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Flt3-Ligand Plasmid Prevents the Development of Pathophysiological Features of Chronic Asthma in a Mouse Model

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Abstract

Airway inflammation and remodeling are primary characteristics of long-standing asthma. A balance between the T_H1/T_H2 cytokines regulates the accumulation and activation of inflammatory cells, including mast cells and eosinophils. Recently, we demonstrated that pUMVC3-hFLex, an active plasmid, mammalian expression vector for the secretion of Flt3-L, reversed established airway hyperresponsiveness (AHR) in a murine model of acute allergic airway inflammation. The present experiments were undertaken to examine the effect of pUMVC3-hFLex in a chronic model of allergic airway inflammation that was established in Balb/c mice by sensitization and challenge with ovalbumin (OVA). pUMVC3-hFLex or the control plasmid, pUMVC3, were administered by injection into the muscle interior tibialis. Treatment with pUMVC3-hFLex completely reversed established AHR ($p< 0.05$), and this effect continued even after several exposures to the allergen ($p< 0.05$). pUMVC3-hFLex treatment prevented the development of goblet cell hyperplasia and subepithelial fibrosis, and significantly reduced serum levels of IL-4 and IL-5, and increased serum IL-10 levels ($p <0.05$) with no effect on serum IL-13. Serum IgE or serum total and anti-OVA IgG1 and IgG2a levels did not change. Total BALF cellularity and BALF IL-5 levels were reduced ($p < 0.05$), but there was no significant effect on BALF IL-10 and IL-13. These results suggest that pUMVC3-hFLex treatment can prevent the development of airway remodeling and maintain airway protection in chronic experimental asthma model, and might provide a novel approach for treating chronic asthma.

Key Words

Allergy
Asthma
Flt3-L
Mouse model of asthma
pUMVC3-hFLex plasmid
T_H1/T_H2 cells

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Introduction

Asthma is characterized by chronic inflammation of the bronchial walls along with infiltration of inflammatory cells including eosinophils, mast cells, and lymphocytes. Some asthma patients develop irreversible airflow obstruction *(1)*. This process has been termed airway remodeling and represents an injury-repair process of the airway tissue. The components of airway remodeling include airway epithelial injury, airway smooth muscle hypertrophy, mucus gland and goblet cell hyperplasia, angiogenesis, and collagen deposition in the airway. Airway remodeling and inflammation have been proposed to be major characteristics of asthma.

We do not exactly understand the association between airway remodeling, inflammation, and non-specific airway hyperresponsiveness (AHR), and their relationship to accelerated decline in lung function in patients with asthma. Inhaled corticosteroids are now a first-line treatment for chronic asthma *(2,3)*. On the other hand, glucocorticoids, particularly at high doses, have significant and severe adverse effects. Furthermore, some patients with severe asthma exhibit poor response to treatment with glucocorticoids and might be glucocorticoid-resistant *(4,5)*. Hence, there is a pressing need to develop new therapeutics for chronic asthma to control the underlying inflammatory and destructive processes.

Recently, we reported that treatment with Flt3-L could prevent the development of *(6)* and reverse *(7)* asthma-like conditions in a mouse model, resulting in complete abolition of AHR to methacholine. We have also shown that pUMVC3-hFLex plasmid has a similar effect. In this study, we examined the effect of pUMVC3-hFLex on the regulation of AHR and airway remodeling in a murine chronic allergic airway inflammation model.

Materials and Methods

Animals

Pathogen-free 4- to 5-wk-old female BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN) and were housed in separate cages. Food and water were provided *ad libitum*. Experiments were carried out in accordance with the National Institutes of Health guidelines. The research protocol of this study was approved by the Institutional Animal Care and Use Committee of Creighton University, Omaha, NE.

Plasmid DNA

The pUMVC3-hFLex plasmid contains the extracellular domain (secreted form) of the human Flt3-L gene. This vector as well as the control plasmid, pUMVC3, were obtained from the Vector Core Laboratory at the University of Michigan (Ann Arbor, MI, USA) and has been described previously *(8)*.

Sensitization and Treatment

Mice were sensitized by intraperitoneal injection of ovalbumin (OVA; 20 µg, Sigma-Aldrich, St Louis, MO) emulsified in 2.25 mg imject alum (Pierce, Rockford, IL) in a total volume of 100 µL. Control animals received alum only. Mice were challenged for 20 min on d 28, 29, 30, and 32 by aerosol nebulization with OVA (1% in PBS) using an UltraNeb 90 nebulizer (De Villbiss, Somerset, PA). The plasmid (200 µg in PBS) either active, pUMVC3-hFLex, or control, pUMVC3, or the vehicle were administered by injection into the muscle interior tibialis three times on d 33, 38, and 41. On d 44, mice were challenged with 1% aerosolized OVA. On d 58, mice were challenged with 1% OVA aerosol. On d 66, mice received a booster OVA ip injection. On d 79 mice were challenged with 1% OVA. Mice were sacrificed on d 80. Non-

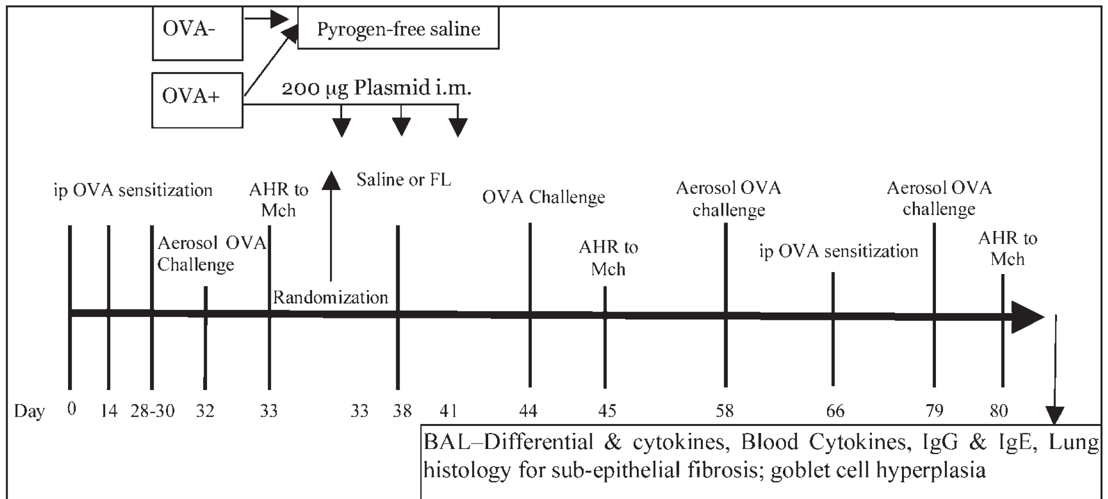

Fig. 1. OVA murine model of asthma and immunotherapy protocol. Balb/c mice were sensitized to OVA by ip injection and subsequently challenged with OVA by aerosol on d 28, 29, and 30. Starting on d 33, OVA-sensitized mice were randomized into three groups: two of the groups received injection into the muscle interior tibialis 200 µg pUMVC3-hFLex or pUMVC3 treatment three times on d 33, 38, and 41, while the OVA group received PBS only. On d 44, mice were challenged with 5% aerosolized OVA and 24 h later AHR to methacholine was measured. On d 58, mice were challenged with 1% OVA aerosol. On d 66, mice received a booster OVA ip injection. On d 79 mice were challenged with 1% OVA, 24 h later AHR to methacholine was measured. Mice were sacrificed after the last methacholine challenge on d 80. Non-sensitized control mice were treated only with the vehicle (PBS).

sensitized control mice were treated only with the vehicle (PBS) (Fig. 1).

Noninvasive Method for Measuring AHR

AHR to increasing concentrations of methacholine (3.1–50 mg/mL) was measured on unrestrained conscious mice by using single-chamber whole-body plethysmograph (Buxco Electronics, Troy, NY) 24 h after OVA challenge on d 45 and 80. Absolute enhanced pause values (P_{enh} units) were directly obtained from these analyses. This method has been demonstrated to accurately reflect airway resistance *(9–11)*.

Cytokine Assays

Serum and BALF cytokines were measured by sandwich ELISA with capture and biotinylated detection antibody pairs for IFN-γ, IL-4, IL-5, IL-10, and IL-12 and avidin–horseradish peroxidase and TMB substrate (Pharmingen, San Diego, CA).

Serum IgE Analysis

Blood collected after sacrifice on d 45 was immediately centrifuged and serum was collected and stored at –80°C for later analysis. Enzyme-linked immunosorbent assay (ELISA) for total IgE was conducted as previously described *(12)* and according to the manufacturer's recommendations using rat anti-mouse IgE (BD Pharmingen), standard IgE (BD Pharmingen), and rat anti-mouse IgE-HRP (Southern Biotechnology Associates, Birmingham, AL) for the total IgE assay. Both cytokine and IgE assays were developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (BD Pharmingen), reactions were

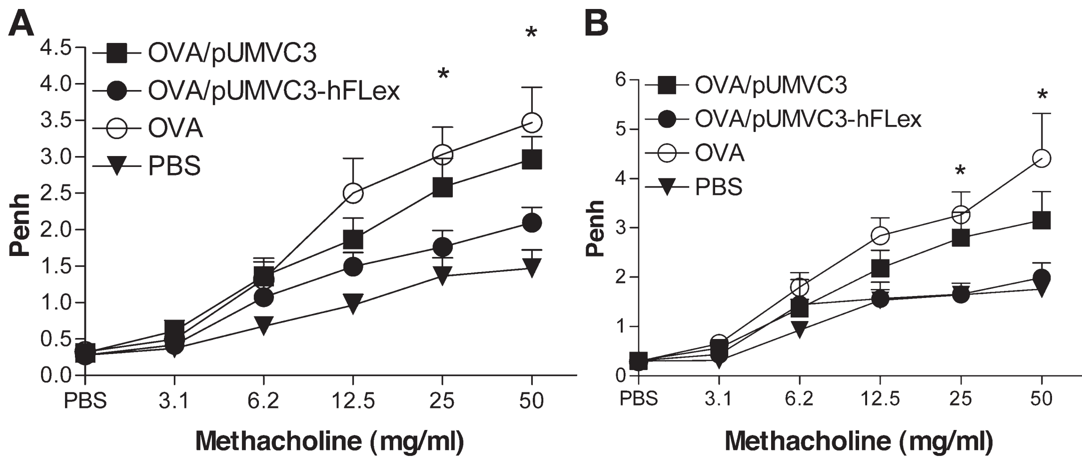

Fig. 2. AHR to methacholine in unrestrained mice. Following OVA sensitization and challenge, AHR to methacholine was established (d 33) followed by treatment with pUMVC3-hFLex or PUMVC3 (200 µg/d, ip) three times. On d 45, AHR to methacholine was again measured and P_{enh} values were recorded. pUMVC3-hFLex treatment abolishes AHR to methacholine in established allergic inflammatory mice. Mice were challenged several times with OVA aerosol. On d 80, AHR to methacholine was again measured and P_{enh} values were recorded. pUMVC3-hFLex treatment abolished AHR to methacholine, and this effect lasted even after repeated exposure to allergen. Data are shown as mean + SEM (n=10 in each group). *p <0.05 compared with OVA group.

stopped with 2 N H_2SO_4, and read at 450 nm using a Bio-Rad microplate reader and software (Bio-Rad, Hercules, CA). Sensitivity for total IgE was 1 ng/mL.

Serum Total and Anti-OVA IgG Isotype Analysis

Total IgG were determined using rat anti-mouse IgG2a or rat anti-mouse IgG1 (BD Pharmingen,), IgG2a or IgG1 standard (BD Pharmingen), and rat anti-mouse IgG2a-HRP or rat anti-mouse IgG1-HRP (BD Pharmingen). Anti-OVA IgGs serum levels were determined as previously described *(13)*. Briefly, microtiter plates were coated with 100 µg/mL chicken egg OVA. The coated plates were washed several times with PBS containing 0.05% Tween (PBS-T) and blocked with 10% FBS for 2 h at room temperature. Diluted serum was incubated in duplicates overnight, washed with PBS-T, incubated with anti-mouse avidin conjugates (IgG1 or IgG2a, BD Pharmingen) for 2 h, and then washed several times with PBS-T. Assays were developed with TMB substrate reagent, reactions were stopped with 2 N H_2SO_4, and read at 450 nm using a Bio-Rad microplate reader and software.

Bronchoalveolar Lavage (BAL) Collection and Lung Histology

Immediately after the last challenge with methacholine, mice were euthanized with a lethal dose of pentobarbital. BALF was collected from each animal via cannulation of the exposed trachea and gentle flushing of the lungs with 1 mL warm PBS, which was centrifuged and the supernatant was collected. The lungs were removed, mounted in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC), and frozen immediately in liquid nitrogen, sectioned, and stained with collagen Masson's trichrome stain (IMEB

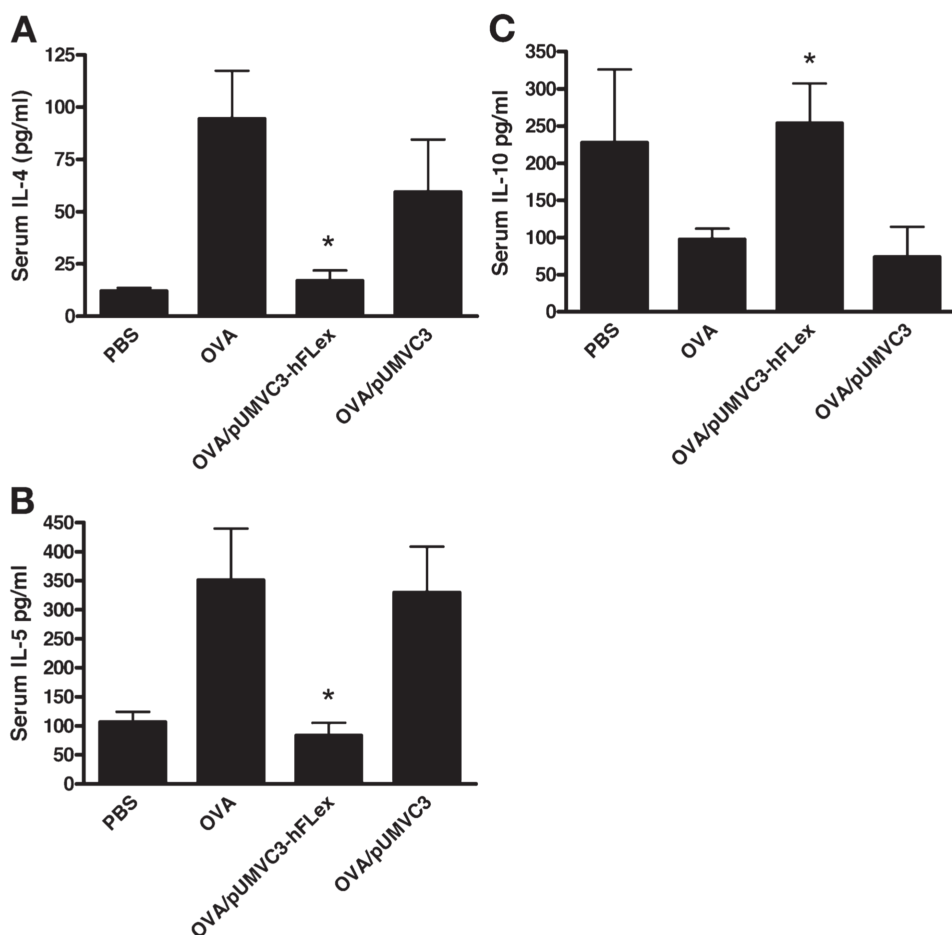

Fig. 3. Effect of pUMVC3-hFLex treatment on serum cytokines. On d 80, after recording pulmonary functions for AHR, blood was collected to measure serum IL-4 **(A),** serum IL-5 **(B),** and serum IL-10 **(C).** Data are shown as mean ±SEM (n=10) (*$p < 0.05$ compared with OVA group). Serum IL-13 was not detected. pUMVC3-hFLex, a plasmid, mammalian expression vector for the secretion of Flt3-L; pUMVC3 the backbone plasmid without the Flt3-L insertion.

Inc., Chicago, IL) Airway mucus was identified by the periodic acid–Schiff (PAS) (Sigma-Aldrich) reaction by using a standard protocol as recommended by manufacturer.

Data Analysis

Data were analyzed using GraphPad PRISM statistical analysis and graphing software. One-way ANOVA Bonferroni's multiple comparison test was used to determine differences between the groups. A p value of <0.05 was considered significant.

Results

Effect of pUMVC3-hFLex Treatment on AHR

We investigated the immunotherapeutic effect of pUMVC3-hFLex on airway responsiveness in mice. Mice were sensitized with ovalbumin/alum and were treated with

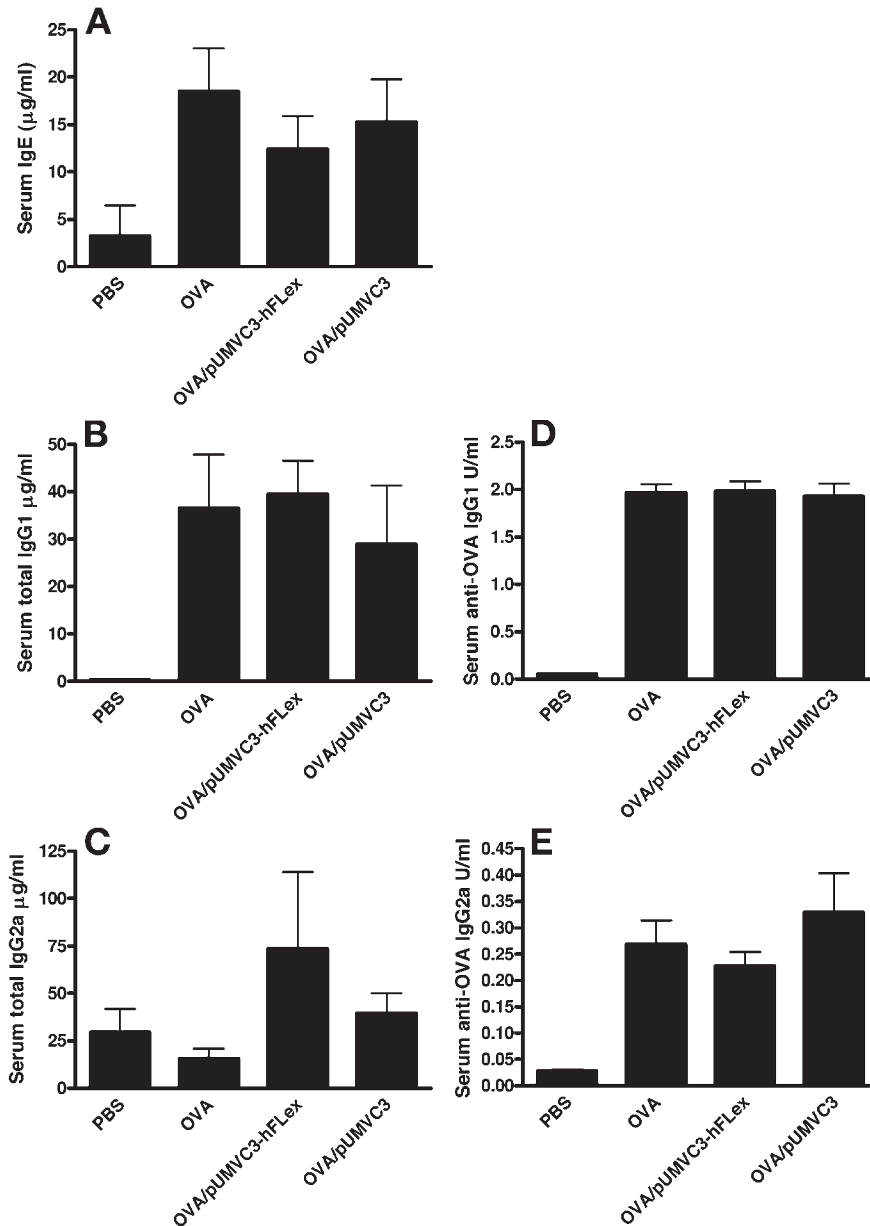

Fig. 4. Effect of pUMVC3-hFLex treatment and Ag sensitization and challenge on the levels of IgG subclasses in mice serum. On d 80, after recording pulmonary functions for AHR, blood was collected to measure IgE **(A),** total IgG1 **(B),** total IgG2a **(C),** anti-OVA IgG1 **(D),** and anti-OVA IgG2a **(E).** Data are shown as mean ± SEM from n=10 animals in each group.

pUMVC3-hFLex, pUMVC3, or PBS. The airway responsiveness to the bronchoconstrictive stimulus methacholine was determined 24 h after OVA challenge on d 45 (Fig. 2A) and d 80 (Fig. 2B). pUMVC3-hFLex immunotherapy significantly suppressed ($p < 0.05$) the airway responsiveness to methacholine compared with OVA-treated mice.

Table 1. Effect of Flt3-L plasmid (FL) Treatment on BALF cells

	Treatment group			
	PBS	OVA	FL	Control
Total cells	48.8±13.9**###	310.6±28.1‡‡	175.0±30.2**‡‡‡	219.0±18.4‡‡‡
Macrophages	38.8±2.2	38.4±7.0	89±5.0***‡‡‡###	30.7±3.5
Eosinophils	9.0±2.0***###	262.8±8.5‡‡‡###	84.0±4.7***###	183.3±4.8***‡‡‡
Neutrophils	0.6±0.4	21.4±0.8	7.0±4.1	2.2±0.9
Lymphocytes	0.4±0.2	2.0±1.2	0.8±0.5	2.2±0.9

BAL Fluid (0.8 mL) was collected from each animal and centrifuged. Recovered total cells were counted (cell/mL × 10^{-3}) and differential analysis was performed using standard morphological criteria on cytospin slides. At least 300 cells were examined in each cytospin slide and absolute cell numbers were calculated per milliliter of the BALF based on the percentage of individual cell in a slide. Shown are means ± SEM for eight animals in each group (**$p< 0.01$; ***$p < 0.001$ compared with OVA group, ###$p < 0.001$ compared with pUMVC3 group, ‡‡$p< 0.01$; ‡‡‡$p< 0.001$ compared with PBS group). pUMVC3-hFLex, an active plasmid, mammalian expression vector for the secretion of Flt3-L; pUMVC3 the backbone plasmid without the Flt3-L insertion.

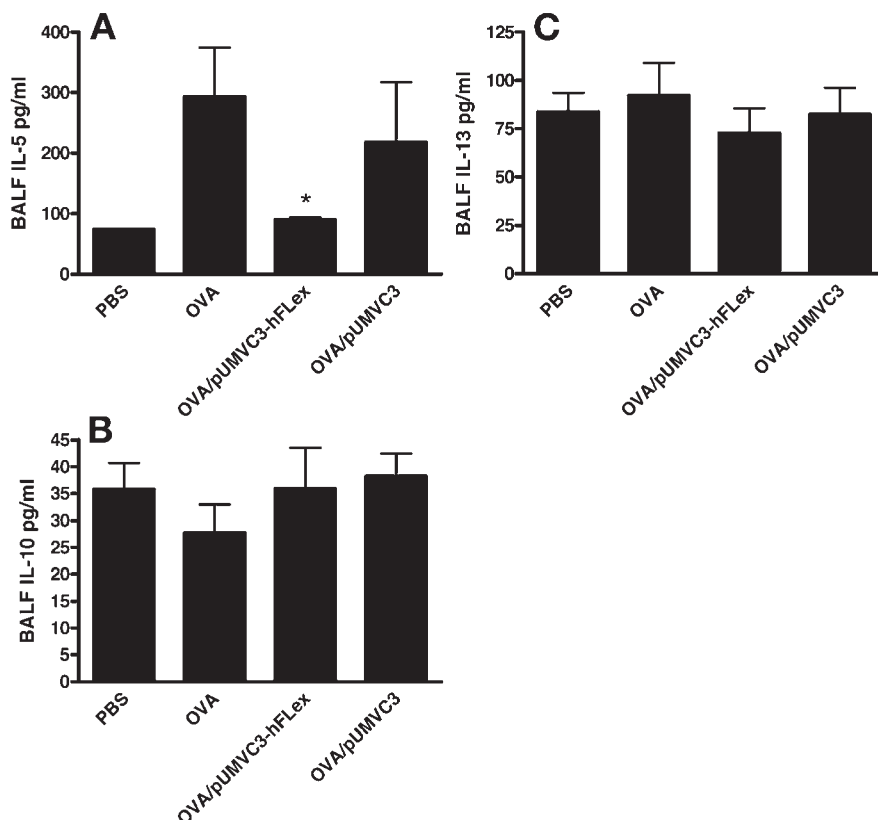

Fig. 5. Effect of pUMVC3-hFLex treatment on BALF cytokines levels in mice. On d 80, after recording pulmonary functions for AHR, BALF samples were immediately centrifuged and cytokines in supernatants were measured: BALF IL-5 **(A),** BALF IL-10 **(B),** and BALF IL-13 **(C).** BALF IL-4 was not detected. Data are shown as mean ±SEM (n=10) (*$p < 0.05$ compared with OVA group).

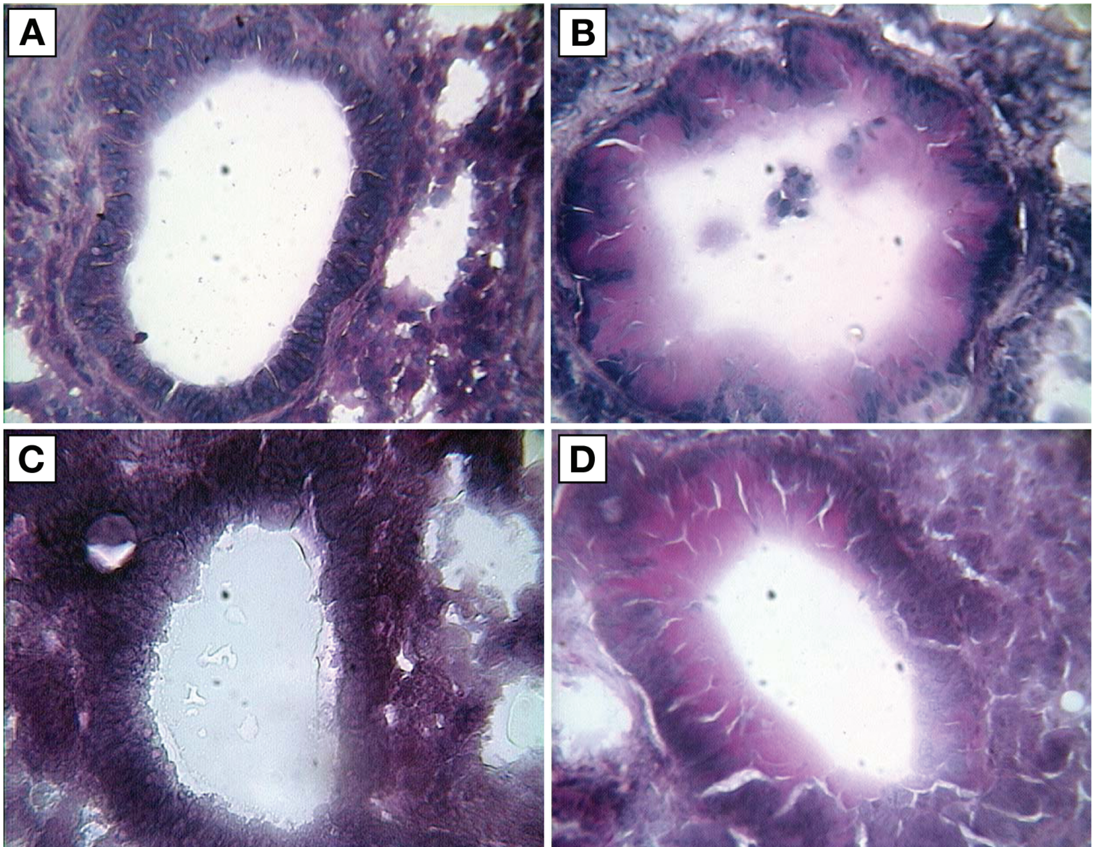

Fig. 6. Effect of pUMVC3-hFLex treatment on goblet cell hyperplasia. Lungs were collected and frozen-mounted in OCT. Sections (8 µm) were cut, stained with PAS, and examined at 40× magnification. This is representative lung histology of PBS-treated mice **(A),** OVA-sensitized and challenged mice **(B),** OVA-sensitized and challenged mice treated with pUMVC3-hFLex **(C),** and OVA-sensitized and challenged mice treated with pUMVC3 **(D).** The mucus substances are stained in magenta by the PAS reaction. In the OVA group, there was a strong staining to PAS, which was significantly reduced by pUMVC3-hFLex treatment, and the histology was comparable to PBS-treated control group.

Interestingly, this effect was maintained even after several exposures to OVA on d 58, d 66, and d 79 (Fig. 1).

Effect of Treatment on Serum Cytokines, Total IgE, and Total and Anti-OVA IgG Subclasses

OVA sensitization significantly increased serum IL-4 and IL-5, and these levels were significantly reduced after treatment with pUMVC3-hFLex (Figs. 3A,B). On the contrary, there was no significant change in serum IL-10 levels after OVA sensitization or treatment with pUMVC3-hFLex or pUMVC3 (Fig. 3C) In addition, OVA sensitization and challenge induced a significant increase in serum total IgE (Fig. 4A), IgG1 (Fig. 4B), IgG2a (Fig. 4C), and anti-OVA IgG1 (Fig. 4D) and anti-OVA IgG2a levels (Fig. 4E). However, treatment with pUMVC3-hFLex or pUMVC3 did not sig-

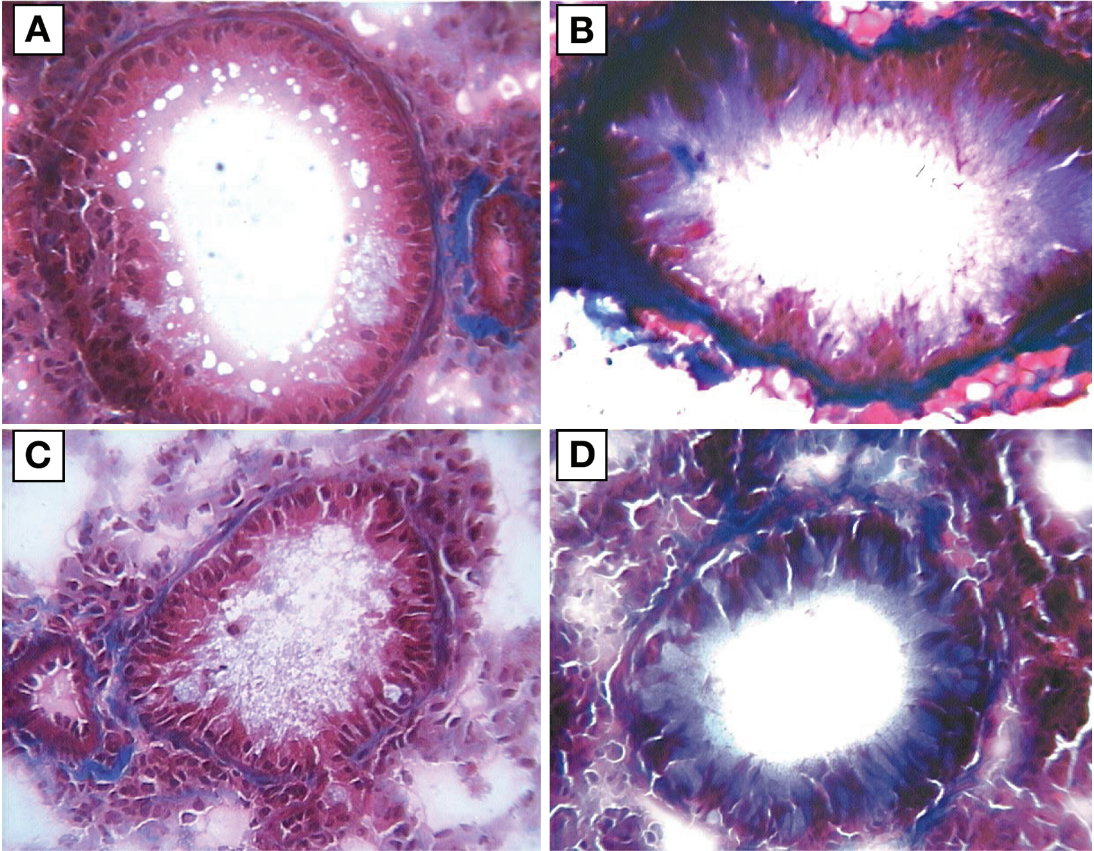

Fig. 7. Effect of pUMVC3-hFLex treatment on collagen matrix deposition. Lungs were collected and frozen-mounted in OCT. Sections (8 µm) were cut, stained with Masson's trichrome, and examined at 40× magnification. This is representative lung histology of PBS-treated mice **(A),** OVA-sensitized and challenged mice **(B),** OVA-sensitized and challenged mice treated with pUMVC3-hFLex **(C),** and OVA-sensitized and challenged mice treated with pUMVC3 control plasmid **(D).** In the OVA group, a massive peribronchial collagen matrix deposition was seen. In contrast, after treatment with pUMVC3-hFLex, collagen matrix deposition was observed and the histology was comparable to PBS-treated group.

nificantly change the levels of the antibody levels.

Effect of pUMVC3-hFLex Treatment on BALF Cytokines

OVA sensitization significantly increased BALF eosinophilia and IL-5 (Table 1; Fig. 5), which was significantly reduced after treatment with pUMVC3-hFLex (Table 1; Fig. 5A). In contrast, there was no significant change in BALF IL-10 and IL-13 levels after OVA sensitization and treatment by pUMVC3-hFLex or by pUMVC3 (Figs. 5B,C).

Effect of Treatment on Goblet Cell Hyperplasia

Excessive mucus secretion is a characteristic feature of the asthmatic airway. In order to determine the extent of mucus cell metaplasia following allergen challenge and treat-

ment, lungs were collected and frozen-fixed in OCT, sections (8 μm) were cut, stained with periodic acid–Schiff stain (PAS). An increase in PAS-positive cells was observed in the bronchial epithelium of OVA-sensitized and challenged mice (Fig. 6B, magenta staining). PAS-positive cells were significantly reduced after treatment with pUMVC3-hFLex (Fig. 6C) to levels comparable to PBS-treated control mice (Fig. 6A). There was no effect of control plasmid on PAS-positive cells (Fig. 6D).

Effect of Treatment on Collagen Matrix Deposition

A prominent feature of airway remodeling observed in the airways of patients with asthma is an increase in collagen deposition in the subepithelial region of the bronchioles. In order to determine the effect of allergen challenge and treatment on matrix deposition, lungs were collected and frozen-fixed in OCT. Sections (8 μm) were cut, stained with Masson's trichrome to show collagen deposition (Fig. 7A, blue represents collagen). PBS-treated control mice showed little collagen staining around the airways. After OVA sensitization and challenge, an increase in collagen matrix was observed in the subepithelial layer of the airways (Fig. 7B). This collagen deposition was not present in pUMVC3-hFLex–treated mice (Fig. 7C). The control plasmid did not decrease the collagen deposition in the airways (Fig. 7D).

Discussion

Recently, we have demonstrated that Flt3-L, administered prior to antigen sensitization, was capable of preventing *(6)* and reversing *(7)* airway inflammation and AHR to methacholine. In this study we present evidence that Flt3-L secreting plasmid is also effective in preventing the features of airway remodeling in a murine model of chronic asthma. Airway remodeling is a result of repeated inhalation of environmental allergens over an extended period of time with repeated airway inflammation. Therefore, it is potentially achievable to prevent structural changes by targeting airway inflammation. In addition, it might be therapeutically effective to target the airway remodeling early in the pathologic processes.

A key characteristic of the airway remodeling in human asthma is an increased thickness of the reticular layer of the basement membrane (subepithelial fibrosis) *(14)*. Subepithelial fibrosis in a murine model has been reported to develop after 6 wk of allergen exposure *(15)*. In our current study we administered plasmid treatment on d 33, 38, and 41 after initial sensitization with antigen. Our results demonstrate that repeated OVA administration induced significant subepithelial collagen deposition and treatment with pUMVC3-hFLex significantly inhibited this fibrosis, and the therapeutic effect of pUMVC3-hFLex on allergic airway remodeling was associated with a significant decrease in AHR. The reduction in collagen deposition is consistent with changes seen in asthmatic human airways *(16,17)*. The induction of subepithelial fibrosis as well as airway inflammation and AHR have been well-documented to be mediated by the overproduction of T_H2 cytokines such as IL-4, IL-5, and IL-13 *(18–20)*.

In murine models, IL-4 plays a crucial role in allergic airway inflammation *(21)* and AHR *(22)*. Increased IL-4 levels have also been observed in murine pulmonary fibrosis model *(23)*. In this model, IL-4–deficient mice exhibited significantly less fibrosis than wild-type controls *(24)*. Several investigators have reported the fibrogenic nature of IL-4, and demonstrated that IL-4 regulates collagen biosynthesis by lung fibroblasts in vitro *(25)*. In our study, levels of IL-4 were dominant in the serum of the OVA-sensitized mice. The intramuscular delivery of pUMVC3-hFLex resulted in a significant decrease in serum IL-4 levels.

Despite promising results in preclinical studies, trials with IL-4 neutralizing agents in patients with asthma have evidently failed to demonstrate benefits for asthmatic subjects and efforts to develop such therapies have stopped, raising questions about the importance of this cytokine in established asthma. However, the significance of this cytokine cannot be ruled out and further studies are warranted.

IL-5 has been reported to play a critical role in allergen-induced subepithelial fibrosis *(26)*. Treatment with anti-IL-5 has been reported to reduce reticulin deposition in a murine model of allergic airway inflammation *(27)*. In addition, anti-fibrotic role for IL-5 has been demonstrated in a recent clinical study using a humanized antibody to IL-5 *(28)*. Moreover, treatment with anti-IL-5 antibody prevented subepithelial and peribronchial fibrosis and eosinophilia *(29)*. In our study, levels of IL-5 in OVA-sensitized mice were also increased. pUMVC3-hFLex significantly inhibited IL-5 levels, suggesting that the ability of pUMVC3-hFLex to reduce IL-5 secretion might be responsible for the reduced recruitment of eosinophils into the lungs.

The therapeutic effect of pUMVC3-hFLex on allergic airway inflammation and pulmonary function was associated with a significant increase in serum IL-10 levels. IL-10 has systemic anti-inflammatory properties, suppressing both T_H1 and T_H2 cytokine responses, and it can induce $CD4^+$ T cells *(30)*, dendritic cells tolerance *(31)*, as well as protection against allergen-induced AHR *(32)*. The role of IL-10 is not completely understood. One plausible explanation is regulatory T cells ($CD25^+CD4^+$, Treg cells), which are suggested to play an important role in maintaining immunological tolerance *(33,34)* by reducing antigen-presenting cell-induced proliferation of $CD4^+$ T cells, suppressing immunoglobulin production, and decreased antigen presentation *(35)*.

Interestingly, levels of BALF IL-13 were not changed. However, this does not rule out IL-13 as a contributor to the pathology of asthma. Transgenic mouse study has shown that lung specific overexpression of IL-13 induced subepithelial fibrosis suggesting a role for IL-13 in the development of airway remodeling *(20)*. However, the pathophysiology of this particular IL-13 model is more similar to that of chronic obstructive pulmonary disease rather than the airway remodeling observed in chronic asthmatics.

Goblet cell hyperplasia in the airway epithelium is a key feature of asthma and contributes to excessive mucus production during asthma exacerbations *(36)*, and is associated with significant asthma mortality and morbidity *(37, 38)*. In our study, we found a significant increase in goblet cell hyperplasia in OVA-sensitized mice, which was inhibited by treatment with pUMVC3-hFLex. Substantial evidence demonstrates the importance of IL-4, IL-5, and IL-13 in goblet cell hyperplasia and mucus production *(39–41)* suggesting that pUMVC3-hFLex most likely inhibit goblet cell hyperplasia and mucus production by down-regulating T_H2-type responses. The decrease in AHR observed in pUMVC3-hFLex treated mice is probably due to the combined result of lowering IL-4 and IL-5 production.

We conclude that although the mechanisms of airway remodeling remain to be fully elucidated, the findings from this study demonstrate that the administration of pUMVC3-hFLex is capable of inhibiting the features of allergic airway inflammation, airway remodeling and AHR, and thus may be of significant benefit in the treatment of chronic asthma.

Acknowledgments

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