared with the naive mice (d), lung tissue from mice treated with siIL-5 (a) had only minimal inflammation; lung tissue from mice treated with cIL-5 (b) and with NS (c) revealed significant inflammation and peribronchiolar mononuclear cell infiltrates consisting of lymphocytes, eosinophils, and some neutrophils. Original magnification 200×

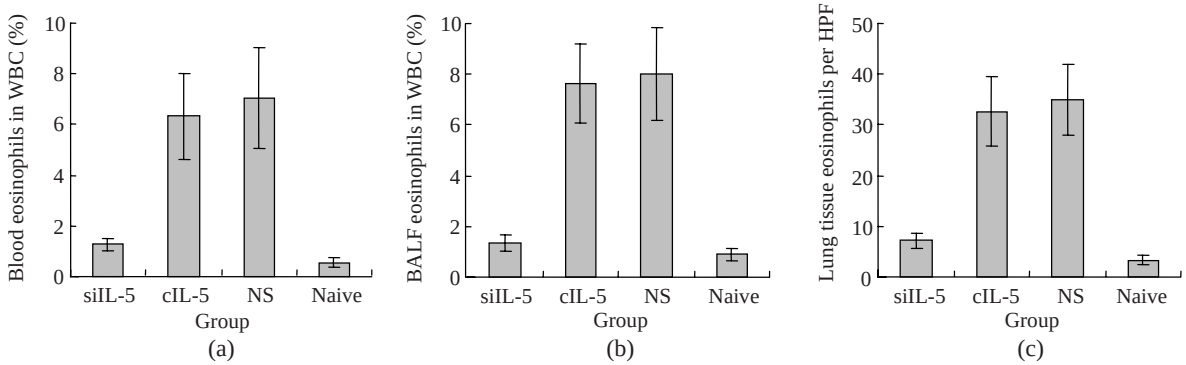

Fig.3 Inhibition of eosinophil infiltration

Blood, BALF, and lung tissue samples were collected at the end of the experiment. Compared with cIL-5 or NS group, the eosinophils of blood (a), BALF (b), or lung tissue (c) in the siIL-5 group were significantly reduced (P<0.001); however, compared with the naive mice, the eosinophils of blood (a), BALF (b), or lung tissue (c) in the siIL-5 group showed no difference (P>0.05). WBC: white blood cell; HPF: high power field. Data are expressed as mean±*SD*

eosinophils were significantly attenuated in the siIL-5-treated mice (P<0.001) compared with the NS-treated mice. These results show that siIL-5 significantly reduced the proportion of eosinophils, confirming the results observed in the lung histology.

Inhibition of AHR development

AHR was measured with a whole-body plethysmograph by challenging with increasing concentrations of β-methacholine at different time intervals. Fig.4 demonstrates the strand of AHR development. The control mice treated with cIL-5 or NS developed significant AHR. However, mice immunized with siIL-5 showed dramatically reduced AHR, comparable to that found in the naive mice (Fig.4). These results indicate that siIL-5 could significantly inhibit the development of AHR in allergic asthma mice.

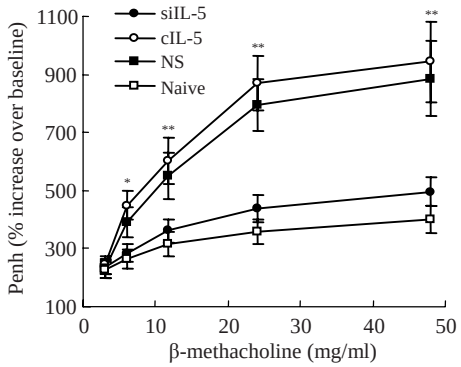

Fig.4 Inhibition of AHR development

Reactivity to β-methacholine was measured by barometric plethysmography, and the data (mean±*SD*) represent the percentage increase in Penh over baseline values from the naive mice at different concentrations of β-methacholine. Heightened reactivity was seen at all concentrations of β-methacholine in the cIL-5 and NS mice, compared with that in the siIL-5 mice: $^{*}P$<0.05, $^{**}P$<0.01

DISCUSSION

Pathophysiologic studies on bronchial asthma have indicated that airway inflammation is the major component of the disease (Busse and Lemanske, 2001; Walter and Holtzman, 2005). Various inflammatory cell types, including $CD4^{+}$ T cells and mast cells, have been found in both the bronchial mucosa and the lumen of the airways, among which activated eosinophils appear to be the predominant cell type (Bentley *et al*., 1992; Gleich, 1990; Humbles *et al*., 2004; Sanderson, 1992). Activated eosinophils produce a number of cytotoxic proteins, such as the major basic protein, eosinophil peroxidase, and eosinophil cationic protein, which have been hypothesized to act in concert and contribute to the tissue edema, epithelial damage, bronchial hyperresponsiveness, mucus secretion, and airway remodeling (Kroegel *et al*., 1994). In addition to airway eosinophilia, asthmatic individuals also show a systemic eosinophilia (Durham *et al*., 1989), which indicates that pulmonary eosinophilia is dependent on increased eosinophil production in bone marrow prior to the release of mature cells into the bloodstream, and their subsequent recruitment to the sites of inflammation. These results demonstrate that inhibitors of either eosinophil production, recruitment to the airway, or subsequent activation could be novel anti-inflammatory approaches to future asthma therapy.

To date, there have been few specific antagonists or molecular targets directed against eosinophils. Several studies have demonstrated that the function of eosinophils is principally under the control of a subset of T helper 2 (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 (Humbert, 1996), IL-5 alone is capable of promoting the terminal differentiation of bone marrow-derived eosinophil precursors into mature eosinophils (Clutterbuck *et al*., 1989). 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 (Sanderson, 1992). Thus, blocking the activity of IL-5 seemed the most effective way of targeting the eosinophils, potentially leading to the highest therapeutic benefit with the least adverse consequences. Several studies have demonstrated that the expression of IL-5 in the lung was inversely correlated with pulmonary dysfunction in asthma patients, and that the level of expression was directly correlated with the number of eosinophils detected in asthmatic airways (Hertz *et al*., 2001; Robinson *et al*., 1992; 1993). Reducing IL-5 levels also reduced AHR independently of IL-5 role in eosinophilia (Tournoy *et al*., 2001), probably through the effects of IL-5 on

airway smooth muscle (Hakonarson *et al*., 1999a; 1999b). At present, although some studies have shown that a monoclonal antibody against IL-5 (Blyth *et al*., 2000; Garlisi *et al*., 1999; Leckie *et al*., 2000) and IL-5 antisense oligonucleotide (Karras *et al*., 2000) could reach satisfied anti-asthma effects, these therapeutic approaches contain many shortages. Treatment with a 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. Oligonucleotide degradation in vivo is a well-known problem that arises as a result of treatment with IL-5 antisense oligonucleotide. Therefore, it is highly recommended to develop an additional, novel approach in the treatment of asthma.

In our current study, we designed a small interfering RNA against IL-5 (siIL-5) and expressed it in a lentivirus system. Our results demonstrate that siIL-5 was capable of inducing therapeutic anti-asthma efficacies in the murine model of asthma. Treatment with siIL-5 significantly reduced airway inflammation and AHR in the pulmonary compartments of mice exposed to OVA sensitization and challenges. These results show that RNA interference with siIL-5 resulted in decreased levels of IL-5 expression in lung tissue. It is likely that the IL-5 mRNA knock-down reduced tissue infiltration of eosinophils and resulted in a remission of inflammation and AHR.

Compared with current experimental approaches, such as monoclonal antibodies and antisense oligonucleotide, siRNA delivered intratracheally is more applicable and economic. Therefore, RNA interference with siIL-5 through the lentivrirus system could be a potential therapeutic approach to the treatment of allergic asthma and possibly other eosinophil-related diseases.

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