

ORIGINAL ARTICLE

Small interfering RNA against interleukin-5 decreases airway eosinophilia and hyper-responsiveness

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Interleukin-5 (IL-5) has been suggested to be involved in the development of airway hyper-responsiveness (AHR). Both clinical and experimental investigations have shown strong correlation between the presence of eosinophils and AHR. In this study, we used small interfering RNA (siRNA) as an approach to inhibiting the expression of IL-5 and reducing AHR. siRNAs targeting IL-5 were characterized *in vitro*, and siRNA-expressing lentiviruses were administered intratracheally to OVA-sensitized BALB/c mice. AHR, cytokine levels, serum levels of OVA-specific antibodies and infiltration of inflammatory cells were analyzed to investigate the

effects of siRNA in an OVA-induced murine model of asthma. Lentivirus-delivered siRNA targeting IL-5 efficiently moderated the characteristics of asthma, including AHR, cellular infiltration of lung tissues, eotaxin levels in the bronchoalveolar lavage fluid and IL-5 mRNA levels in lungs in the mouse model of asthma. However, there was no effect on OVA-specific IgE level. These data demonstrate that siRNA delivered by the lentiviral system is an efficacious therapeutic strategy for asthma.

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Introduction

Asthma is a chronic airway inflammatory disease characterized by eosinophil infiltration and airway hyper-responsiveness (AHR). Both clinical and experimental studies have shown strong correlation between the presence of eosinophils and their products and airway hyper-reactivity.¹ Eosinophils induce airway inflammation and AHR by secreting granular basic proteins and membrane-derived lipid mediators. Granular basic proteins damage the bronchial epithelium, and membrane-derived lipid mediators contract smooth muscle and increase mucus secretion.² Eosinophils may also play a role in the airway remodeling by releasing the potent profibrotic cytokines, transforming growth factor- β and other fibrogenic factors.³ Herein, eosinophil has been a potential therapeutic target for asthma.

Eosinophilia in atopic diseases and hyper-eosinophilic syndrome is often associated with a high level of interleukin-5 (IL-5). IL-5 is the key cytokine in eosinophil growth and differentiation in bone marrow^{4–6} and release into the peripheral circulation.^{7,8} In addition, IL-5 enhances eosinophil differentiation, maturation, recruitment, activation and survival in the inflammation site.⁹ IL-5 is mainly produced by activated T cells, CD8 T cells,

mast cells, eosinophils and airway epithelium cells.^{10–12} In asthmatic patients, concentration of IL-5 is correlated with clinical features¹³ and IL-5 mRNA is upregulated in the bronchial mucosa after allergen provocation.¹⁴ IL-5-deficient mice do not respond with increased numbers of eosinophils on challenge.^{15,16} Therefore, inhibiting or reducing IL-5 with an antibody is a promising therapeutic strategy in eosinophilic diseases.

Anti-IL-5 antibody therapy has resulted in dramatic changes in both circulating and airway luminal eosinophil numbers.¹⁷ For example, in patients with bronchial asthma or in the primate model of allergic airway inflammation, reduced reactions of early and late phase and on AHR have been observed.^{17–19} Moreover, patients with atopic dermatitis have shown a significant decrease in peripheral blood eosinophils but only a modest improvement of their skin lesions and pruritus scoring.²⁰ According to these studies, anti-IL-5 therapy induced a rapid and sustained decrease of peripheral blood eosinophil. However, the decrease of tissue eosinophils is less dramatic.

Recently, pharmacological modulation of gene expression has been received increasing attention in allergic diseases. Manipulation of gene expression in mRNA level is more efficient than in protein level because multiple copies of a protein (approximately 5000 copies) are produced by each mRNA.²¹ Therefore, hybridization-based antisense oligonucleotides and RNA interference are investigated for their therapeutic effect on asthma and other allergic diseases.

In this study, we aimed to reduce the IL-5 expression in tissue by directly using small interfering RNA

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(siRNA) against IL-5 in an ovalbumin (OVA)-induced murine model of airway inflammation.

Results

In vitro characterization of siRNA targeting to murine IL-5

Three siRNA fragments were designed against IL-5. Before the administration of siRNA-expressing cassette (SEC) to mice, we examined whether siRNA could suppress IL-5 expression efficiently. It has been reported that EL4 cells could secrete IL-5 after stimulation with phorbol 12-myristate 13-acetate (PMA) and cyclic AMP (cAMP).²² We infected EL4 cells with lentiviruses containing SEC1, -4, -7 or enhanced green fluorescence protein to screen which one had the best inhibitory function. SEC1 targets the coding region of IL-5 mRNA while SEC4 and SEC7 target to 5'UTR region of IL-5 mRNA. Lentivirus-infected cells were then treated with PMA and cAMP for 24 h, and IL-5 levels in supernatant were assayed by enzyme-linked immunosorbent assay (ELISA). The IL-5 expression of polybrene-treated EL4 cells was regarded as 100%. The data demonstrated that SEC4 and SEC7 decreased the PMA- and cAMP-stimulated IL-5 protein expression to 30.3 ± 0.8 and $33.2 \pm 1.4\%$ of polybrene-treated EL4 cells, respectively (Figure 1a).

IL-5 mRNA level knockdown in vivo

We further investigated the suppressive effect of SEC4 *in vivo*. BALB/c mice were sensitized with OVA, administered intratracheally with SEC4-containing lentiviruses, and finally challenged with OVA as the protocol described in Materials and methods. At 48 h after the last of three sequential OVA challenges (days 40–42), we analyzed the levels of IL-5 mRNA in the lungs by a quantitative real-time PCR analysis. The IL-5 mRNA expression of OVA-sensitized mice treated with normal saline had a 7.96 ± 2.33 -fold increase compared to naive mice. IL-5 mRNA levels of mice treated with different doses of SEC4 were inhibited efficiently, with 1.76 ± 1.09 -, 2.03 ± 0.66 - and 1.52 ± 0.50 -fold, respectively, compared to that of naive mice (Figure 1b). The control SEC (SECneg) containing a fragment, which lacked homology to any genes of mouse, slightly reduce IL-5 mRNA expression. However, 50% reduction of IL-5 mRNA was noted in mice treated with SEC4 compared to that of control SEC (SECneg) (Figure 1b). Taken together, inhibition by SEC4 was sequence specific.

Reduction of AHR and eosinophilia

To investigate the effect of the SEC on AHR and eosinophilia in the murine model of asthma, we intratracheally administered SEC4-containing lentiviruses to BALB/c mice on day 37 after OVA sensitization and challenged mice with OVA intranasally on days 40, 41 and 42.

First, we examined the serum immunoglobulin levels of these mice. Compared with naive group, OVA-specific IgE in OVA-sensitized groups, including phosphate-buffered saline (PBS)-, SECneg-, SEC4L-, SEC4M- and SEC4H-treated groups, was significantly increased (Figure 2). However, OVA-specific IgE levels were not significantly different among the SEC4-, SECneg- and

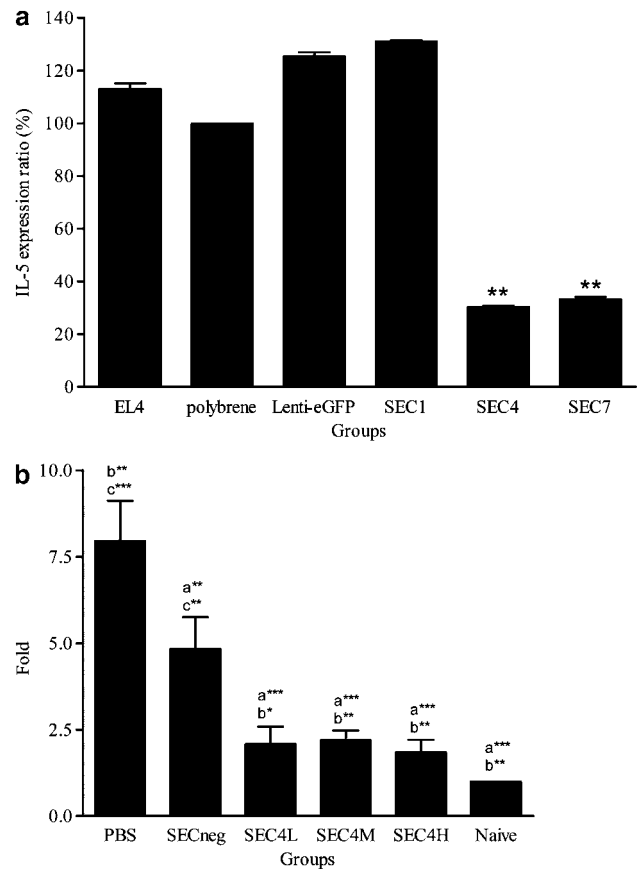


Figure 1 The suppression effect of IL-5 siRNAs *in vitro* and *in vivo*. (a) EL4 cells were infected with siRNA-expressing or control lentivirus for 72 h and then stimulated with PMA and cAMP for 24 h. IL-5 concentrations in the supernatant were analyzed by ELISA. IL-5 expression of the control group (polybrene only) was taken as 100%. Results were expressed as mean $\pm$ s.e.m. $**P < 0.001$ compared with the IL-5 production of EL4 cells infected with eGFP-expressing control virus. (b) SEC4 inhibited IL-5 mRNA level of lungs. After BAL fluid was obtained, the partial lungs of mice were cut, froze in liquid nitrogen immediately and stored at -80°C . The IL-5 mRNA level was analyzed by quantitative real-time PCR ($n = 4-6$ per group). IL-5 mRNA level of non-sensitized mice (naive) was as 1 and those of other groups mice were relatively compared to non-sensitized mice. The results were expressed as mean $\pm$ s.e.m. $a**P < 0.01$, $a***P < 0.001$ compared with PBS group. $b*P < 0.05$, $b**P < 0.01$ compared with SECneg group. $c**P < 0.01$, $c***P < 0.001$ compared with naive group. IL-5, interleukin-5; siRNA, small interfering RNA; cAMP, cyclic AMP; ELISA, enzyme-linked immunosorbent assay; eGFP, enhanced green fluorescence protein; SEC, siRNA-expressing cassette; BAL, bronchoalveolar lavage; PBS, phosphate-buffered saline.

PBS-treated mice (Figure 2). The OVA-specific IgG2a and IgG1, Th2 and Th1 response-related immunoglobulins, were also not different among those groups of mice.

To determine the effect of IL-5 siRNA in airway function, the mice were exposed to methacholine (MCh) aerosols to measure the AHR. Airway responsiveness was assessed by non-invasive whole-body plethysmography. As shown in Figure 3a, relative enhanced pause (Penh) values of mice treated with three different doses of SEC4 were significantly decreased compared to the PBS group and that of mice treated with medium and high doses of SEC4 were significantly reduced compared

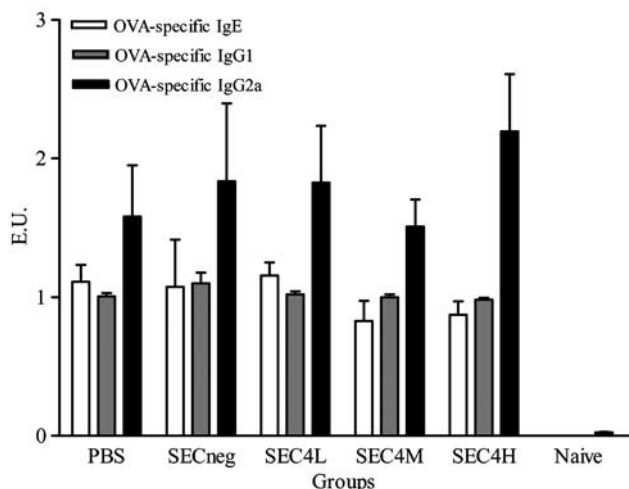


Figure 2 Serum OVA-specific IgE, IgG1 and IgG2a levels had no differences among the different dosages of SEC4-treated mice. On day 44, blood samples were obtained and OVA-specific IgE, IgG1, IgG2a concentrations were measured by ELISA ($n = 7-8$ per group). SEC, siRNA-expressing cassette; ELISA, enzyme-linked immunosorbent assay.

to mice treated with control siRNA (SECneg) when mice were exposed to 50 mg ml⁻¹ MCh.

We also examined airway function using invasive technique in anesthetized mice to confirm the decrease of airway resistance in SEC4-treated mice. Intratracheal administration of PBS and SECneg led to significant increase in lung resistance (RL) to inhaled MCh when compared to mice that were not sensitized but challenged with OVA on days 41–42. Administration of SEC4-containing lentiviruses resulted in significant decreases in RL to inhaled MCh when compared with administration of PBS or SECneg (Figure 3b). As shown in Figure 3b, administration of three different doses of SEC4-containing lentiviruses resulted in similar decrease of RL levels.

We likewise examined the influx of inflammatory cells into the bronchoalveolar lavage (BAL) 48 h after the last OVA challenge. In BAL fluid of PBS- or SECneg-treated mice, eosinophil and neutrophil infiltration significantly increased compared to that of naive mice (Figure 4). Administration of SEC4-containing lentiviruses could efficiently inhibit eosinophil infiltration into the lungs (Figure 4).

Histopathologically, inflammatory cells infiltrated around airways were noted in PBS and SECneg groups. However, inflammatory cell infiltrates were less in SEC4-treated mice (Figure 5).

SEC4 suppresses eosinophil chemoattractive chemokine, eotaxin, in BAL fluid

Eotaxin is an important chemokine for recruitment of eosinophils, basophils and Th2 lymphocytes in the lungs; therefore, we evaluated the eotaxin levels in the BAL fluid. Results showed that different doses of SEC4 treatments could significantly suppress eotaxin production to 37–50% compared to that of control siRNA-expressing virus (SECneg) (Figure 6).

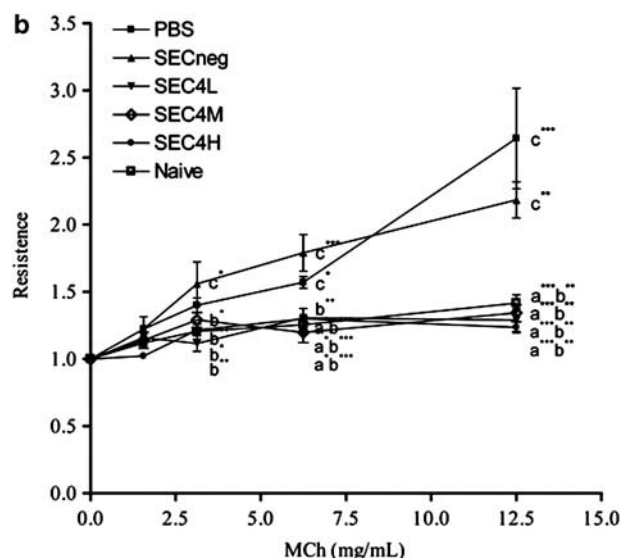
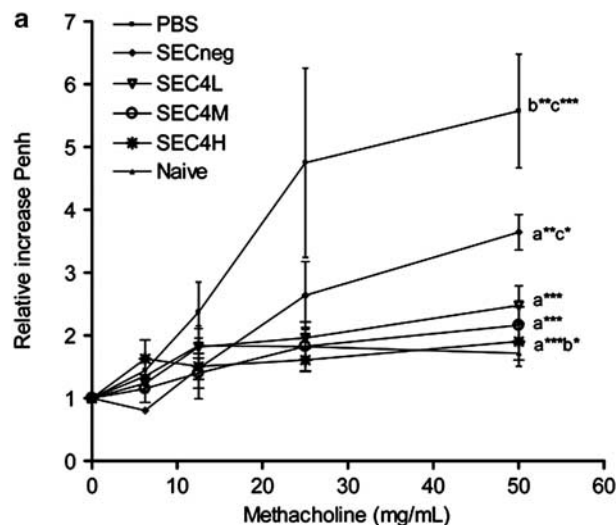


Figure 3 Local SEC4-containing lentivirus delivery reduced AHR. Airway function was measured by non-invasive whole-body plethysmography (a) or invasive body plethysmography (b). Results were expressed for each MCh concentration as the percentage of the baseline Penh value after normal saline exposure (a) or as mean $\pm$ s.e.m. of the RL in the ratio of RL after PBS nebulization (b) of three independent experiments ($n \geq 5$). The results were expressed as mean $\pm$ s.e.m. $a^*P < 0.05$, $a^{**}P < 0.01$, $a^{***}P < 0.001$ compared with the value of PBS group. $b^*P < 0.05$, $b^{**}P < 0.01$, $b^{***}P < 0.001$ compared with the value of SECneg group. $c^*P < 0.05$, $c^{**}P < 0.01$, $c^{***}P < 0.001$ compared with the value of naive group. SEC, siRNA-expressing cassette; AHR, airway hyper-responsiveness; MCh, methacholine; RL, lung resistance; PBS, phosphate-buffered saline; SECneg, control SEC.

Discussion

Th2 cytokines, such as IL-4, IL-5 and IL-13, are important in the development of allergic asthma. Among them, IL-5 plays an important role in the development, recruitment, activation and survival of eosinophils.^{5,6,23} The decreased number of eosinophils in the airway lumen of SEC4-treated mice may be caused by the suppression of differentiation and recruitment of eosinophils from the bone marrow, or the influence on survival of eosinophils

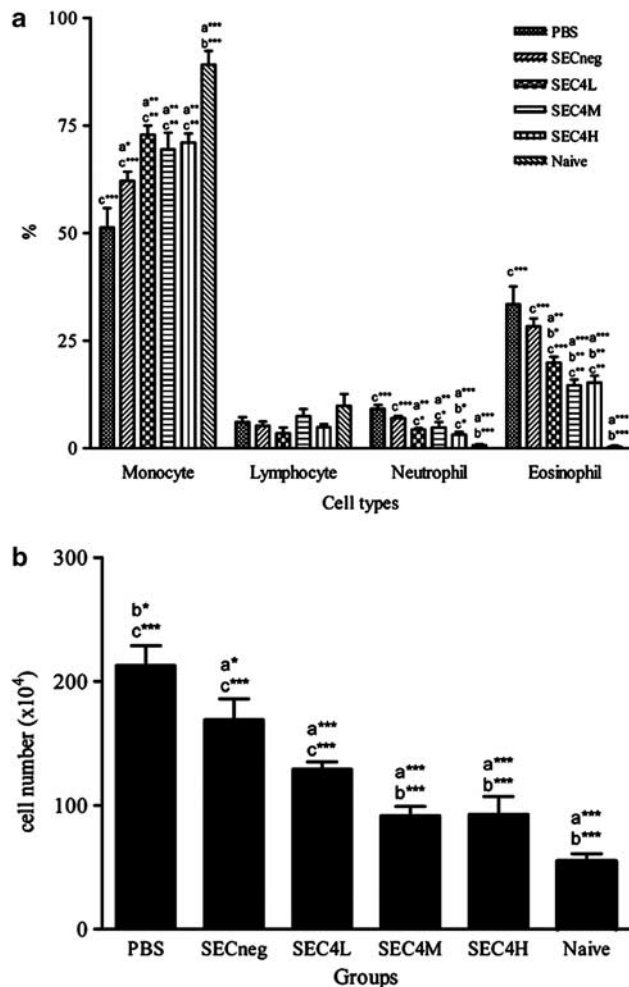


Figure 4 Local SEC4-containing lentivirus delivery moderated airway eosinophilia. BAL fluid was obtained and the cell population was assayed as previously described ($n=5-7$ per group). (a) The cell composition of BAL fluid. (b) Total cell number of BAL fluid. The results were expressed as mean $\pm$ s.e.m. $a^*P<0.05$, $a^{**}P<0.01$, $a^{***}P<0.001$ compared with the value of PBS group. $b^*P<0.05$, $b^{**}P<0.01$, $b^{***}P<0.001$ compared with the value of SECneg group. $c^*P<0.05$, $c^{**}P<0.01$, $c^{***}P<0.001$ compared with the value of naive group. SEC, siRNA-expressing cassette; BAL, bronchoalveolar lavage; SECneg, control SEC.

in local tissues. However, the number of airway eosinophils in SEC4-treated mice reduced only about 45% compared to that of SECneg-treated mice. This implies that other cytokines, such as IL-3²⁴ and granulocyte-macrophage colony-stimulating factor,²⁵ might also be important in eosinophil survival and persistence in tissues.

Allergic asthmatic responses can be divided into early and late phases. Cross-linking of allergic specific IgE bound to the surface of mast cells by allergen, degranulation of these cells and mediator release, all of which are responsible for the early allergic response. In our study, circulating IgE levels were not different between SEC4-treated mice and control mice (Figure 2), but other late allergic responses were significantly improved. Although the early-phase response was induced in SEC4-treated mice, the subsequent recruitment of inflammatory cells,

Th2 lymphocytes and eosinophils might play the major roles in late-phase response.

According to our results, we proposed that the SEC4-containing lentivirus efficiently suppressed local expression of IL-5 and then further reduced the differentiation, maturation of eosinophils in the bone marrow, and recruitment and activation of eosinophils in the airway. In the study, we did not observe decreased OVA-specific IgE level in SEC-treated mice. It is possible that high IgE level in SEC-treated mice was the time point of OVA-specific antibody assay, only 7 days after SEC treatment. Another reason for the high IgE level was the local single-dose delivery of SEC4. In the future, systemic or multiple-dose delivery of SEC4 should be performed to test this hypothesis. Eosinophil is also a source of eotaxin. Less eosinophil infiltration and activation of eosinophils in the lungs of SEC4-treated mice resulted reduction of eotaxin level in BAL fluid.

The improvement of the asthmatic syndrome of mice treated with different dosages of SEC4 did not show any dose-dependent effect. Administration of low dose of SEC4 (3×10^5 infectious unit (IFU) per mice) might already have achieved the maximum suppression *in vivo*, so the delivery of more SEC4 did not show more efficient inhibitory effect.

The contribution of IL-5 on AHR is controversial. Some studies on murine models of experimental asthma revealed that IL-5^{-/-} or blockage of IL-5 by anti-IL-5 antibodies prevents AHR and airway inflammation characterized by an influx of Th2 cells and eosinophils,^{15,26} while others not.²⁷ Clinical trials have demonstrated that anti-IL-5 therapy results in a rapid decrease in peripheral blood eosinophil numbers. Moreover, anti-IL-5 therapy results in improvement of symptoms in patients with lymphocytic variants of hypereosinophilic syndromes, in eosinophilic esophagitis²⁸ and chronic rhinitis with nasal polyposis.²⁹ In contrast, in patients with bronchial asthma or atopic eczema, anti-IL-5 therapy showed only moderate or no clinical effects. Studies on asthmatic patients showed dramatic suppression of circulating eosinophils and reduction of sputum eosinophilia after allergen challenge following a single intravenous administration of mepolizumab, an anti-IL-5 antibody, but have no significant effects on the late asthmatic response or on AHR induced by histamine.¹⁷⁻¹⁹

These results suggested that anti-IL-5 treatment had local effects but did not affect Th1/Th2 lymphocyte function and cytokine secretion in peripheral blood.¹⁹ In this study, we applied siRNA as a therapeutic strategy to suppress the IL-5 expression locally and successfully reduced the inflammation, infiltration of eosinophils and hyper-responsiveness in airways but not serum levels of OVA-specific antibodies. The local effects of IL-5 might explain why the levels of OVA-specific IgE, IgG1 and IgG2a had no significant differences among the different groups in this study.

Karras *et al.*³⁰ treated the OVA-sensitized BALB/c mice with an intravenous IL-5 antisense oligonucleotide daily for 14 days during sensitization period and they found that IL-5 antisense oligonucleotide could inhibit antigen-induced eosinophilia and late-phase AHR (from 2 to 9 h after challenge). Our siRNA, expressed by lentiviral system, could avoid the oligonucleotide degradation *in vivo*, so we only intratracheally delivered

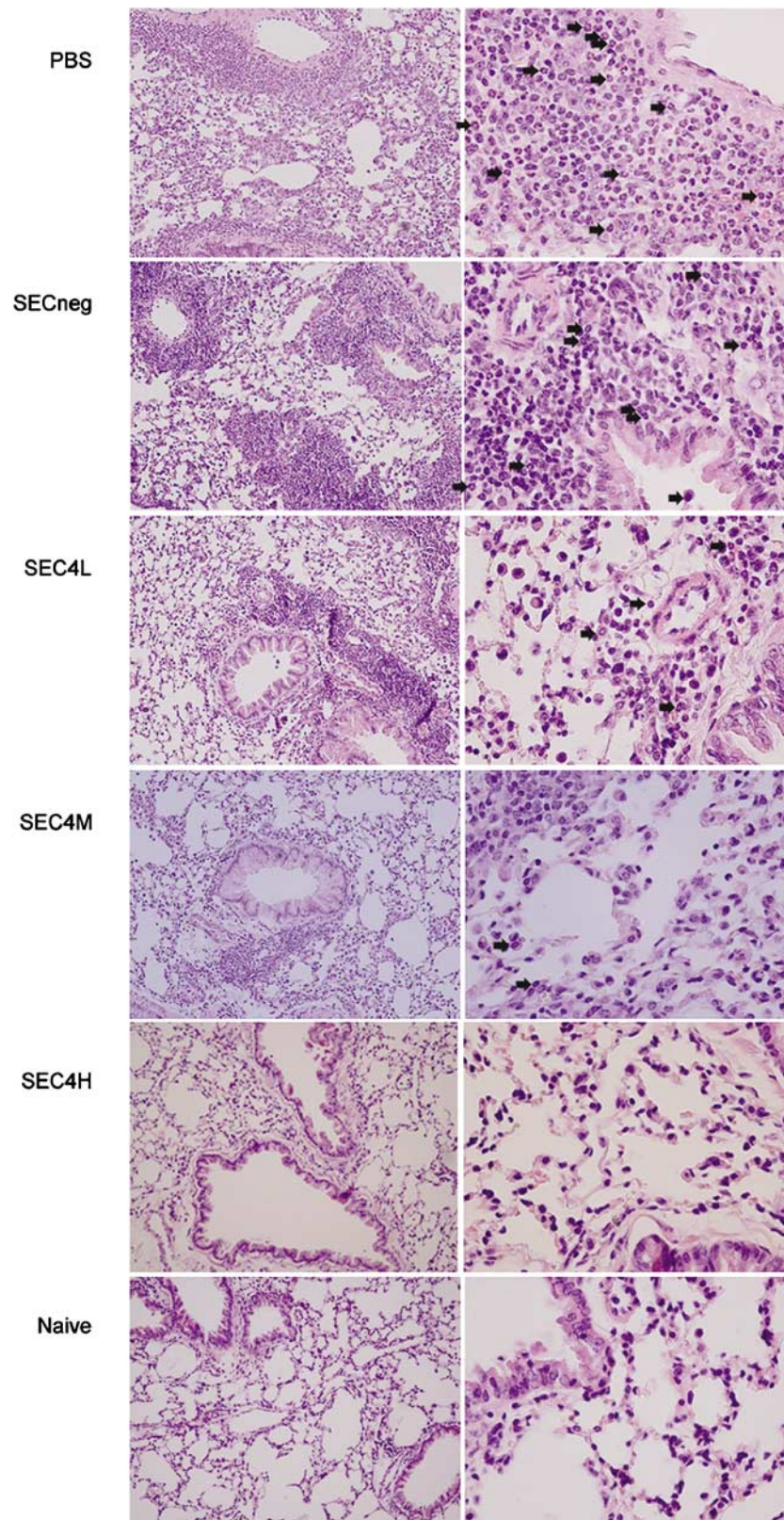


Figure 5 Histological examination of lung sections. On day 44, lungs of different groups of mice were excised after BAL fluid collection and fixed with 10% buffered formalin. Sections (5 μ m thick) were prepared, subjected to H&E staining (black arrow, eosinophil; magnification: left: $\times 100$, right: $\times 400$). BAL, bronchoalveolar lavage; H&E, hematoxylin and eosin stain.

SEC once before OVA challenge and eosinophil infiltration and AHR were efficiently reduced at 24 h after challenge.

One potential problem of RNA interference-based drugs is induction of interferon (IFN) response, which causes nonspecific inhibition of gene expression.

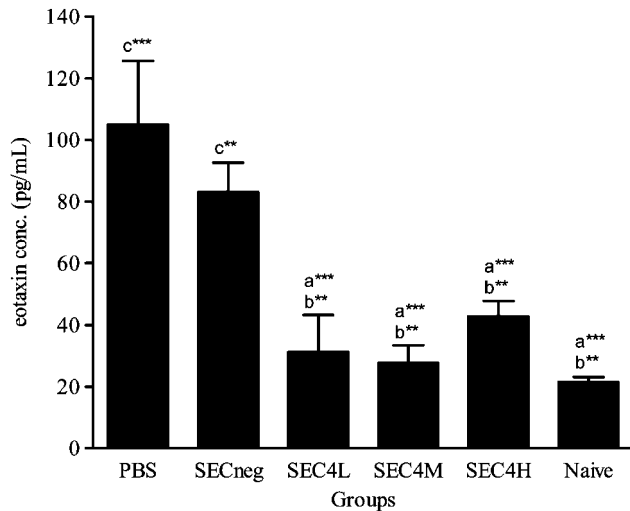


Figure 6 The eotaxin level in the BAL fluid decreased in SEC4-treated mice. Eotaxin level in the BAL fluid was measured by ELISA ($n=5-7$ per group). The results were expressed as mean $\pm$ s.e.m. a*** $P<0.001$ compared with PBS group. b** $P<0.01$ compared with SECneg group. c** $P<0.01$, c*** $P<0.001$ compared with naive group. BAL, bronchoalveolar lavage; SEC, siRNA-expressing cassette; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; SECneg, control SEC.

Previous studies have also shown that lentiviral-mediated delivery of siRNA could induce a subset of IFN-stimulated genes.³¹ Our results showed that SECneg-treated mice showed some reduction in AHR and IL-5 mRNA in the lungs (Figures 1b and 3) compared to PBS-treated mice. To clarify whether the SEC-containing lentiviruses induce side effects of IFN response, we assayed the IFN- γ level in BAL fluid. The levels of IFN- γ were not different among PBS-treated, SEC-treated and naive mice (Supplementary Figure 1). In our previous results, lentiviruses used to deliver siRNA did not increase the expression of IFN response genes, including 2'5'-oligoadenylate synthetase (OAS-1) and myxovirus-resistance protein A, in lungs of lentivirus-treated mice compared with naive mice.³² Therefore, we exclude the possibility that IFN response induced by lentivirus reduced AHR. Previous studies also demonstrated that siRNA would cause nonspecific gene regulation by partial homology.^{33,34} However, it was not clear whether unanticipated off-target effects occurred in our study. More studies are needed in understanding the mechanisms of nonspecific off-target effects.

In summary, we have demonstrated that a single treatment of SEC4-containing lentivirus against IL-5 in an OVA-induced asthma murine model can efficiently reduce IL-5 mRNA level in the airway, late-phase AHR, eosinophil chemoattractive cytokine, eotaxin in the BAL fluid and eosinophil infiltration.

Materials and methods

Construction of SEC-IL5

The SECs targeting mouse IL-5 gene were synthesized by a siRNA expression cassette kit (Ambion, Houston, TX, USA). The SEC1, SEC4 and SEC7 targeting cDNA sequences were 5'-AAGAATCAAAGTGTCCGTGGG-3',

5'-AAGAAATTCCTGTAGCGCAGG-3' and 5'-AATCA GACTGTGCCATGACTG-3', respectively. The control siRNA sequence of the mock virus (SECneg) was 5'-GTCAGACTGTGCCATGACTG-3'. The control siRNA was provided by the siRNA expression cassette kit, which lacked homology to any mouse gene. The SECs were digested with *EcoRI* and *HindIII* and ligated into the pSEC hygro vector (Ambion). The SECs were then changed into pTY-linker vector for the lentivirus-delivering system.

Preparation of SEC-expressing lentiviruses

Plasmids for producing lentivirus were provided by Dr Lih-Hwa Hwang (Graduate Institute of Microbiology, National Taiwan University, Taiwan). 293T cells were seeded in 10 cm dish the day before transfection of the plasmids. The lentivirus vectors, containing the SEC or enhanced green fluorescence protein with three other plasmids for virus generation, pHP-dl.Nde/Ase, pCEP4-tat and pHEF-VSV-G, were mixed at ratios of 3, 3, 2 and 1, respectively, and co-transfected into 293T cells. After 18–24 h, fresh complete medium was changed. Forty-eight hours after transfection, lentivirus particles were produced into the supernatant. Filtrated lentivirus-containing supernatant was concentrated with Centricon 100 kDa centrifuge tube and stored at -80°C .

Determination of cytokine expression

IL-5, eotaxin and INF- γ levels were assayed by ELISA according to the manufacturer's recommended protocols (R&D, Minneapolis, MN, USA).

Determination of suppression efficiency of SECs in vitro

Mouse thymoma cell line (EL4 cells) was transduced with three SEC-containing lentiviruses or enhanced green fluorescence protein-expressing lentiviruses (multiplicity of infection = 12.5) for 72 h. Then, infected EL4 cells ($4 \times 10^5 \text{ ml}^{-1}$) were treated with PMA (5 ng ml^{-1}) and cAMP ($500 \mu\text{M}$) for 24 h. IL-5 levels in the supernatants were measured by ELISA.

OVA-induced allergic airway inflammation

Six- to eight-week-old BALB/c mice were obtained from the Animal Center of the College of Medicine, National Taiwan University, and sensitized by an intraperitoneal injection of $20 \mu\text{g}$ of OVA emulsified in 2 mg of aluminum hydroxide in a total volume of $200 \mu\text{l}$ PBS on day 0, and boosted with $50 \mu\text{g}$ of OVA emulsified in $2 \mu\text{g}$ of aluminum hydroxide on days 14 and 28. On day 37, different dosage of lentivirus (low: 3×10^5 IFU, SEC4L; medium: 1.5×10^6 IFU, SEC4M; high: 3×10^6 IFU, SEC4H; and SECneg: 3×10^6 IFU) was administered intratracheally into the anesthetized animals, and positive control mice were administered PBS. Naive group was not sensitized with OVA and administered with nothing.

For challenge, all mice were treated intranasally with OVA ($100 \mu\text{g}$ in a total volume of $40 \mu\text{l}$ PBS) on days 40, 41 and 42. Twenty-four hours after last OVA challenge, AHR was assessed. On day 44, sera and BAL fluid were collected. The partial lungs were cut and fixed with 10% neutralized buffered formalin. Sections ($5 \mu\text{m}$ thick) were prepared, subjected to H&E staining and examined by

light microscopy. Another part of lungs was frozen in liquid nitrogen immediately and then stored at -80°C for RNA extraction.

Determination of the airway function

Airway function was measured by the whole-body plethysmography and the RL. The whole-body plethysmography was measured with the Penh system (Penh = pause $\times$ (peak expiratory box flow/peak inspiratory box flow)) as described previously.^{35,36} Briefly, mice were placed in the main chamber of a whole-body plethysmography and challenged with aerosolized normal saline, and later challenged with increasing doses of MCh (6.25–50 mg ml⁻¹; Sigma, St Louis, MO, USA). Results were expressed for each concentration of MCh as a percentage of the baseline Penh value after exposure to normal saline.

We also measured changes in RL in response to increasing doses of aerosolized MCh in anesthetized mice using a modification of the techniques described by Glaab *et al.*³⁷ Mice were anesthetized with 70–90 mg kg⁻¹ pentobarbital sodium (Sigma), tracheostomized and mechanically ventilated at a rate of 150 breaths per min, a tidal volume of 0.3 ml and a positive end-expiratory pressure of 3–4 cmH₂O with a computer-controlled small animal ventilator (Harvard Rodent Ventilator, model 683; Harvard Bio-Science, Southnatick, MA, USA). PE-50 tubing was inserted into the esophagus to the level of the thorax, coupled with a pressure transducer (LDS GOULD, Valley View, OH, USA). Flow was measured by electronic differentiation of volume signal. Pressure, flow and volume change were recorded. Pulmonary resistance was calculated by the software program (Model PNM-PCT100W, LDS PONEMAH Physiology Platform; LDS GOULD). MCh aerosol was generated with an in-line nebulizer and administered directly through the ventilator. The resistance of the orotracheal tube (0.45 cmH₂O s ml⁻¹) was subtracted from all airway resistance measurements. Data were expressed as RL in the ratio of RL after PBS nebulization of three independent experiments.

OVA-specific antibody assay

Sera anti-OVA IgE, IgG1 and IgG2a antibody titers were determined by ELISA. Briefly, 96-well microtiter plates were coated with 1 μg per well OVA in NaHCO₃ buffer (pH 9.6). After overnight incubation at 4°C , plates were washed and blocked with 3% bovine serum albumin in PBS for 2 h at room temperature. Serum samples were diluted and added to each well overnight at 4°C . Plates were then washed. Biotin-conjugated anti-mouse IgE or IgG1 or IgG2a (0.5 mg ml⁻¹; Pharmingen, San Diego, CA, USA), diluted in 3% bovine serum albumin–PBS buffer (1:500), was added for 45 min at room temperature. Avidin-conjugated horseradish peroxidase (1:5000; Pierce Biotechnology, Rockford, IL, USA) was then added for an additional 30 min at room temperature. Finally, the reaction was developed by peroxidase substrate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (0.5 mg ml⁻¹), and the absorbance determined at 420 nm in a microplate reader. The levels of antibody were compared to standard serum and the concentration of IgG1, IgE and IgG2a in standard serum was arbitrarily assigned 1 ELISA unit (1 EU).

BAL fluid

Bronchoalveolar lavage fluid was performed by cannulating the trachea of each mouse and washing the airways with 1 ml Hank's balanced salt solution. The BAL fluid was centrifuged at 1500 r.p.m. for 10 min at 4°C . The supernatant was stored at -20°C to determine the production of eotaxin and INF- γ by ELISA. The pellets of cells were resuspended with secondary collected BAL fluid, washed with Hank's buffered salt solution and resuspended in 1 ml Hank's buffered salt solution. Appropriate cells of BAL fluid (about 2×10^4) were cytopspined and stained with Liu's stain. Based on morphology, a minimum of 200 cells were counted and classified as macrophages, lymphocytes, neutrophils or eosinophils to analyze the inflammatory cell population in BAL fluid.

Real-time quantitative PCR

The lungs of mice were homogenized by MagNA Lyser Green Beads (Roche, Mannheim, Germany) with 1 ml TRIzol reagent (Invitrogen, Gaithersburg, MD, USA) and the total RNA was extracted according to the manufacturer's instructions. The total RNA was treated with rDNase I (Ambion) for 30 min. cDNA synthesis was performed with random hexamer primers and SuperScript II RNase H⁻ reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed in an ABI PRISM 7700 cycler (Applied Biosystems, Foster City, CA, USA) using the murine IL-5 TaqMan gene expression assays (Applied Biosystems). All reported mRNA levels were normalized to the hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA level.

Statistical analysis

Differences between experimental groups were assessed by one-way analysis of variance followed by the Newman–Keuls multiple comparison test. Significance was assumed at a *P*-value less than 0.05. Values for all measurements are expressed as means $\pm$ s.e.m.

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