

Treatment with Flt3 ligand plasmid reverses allergic airway inflammation in ovalbumin-sensitized and -challenged mice

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Abstract

We have previously reported that fms-like tyrosine kinase 3 ligand (Flt3-L) prevents and reverses established allergic airway inflammation in an ovalbumin (OVA) induced mouse model of asthma. In this study, we investigated the effect of pUMVC3-hFLex, a plasmid, mammalian expression vector for the secretion of Flt3-L on the same mouse model as well as the duration of the effect of the treatment. Allergic airway inflammation to OVA was established in BALB/c mice. OVA-sensitized mice received three intramuscular (i.m.) injections of 200 µg pUMVC3-hFLex over 10 days. The response to pUMVC3-hFLex therapy was assessed based on airway hyperresponsiveness (AHR) to methacholine and inflammation, measured as serum cytokine and immunoglobulins (Ig) levels, and the total and differential cells in bronchoalveolar lavage fluid (BALF). pUMVC3-hFLex treatment completely reversed established AHR ($P<0.01$) and this effect lasted for at least 24 days after the last treatment injection ($P<0.001$). pUMVC3-hFLex treatment significantly increased BALF interferon-gamma (IFN- γ) ($P<0.01$), serum interleukin (IL)-10 ($P<0.01$) and anti-OVA IgG2a levels ($P<0.01$). In contrast, serum IL-4 and IgE levels were significantly reduced ($P<0.05$). Total BALF cellularity, eosinophiles counts and BALF IL-5 levels were also reduced

Abbreviations: Ab, antibody; Ag, antigen; AHR, airway hyperresponsiveness; ACT, ammonium chloride Tris; APCs, antigen presenting cells; DC, dendritic cells; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; Flt3-L, fms-like tyrosine kinase 3 ligand; IFN- γ , interferon-gamma; Ig, immunoglobulin; IL, interleukin; i.m., intramuscular; i.p., intraperitoneal; OVA, ovalbumin; pDCs, plasmacytoid DCs; PBS, phosphate buffer saline; TGF- β , transforming growth factor-beta; TH, T helper.

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($P < 0.01$). pUMVC3-hFLex treatment can reverse established experimental asthma and might provide a novel approach for treating asthma.

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1. Introduction

Asthma is a multifactorial disease characterized by episodes of reversible airway inflammation and edema, as well as mucus secretion, which result in bronchial constriction and airway obstruction. Several immune cells and mediators are responsible for the development of allergy and asthma [1]. As a result of this heterogeneity, there are many treatment modalities used clinically [2–7]. Overall, recent therapeutic approaches have focused on modulating antigen (Ag) presentation to T cells, inhibition of Ag-specific T helper (TH) 2 responses and/or increasing the TH1 response [8]. In recent years, various immunomodulators have shown therapeutic activity for allergen-induced asthma, including anti-IgE [9], anti-cytokine antibodies (Abs) [10,11], probiotics [12], CpG [13], synthetic YpG, CpR and YpR immunostimulatory motifs [14], and mycobacterial Ags [15]. Vaccination using DNA-encoded Ags induce cellular and humoral immunity against microbial pathogens [16–18]. Plasmid vectors encoding fms-like tyrosine kinase 3 ligand (Flt3-L), the novel human hematopoietic growth factor, have been shown to provide adjuvant activity for the induction of Ag-specific immunity in mice [19–21]. The adjuvant activity is due, in part, to the ability of Flt3-L to expand dendritic cell (DC) numbers in the circulation and organs. [22] It has recently been demonstrated that hypervolemic injections of a Flt3-L plasmid vector could result in the expansion of DCs [23]. In contrast, Flt3-L can also promote tolerance to orally administered Ags [24]. Thus, DCs have a regulatory role in the induction of both tolerance and immunity, although both processes can be regulated by other cell types [25].

Recently, we reported that treatment with Flt3-L could prevent the development [26] and reverse [27] asthma-like conditions in a mouse model, resulting in the complete abolition of airway hyperresponsiveness (AHR) to methacholine. However, in these studies, mice were injected daily with recombinant Flt3-L for

10 days. Because of the adjuvant activity by Flt3-L protein, plasmids and viral vectors for vaccines [28,29], and our finding of therapy for asthma with Flt3-L protein, we wished to examine the therapeutic activity of Flt3-L cDNA for asthma as well as the duration of the effect of the treatment. Further, because of the therapeutic activity of CpG against asthma, there is the potential for multiple mechanisms with a Flt3-L plasmid, including Flt3-L protein activity and the CpG motif. Thus, we compared the ability of Flt3-L secreting plasmid (pUMVC3-hFLex) to a control plasmid using mice with preexisting AHR and allergic airway inflammation.

2. Methods

2.1. Animals

Four- to five-week-old female BALB/c mice were obtained from Harlan Laboratories (Indianapolis, IN) and were housed in separate cages according to treatment. Food and water were provided ad libitum. According to the National Institutes of Health guidelines, the research protocol of this study was approved by the Animal Research Committee of Creighton University (Omaha, NE).

2.2. 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 [30].

2.3. Sensitization and treatment

Allergic airway inflammation to ovalbumin (OVA) was induced intraperitoneal (i.p.) injection of 20 μ g

grade V chicken egg OVA (Sigma-Aldrich, St. Louis, MO) emulsified in 2.25 mg inject Alum (Pierce, Rockford, IL) in total volume of 100 μ l on days 1 and 14 (Fig. 1), followed by aerosol sensitization with 1% OVA for 20 min using an Ultra-Neb 90 nebulizer (De Villbiss, Somerset, PA) on days 28, 29 and 30. Starting on day 33, OVA-sensitized mice were randomized into three groups: two of the groups received injection into the muscle interior tibialis of 200 μ g pUMVC3-hFLex or pUMVC3 treatment three times on days 33, 38 and 41, and the OVA group received phosphate buffer solution (PBS) only. On day 44, mice were challenged with 5% aerosolized OVA. After methacholine challenge on day 45, half the mice in each group were sacrificed. On day 64, mice were challenged with 5% aerosolized OVA and sacrificed after the last methacholine challenge on day 65. Non-sensitized control mice were treated only with the vehicle (PBS).

2.4. Noninvasive method for measuring pulmonary function

Single-chamber whole-body plethysmograph (Buxco Electronics, Troy, NY), without the use of anesthesia or restraint, were used to measure pulmonary functions. This method has been dem-

onstrated to accurately reflect airway resistance [5,31], expressed as the P_{enh} units [32]. Twenty-four hours following OVA challenge, mice were placed within the chambers and challenged with increasing doses of methacholine (Sigma-Aldrich) to measure AHR, and P_{enh} in response to methacholine challenge were recorded and expressed as the absolute P_{enh} units.

2.5. Serum IgE analysis

Blood collected after sacrifice on day 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 [33] and according to the manufacturer's recommendations using rat anti-mouse IgE (BD PharMingen, San Diego, CA), 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 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.

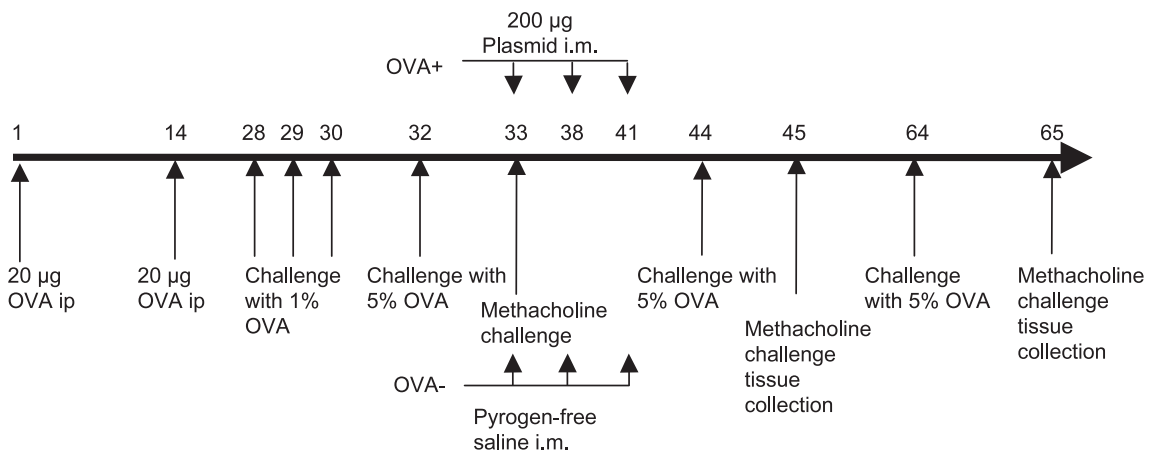


Fig. 1. OVA murine model of asthma and immunotherapy protocol. Balb/c mice were sensitized to OVA by i.p. injection and subsequently challenged with OVA by aerosolization on days 28, 29 and 30. Starting on day 33, OVA-sensitized mice were randomized into three groups: two of the groups received injection into the muscle interior tibialis 200 μ g pUMVC3-hFLex or pUMVC3 treatment three times on days 33, 38 and 41, while the OVA group received PBS only. On day 44, mice were challenged with 5% aerosolized OVA. Half the mice in each group were sacrificed on day 45. On day 64, mice were challenged with 5% aerosolized OVA and sacrificed after the last methacholine challenge on day 65. Non-sensitized control mice were treated only with the vehicle (PBS).

2.6. 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 [34]. 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 H₂SO₄ and read at 450 nm using a Bio-Rad microplate reader and software.

2.7. Bronchoalveolar lavage (BAL) collection

Immediately after methacholine challenge, mice were euthanized with a lethal dose of pentobarbital. Bronchoalveolar lavage fluid (BALF) was collected from each animal via cannulation of the exposed trachea and gentle flushing of the lungs with 1 ml warm PBS (0.8 ml) was recovered, which was centrifuged and the supernatant was collected.

2.8. Cytokine assays

Serum and BALF cytokines were measured by sandwich ELISA with capture and biotinylated detection antibody (Ab) pairs for interferon-gamma (IFN-γ), interleukin (IL)-4, IL-5, IL-10 and IL-12 and avidin-horseradish peroxidase and TMB substrate (BD PharMingen).

2.9. Phenotype of lung dendritic cells

DCs were isolated from the lungs of animals. After removing the blood, lungs were excised, washed with PBS, and digested with 5 ml of 1 mg/ml collagenase D in RPMI media, digested lungs were then centrifuged and incubated with ammonium chloride Tris (ACT) to lyse red blood cells. Cells were incubated

in 4% FBS to block FcγR non-specific binding. Cells were stained with anti CD11c-PE, anti CD11b-FITC, anti CD8α PerCP and anti CD45R–biotin–antigen presenting cell (APC). The cell profiles were gated on DCs (based on high forward and 90° light scatter). The DCs gate was then sub-gated on CD11c⁺ cells, further on the CD11c⁺ gate CD11b, CD8α and B220 profiles were examined.

2.10. Data analysis

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

3. Results

3.1. Effect of pUMVC3-hFLEX treatment on AHR in OVA-presensitized and -challenged mice

On day 33 of the sensitization protocol (Fig. 1), mice were placed in a whole body plethysmograph and enhanced pause response (*P*_{enh}) readings recorded to plot baseline dose response curves to increasing methacholine concentrations and to confirm that OVA-sensitized mice have established AHR (data not shown). Treatment with pUMVC3-hFLEX significantly reduced airway hyperresponsiveness in OVA-sensitized mice to levels comparable to PBS-treated mice (Fig. 2A). This effect lasted for at least 24 days after the last pUMVC3-hFLEX injection (Fig. 2B). In contrast, none of the OVA-sensitized pUMVC3-treated and -non-treated mice exhibited changes in AHR.

3.2. Effect of treatment on serum cytokines, total IgE and total and anti-OVA IgG subclasses in OVA-presensitized and -challenged mice

In the serum samples collected on day 45, OVA sensitization significantly increased serum IL-12 and total IgE levels. Further, these levels were significantly reduced after treatment with pUMVC3-hFLEX and pUMVC (Fig. 3C,D). Additionally, serum IL-4 levels were significantly reduced after treatment with

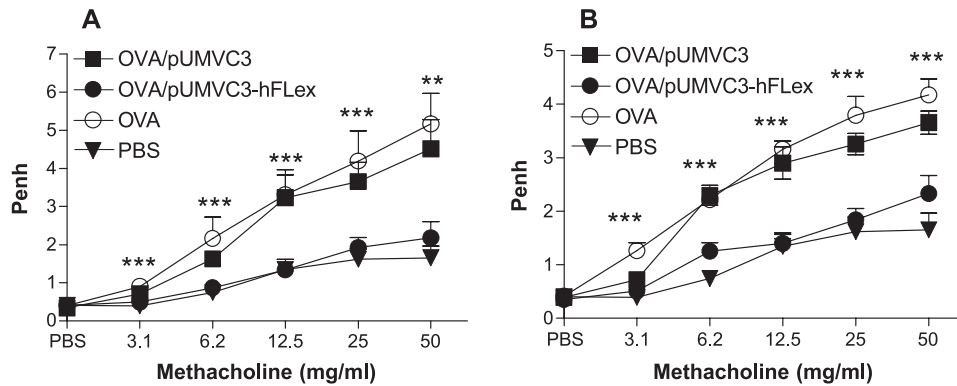


Fig. 2. AHR to methacholine in unrestrained mice. Following OVA sensitization and challenge, AHR to methacholine was established (day 33) (see Fig. 1) followed by treatment with pUMVC3-hFLex (200 μ g/day, i.p.) three times. On day 45, AHR to methacholine was again measured and P_{enh} values were recorded. pUMVC3-hFLex treatment abolishes AHR to methacholine in established allergic inflammatory mice (left panel A). On day 65, AHR to methacholine was again measured and P_{enh} values were recorded. pUMVC3-hFLex treatment abolishes AHR to methacholine and this effect lasts for at least 24 days after final dose of treatment of established allergic inflammation in mice (right panel B). Data is shown as mean $\pm$ S.E.M. ($N=8$ in each group). ** $p<0.01$, *** $p<0.001$ compared with OVA group.

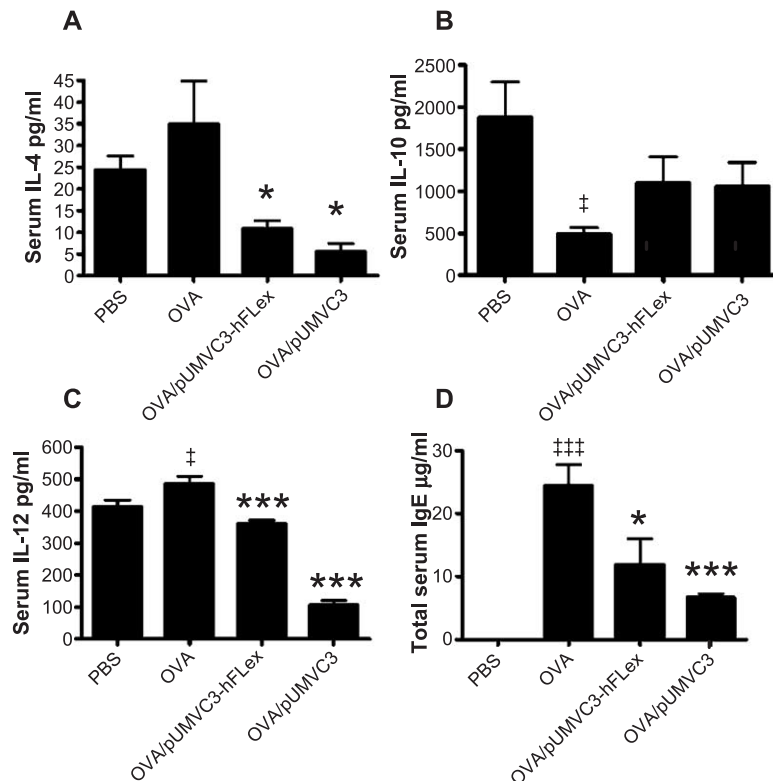


Fig. 3. Effect of pUMVC3-hFLex treatment on serum cytokines and total IgE levels in mice. On day 45, after recording pulmonary functions for AHR, blood was collected to measure serum IL-4 (A), serum IL-10 (B), serum IL-12 (C) and serum total IgE concentration (D). Data is shown as mean $\pm$ S.E.M. ($n=8$). (* $p<0.05$, *** $p<0.001$ compared with OVA group; † $p<0.05$, ‡‡‡ $p<0.001$ compared with PBS group), serum IL-5 and IFN- γ were not detected. pUMVC3-hFLex, a plasmid, mammalian expression vector for the secretion of Flt3-L, pUMVC3 the backbone plasmid without the Flt3-L insertion.

pUMVC3-hFLex and pUMVC (Fig. 3A). In contrast, OVA sensitization significantly decreased serum IL-10 levels; however, there was no effect of pUMVC3-hFLex or pUMVC3 on serum IL-10 level (Fig. 3E). On day 65, serum IL-4 levels were significantly higher than PBS control mice (Fig. 4A) and serum IL-10 levels in OVA-presensitized mice were still significantly lower than PBS control mice (Fig. 4B). In contrast, serum IL-12 levels in OVA-presensitized mice were significantly lower than PBS control mice (Fig. 4C).

3.3. Effect of pUMVC3-hFLex treatment on BALF cytokines in OVA-presensitized and -challenged mice

On day 45, OVA sensitization significantly increased BALF IL-5 and IL-12 levels, which were significantly reduced after treatment with pUMVC3-hFLex and pUMVC (Fig. 5B,C). In contrast, OVA sensitization significantly decreased BALF IL-10 and IFN- γ levels (Fig. 5D,E). Further, the BALF levels of IL-4 were unchanged, which differed from the serum IL-4 levels that were increased by pUMVC3-hFLex injections. On day 65, BALF IL-5 levels were significantly higher than PBS control mice (Table 2), in contrast to levels on day 45, BALF IL-5 levels increased to levels comparable to those in OVA-sensitized mice. However, BALF IL-10 levels in OVA-presensitized mice were still significantly

decreased and treatment with pUMVC3-hFLex has increased that levels (Table 2).

3.4. Effect of treatment on serum total and anti-OVA IgG isotypes in OVA-presensitized and -challenged mice

OVA sensitization significantly increased total and anti-OVA serum IgG1 and IgG2a levels. The injection of pUMVC3-hFLex had no effect on total serum IgG2a and IgG1 levels (Fig. 6A,B). However, in serum samples collected on day 45 treatment with pUMVC3-hFLex induced a significant increase in the serum levels of anti-OVA IgG2a (Fig. 6C). In addition, unlike most other Ag responses, there was no significant effect of pUMVC3-hFLex injection on anti-OVA IgG1 levels (Fig. 6D). In contrast to the initial increase in anti-OVA IgG2a levels, on day 65, pUMVC3-hFLex caused a significant reduction in anti-OVA IgG2a levels (Fig. 7C).

3.5. Effect of treatment on BALF inflammatory cells in OVA-presensitized and -challenged mice

On days 45 and 65, sensitization and challenge with OVA induced a significant influx of cells into the airways (Tables 1 and 2). Treatment with pUMVC3-hFLex significantly reduced the total cellular infiltration (Tables 1 and 2). The reduction in the frequency

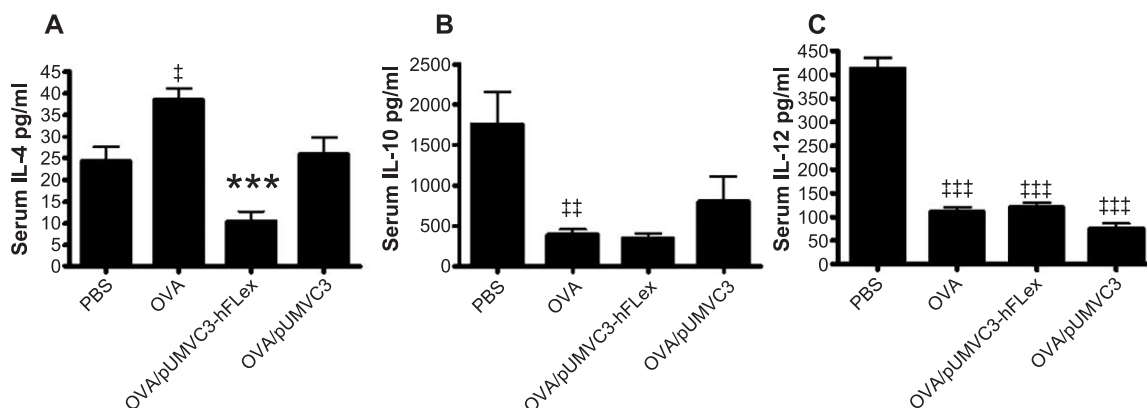


Fig. 4. Effect of pUMVC3-hFLex treatment on serum cytokines in mice. On day 65, after recording pulmonary functions for AHR, blood was collected to measure serum IL-4 (A), serum IL-10 (B) and serum IL-12 (C). Data is shown as mean $\pm$ S.E.M. ($n=8$). (*** $p<0.001$ compared with OVA group; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ compared with PBS group), serum IL-5, IFN- γ and IgE were not detected. pUMVC3-hFLex, a plasmid, mammalian expression vector for the secretion of Flt3-L, pUMVC3 the backbone plasmid without the Flt3-L insertion.

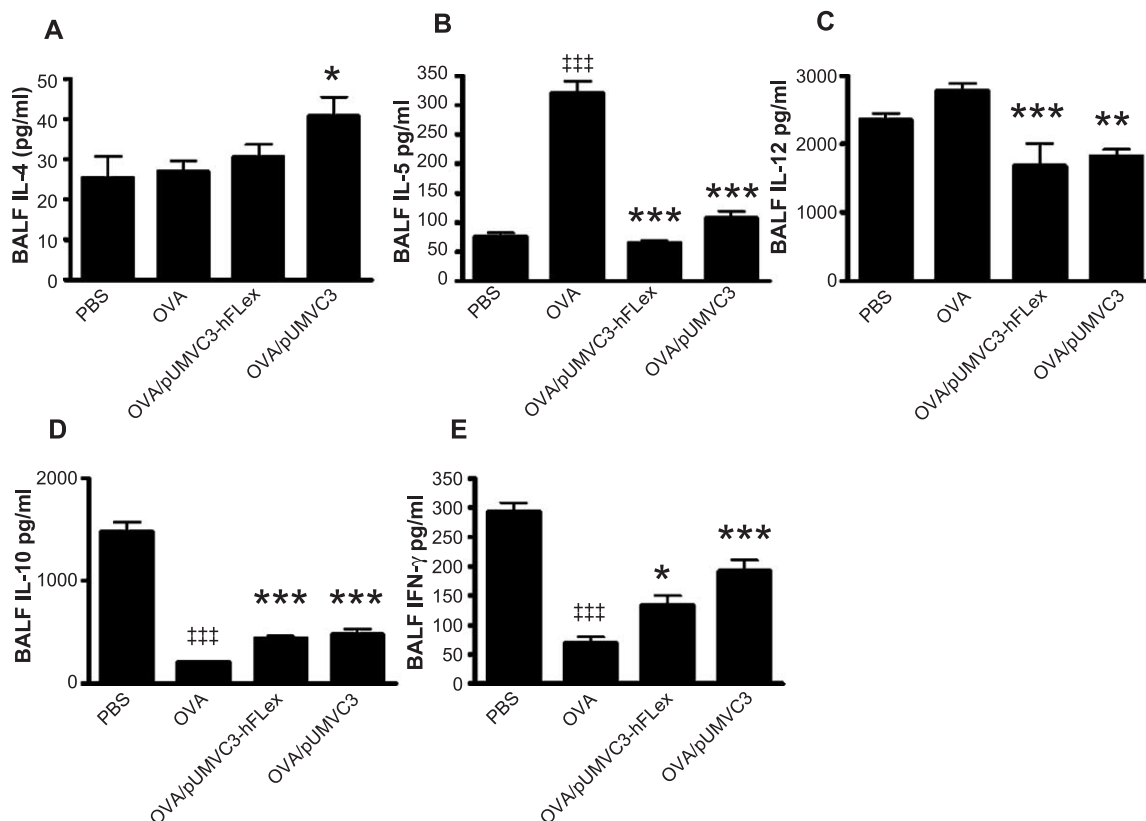


Fig. 5. Effect of pUMVC3-hFLex treatment on BALF cytokines levels in mice. On day 45, after recording pulmonary functions for AHR, BALF samples were immediately centrifuged and cytokines in supernatants were measured: BALF IL-4 (A), BALF IL-5 (B), BALF IL-12 (C), BALF IL-10 (D) and BALF IFN- γ (E). Data is shown as mean $\pm$ S.E.M. ($n=8$). (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with OVA group; ‡ $p<0.05$, ‡‡ $p<0.01$ compared with PBS group).

of infiltrating eosinophils was also significant. Total number of macrophages was significantly increased in both pUMVC3-hFLex-treated and pUMVC3-treated animals (Tables 1 and 2). There was no significant effect on the number of neutrophils or lymphocytes in the BALF.

3.6. Effect of pUMVC3-hFLex administration on lung DCs

pUMVC3-hFLex treatment significantly altered the profile of CD11c⁺ in the lungs with an increase in the total number of CD11c⁺ cells, including CD11c⁺ CD11b⁺ ($p<0.05$) and CD11c⁺ CD8 α ⁺ cells (Table 3). In addition, there was a significant increase in CD11c⁺ B220⁺ ($p<0.05$) as well as (CD11c⁺ CD8 α ⁺) ($p<0.01$) cells as compared to the PBS-treated OVA-sensitized group (Table 3).

3.7. Discussion

This report presents evidence that plasmid containing Flt3-L gene can shape and protect against the immune allergic response to OVA in OVA-prensensitized and -challenged mice with established AHR. This model of allergic airway inflammation has been reported to elicit type 2 T-cell responses and is characterized by eosinophilic infiltration of the airways and nonspecific AHR. Previously, we demonstrated that Flt3-L, administered prior to Ag sensitization, was capable of preventing [26] and reversing [27] airway inflammation and hyperresponsiveness.

In this study, we report the therapeutic activity of a plasmid with the Flt3-L transgene (pUMVC3-hFLex). We report for the first time that the injection of this plasmid can reverse established AHR and this reversal lasts for at least 24 days after the final

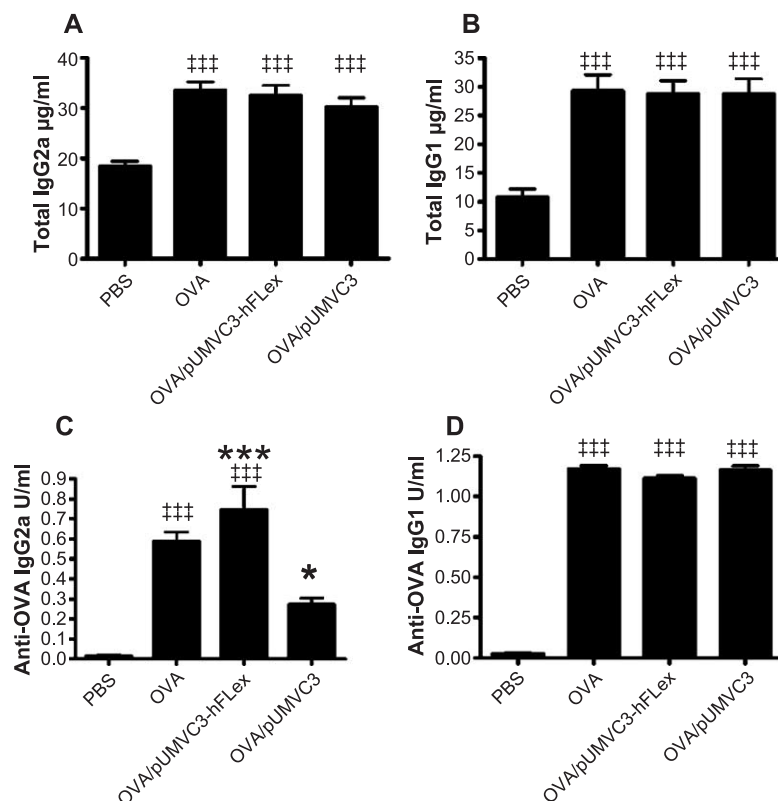


Fig. 6. Effect of pUMVC3-hFLex treatment and Ag sensitization and challenge on IgG subclasses in mice serum. On day 45, after recording pulmonary functions for AHR, blood was collected to measure: total IgG2a (A), total IgG1 (B), anti-OVA IgG2a (C) and anti-OVA IgG1 (D). Data are shown as mean $\pm$ S.E.M. from eight animals in each group. (* $p < 0.05$, *** $p < 0.001$ compared with OVA group; ††† $p < 0.001$ compared with PBS group).

pUMVC3-hFLex injection. BALF IL-5 and eosinophil levels, and serum IL-4 and IgE levels were all reduced. In contrast, the therapeutic effect of Flt3-L on allergic airway inflammation and pulmonary function was associated with a significant increase in BALF IFN- γ and IL-10 levels, and serum anti-OVA IgG2a levels.

The development of allergen-induced asthma is mediated by the production of TH2 cytokines such as IL-4, IL-5 and IL-13. Several studies have shown that type 1 immunomodulators have therapeutic activity for allergen-induced asthma. Some of these not only boost the production of TH1 cytokines, but also block the induction of TH2 immunity. One of these cytokines, IL-4, has been identified as important to the development of allergic inflammation. The role of IL-4 in asthma is supported by the augmented levels of IL-4 in BALF [35] and increased expression of IL-

4 mRNA in broncho-alveolar lavage cells [36,37] reported in patients with allergic inflammation. In murine models, IL-4 has been demonstrated to play a crucial role in allergic airway inflammation [38–40] and AHR [41,42]. These observations are supported by our findings that the i.m. delivery of pUMVC3-hFLex results in a significant decrease in serum IL-4 levels. Interestingly, injections of the control vector, pUMVC3, also decreased serum IL-4 levels, suggesting that the reversal of AHR might be independent of a reduction in IL-4 levels. It is also noteworthy that the pUMVC3 backbone is a pUC19 plasmid containing methylated CpG motifs [43], which might be responsible for the decreased IL-4 levels. Alone, however, it appears not to be able to reverse the AHR, which requires the Flt3-L transgene. Even though trials with IL-4 neutralizing agents in patients with asthma have been reported to be non-therapeutic,

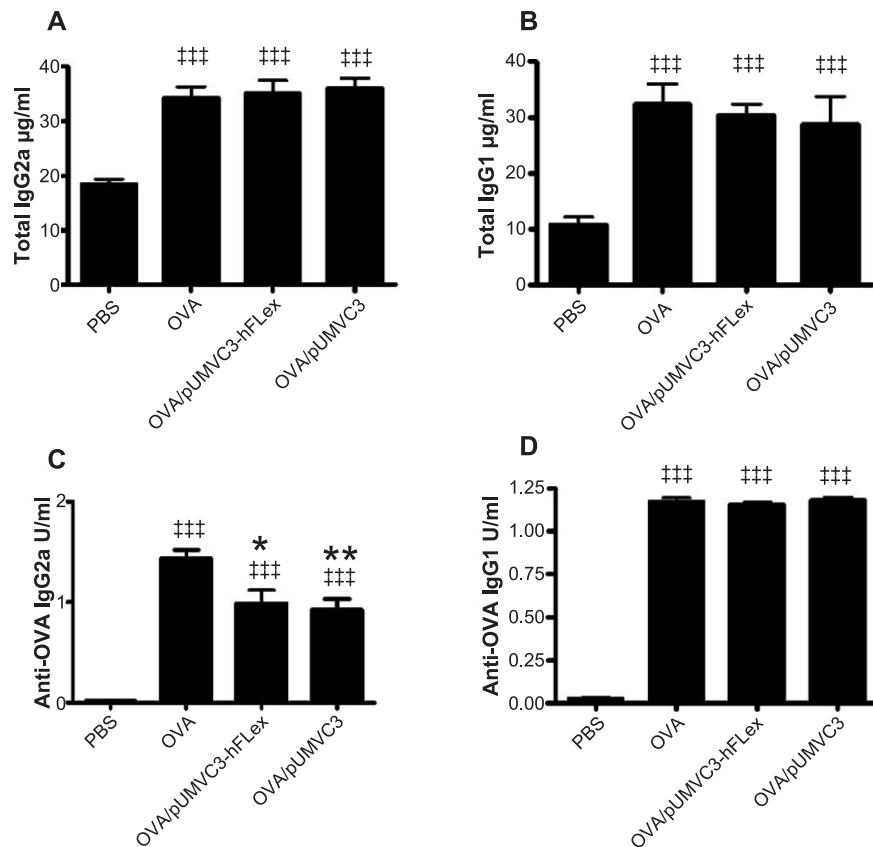


Fig. 7. Effect of pUMVC3-hFLex treatment and Ag sensitization and challenge on IgG subclasses in mice serum. On day 65, after recording pulmonary functions for AHR, blood was collected to measure: total IgG2a (A), total IgG1 (B), anti-OVA IgG2a (C) and anti-OVA IgG1 (D). Data are shown as mean $\pm$ S.E.M. from eight animals in each group. (* p < 0.05, ** p < 0.01 compared with OVA group; ††† p < 0.001 compared with PBS group).

it has raised the question of the importance of this cytokine in established asthma, although the significance of this cytokine cannot be ruled out and further studies are warranted.

IL-5 knockout mice have been reported to be unable to develop airway eosinophilia and hyperresponsiveness [44]. Similarly, Ab neutralization of systemic IL-5 reduces airway inflammation and hyperresponsiveness [45]. In addition, as IL-5 is able to recruit eosinophils to the lung; the observation that treating asthmatics with an anti-IL-5 Ab results in decreased airway eosinophil numbers is expected. Therefore, the effect of pUMVC3-hFLex injections on IL-5 levels and the recruitment of eosinophils to the airways are expected. We report that pUMVC3-hFLex significantly inhibited IL-5 levels and inflammatory cell infiltration as well as lung eosinophilia. We suggest that the ability of

pUMVC3-hFLex to reduce IL-5 secretion may directly reduce the recruitment of eosinophils into the lungs; thereby, regulating, at least, the inflammatory cellular components of asthma. 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.

One mechanism of vaccine adjuvant activity is the recruitment of professional Ag presenting cells (APCs) to the site(s) of infection, and by directly stimulating these APCs to express costimulatory molecules [46,47]. In the lungs, immature DCs act as a sentinel against infectious diseases. After interacting with a foreign Ag, they migrate to the draining lymph nodes, where they present the captured and processed Ag in the context of co-stimulatory molecules to Ag specific naïve T cells, and initiate the development of a primary effector T cell response [48].

Table 1

BALF total and differential cell counts ($\times 10^{-3}$) on day 45

Treatment group	PBS	OVA	OVA/pUMVC3-hFLex	OVA/pUMVC3
Total cells	65.00 $\pm$ 35.06	515.00 $\pm$ 86.72 ^{†††}	176.25 $\pm$ 65.43 ^{***,##}	475 $\pm$ 49.35 ^{†††}
Macrophages	54.58 $\pm$ 28.43	63.73 $\pm$ 11.58	133.51 $\pm$ 15.13 [‡]	130.63 $\pm$ 11.06 [‡]
Eosinophils	10.38 $\pm$ 7.28	436.46 $\pm$ 14.16 ^{†††}	37.45 $\pm$ 13.96 ^{***}	331.31 $\pm$ 6.25 ^{***,†††}
Neutrophils	0.00 $\pm$ 0.00	11.59 $\pm$ 6.78	2.20 $\pm$ 0.84	10.69 $\pm$ 6.25
Lymphocytes	0.05 $\pm$ 0.05	3.22 $\pm$ 1.93	3.08 $\pm$ 1.11	2.38 $\pm$ 1.37

BAL fluid (0.8 ml) was collected from each animal and centrifuged. Recovered total cells were counted (cell/ml $\times 10^{-3}$) 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 $\pm$ S.E.M. for six animals in each group. (* p <0.05 compared with OVA group; ^{###} p <0.001 compared with pUMVC3 group). pUMVC3-hFLex, a plasmid, mammalian expression vector for the secretion of Flt3-L, pUMVC3 the backbone plasmid without the Flt3-L insertion.

^{**} p <0.01 compared with OVA group.

^{***} p <0.001 compared with OVA group.

[‡] p <0.05 compared with PBS group.

^{†††} p <0.001 compared with PBS group.

^{##} p <0.01 compared with pUMVC3 group.

It has recently been demonstrated that a distinct subset of DCs with a CD11c⁺ B220⁺ phenotype, which are identified as plasmacytoid DCs (pDCs), have the ability to secrete IL-10 following activation and regulate T regulatory (Treg) cell differentiation [49]

and expansion [50–52]. In addition, pDCs do not express co-stimulatory molecules, which can contribute to their tolerance inducing properties [53]. Treg cells produce immunosuppressive cytokines, including IL-10 and transforming growth factor-beta (TGF- β)

Table 2

BALF total and differential cell counts ($\times 10^{-3}$) and cytokines on day 65

Treatment group	PBS	OVA	OVA/pUMVC3-hFLex	OVA/pUMVC3
Total cells	64 $\pm$ 13.7	209 $\pm$ 36.6 [‡]	112 $\pm$ 19.4	204 $\pm$ 36.5 [‡]
Macrophages	50.9 $\pm$ 2.9	25.9 $\pm$ 4.7	84.8 $\pm$ 9.6 ^{‡,***,##}	56.1 $\pm$ 4.7*
Eosinophils	11.8 $\pm$ 2.7	177.1 $\pm$ 5.7 ^{†††}	23.8 $\pm$ 8.9 ^{***,##}	142.3 $\pm$ 2.7 ^{†††,***}
Neutrophils	0.8 $\pm$ 0.5	4.7 $\pm$ 2.7	1.4 $\pm$ 0.5	4.6 $\pm$ 2.7
Lymphocytes	0.5 $\pm$ 0.3	1.3 $\pm$ 0.8	1.96 $\pm$ 0.7	1.0 $\pm$ 0.6
IL-4	43.4 $\pm$ 4.0	37.4 $\pm$ 1.9	31.9 $\pm$ 3.4	46.0 $\pm$ 4.6
IL-5	75.5 $\pm$ 6.1	365.8 $\pm$ 19.4 ^{†††}	427.5 $\pm$ 15.3 ^{†††}	502.7 $\pm$ 18.7 ^{†††}
IL-12	2354.2 $\pm$ 95.7	3130 $\pm$ 217.2	3261.7 $\pm$ 183.7 [‡]	3550.4 $\pm$ 135.1 ^{††}
IL-10	1473.9 $\pm$ 98.7	211.5 $\pm$ 17.8 ^{†††}	423.3 $\pm$ 68.1 ^{†††,*}	357.4 $\pm$ 23.5 ^{†††}
IFN- γ	293.4 $\pm$ 15.2	37.2 $\pm$ 10.4 ^{†††}	71.0 $\pm$ 15.9 ^{†††}	74.5 $\pm$ 30.9 ^{†††}

BAL fluid (0.8 ml) was collected and the total cells (cells/ml $\times 10^{-3}$) were counted. One hundred microliters of BALF was stained to differentiate cell types. Differential cell counts were calculated as a product of the percentage of each cell type and the total number of cells per milliliter. Samples were centrifuged and supernatants were analyzed for cytokines by ELISA (pg/ml), n =6 per group, data are shown as mean $\pm$ S.E.M. pUMVC3-hFLex, a plasmid, mammalian expression vector for the secretion of Flt3-L, pUMVC3 the backbone plasmid without the Flt3-L insertion.

* p <0.05 compared with OVA group.

** p <0.01 compared with OVA group.

*** p <0.001 compared with OVA group.

[‡] p <0.05 compared with PBS group.

^{††} p <0.01 compared with PBS group.

^{†††} p <0.001 compared with PBS group.

[#] p <0.05 compared with pUMVC3 group.

^{##} p <0.001 compared with pUMVC3 group.

Table 3
Phenotypic characteristics of lung dendritic cells

Experimental group	Total CD11c ⁺ (% of total cells)	CD11c ⁺ CD11b [−] CD8α ⁺ (DC1 cells)	CD11c ⁺ CD11b ⁺	CD11c ⁺ CD11b [−] B220 ⁺	CD11c ⁺ CD11b [−]
			(DC2 cells)	(plasmacytoid DCs)	CD8α [−] B220 [−]
			% total CD11c cells		
PBS	2.00±0.23	1.74±0.25	57.25±4.06	23.72±2.35	17.29±3.93
OVA	3.07±0.22	0.93±0.25	73.11±2.00 [†]	12.69±1.14 [†]	13.27±1.09
OVA/pUMVC3-hFLex	4.90±0.43** ^{†††}	3.32±0.40**	65.08±2.50	23.60±2.69 ^{†††}	7.99±0.50
OVA/pUMVC3	2.76±0.21	1.87±0.53	59.76±3.05*	23.43±2.87*	14.48±2.55

Effect of sensitization with OVA and treatment on the phenotype of CD11c⁺ cells in the lungs of mice. Percent of total CD11c⁺ cells in the DCs gate and percentages of subtypes within CD11c⁺ population are shown as mean±S.E.M. from five animals in each group. pUMVC3-hFLEX, a plasmid, mammalian expression vector for the secretion of Flt3-L, pUMVC3 the backbone plasmid without the Flt3-L insertion.

* $p < 0.05$ compared to OVA group.

** $p < 0.01$ compared to OVA group.

† $p < 0.05$ compared to PBS group.

††† $p < 0.001$ compared to PBS group.

[50–52] and have an important role in decreasing an exuberant immune response. Therefore, it is reasonable to speculate that pUMVC3-hFLEX-induced effect on AHR and allergic airway inflammation is mediated via the induction of pDCs in the lungs. This is supported by our findings where the i.m. administration of pUMVC3-hFLEX increased CD11c⁺ B220⁺ cells in the lungs five fold. This suggests that marked proliferation of immature DCs, expressing few or no co-stimulatory molecules in response to pUMVC3-hFLEX treatment might have the potential to induce tolerance. This could occur either directly or indirectly by their expansion of Treg⁺ cells.

In our studies, we observed no negative systemic or local effects of i.m. delivered pUMVC3-hFLEX. Nonetheless, we cannot rule out the possibility that unregulated, systemic expression of Flt3-L may have consequences on normal immune function. It should be noted that we did not monitor serum levels of Flt3-L, although previous reports have suggested that the injection of plasmid with the Flt3-L transgene does not result in systemic Flt3-L levels [21].

While the relevance of the murine, induced airway hypersensitivity model to human asthma is somewhat controversial [54,55], mice can never completely replicate the features of human asthma. Nonetheless, our studies using mice injected with an pUMVC3-hFLEX plasmid suggest a potential clinical application for gene therapy in the treatment of asthma, although many questions remain unresolved. Other delivery modalities, such as gene guns to facilitate uptake of the DNA, direct delivery into the trachea or intranasally to

enhance local uptake may also result in higher or localized production of Flt3-L, and then provide better therapeutic benefits. Indeed, recent studies have revealed multiple mechanisms of RNA and DNA regulation of immunity raise the question that the Flt3-L plasmid is independent of the Flt3-L transgene [24]. Overall, these studies demonstrate that the administration of pUMVC3-hFLEX is capable of inhibiting the production of asthma mediators (IL-4 and IL-5), and thus may be of significant benefit in the treatment of asthma.

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