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Research Article

Synergistic therapeutic effects of combined adenovirus-mediated interleukin-10 and interleukin-12 gene therapy on airway inflammation in asthmatic mice

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Keywords

adenoviral vector • airway inflammation • asthma • interleukin-10 • interleukin-12

Abstract



Background

Asthma is a chronic disease characterized by airway inflammation caused by the dysregulated production of cytokines secreted by allergen-specific type 2 T helper (Th2) cells. Although the Th1-promoting cytokine, interleukin (IL)-12, is capable of inhibiting Th2-driven allergen-induced airway changes in mice, IL-12 also aggravates the Th1-driven inflammatory pulmonary pathology. Further, IL-10 was found to exert both anti-inflammatory and immunoregulatory activities. To avoid the side-effects of IL-12, we hypothesized that the low-dose expression of IL-10 with concomitant IL-12 administration in the airway may represent a more effective therapy for allergic airway diseases. Thus, the present study explored the immunomodulatory and therapeutic effects of IL-10 combined with IL-12 in airway inflammation in allergic asthma.

Methods

Adenovirus-expressing murine IL-10 (Ad-IL-10) and IL-12 (Ad-IL-12) were co-administrated in an established murine model of ovalbumin (OVA)-induced asthma.

Results

We found that a single combined treatment of low doses of Ad-IL-10 and Ad-IL-12 efficiently inhibited the development of airway hyper-responsiveness compared to Ad-IL-10 or Ad-IL-12 treatment alone. Moreover, both Ad-IL-10 and Ad-IL-12 treatment reduced pulmonary infiltration of eosinophils and neutrophils. In addition, histological studies showed that combined treatment was able to reduce tumor necrosis factor- α -mediated airway inflammation induced by IL-12 treatment. Suppression of IL-4, IL-5, Keratinocyte-derived chemokine (KC) and eotaxin in bronchoalveolar lavage fluid was also noted in OVA-immunized mice with combined Ad-IL-10 and Ad-IL-12 treatment.

Conclusions

Taken together, the results obtained in the present study indicate that co-administration of IL-12 and IL-10 may have therapeutic potential for the immunomodulatory treatment of allergic asthma. Copyright © 2009 John Wiley & Sons, Ltd.

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Article Text

Introduction



Asthma is a global health problem that results from a complex interplay between genetic and environmental factors. The incidence, morbidity, and mortality from asthma have been increasing worldwide over the last decade. It is recognized that CD4⁺ T-helper type 2 (Th2) cells and their cytokines [interleukin (IL)-4, IL-5, and IL-13] are responsible for the initiation and maintenance of allergic disorders [1]. These Th2 cytokines induce, prolong, and amplify the inflammatory response by enhancing the production of allergic-specific immunoglobulin E; enhancing the recruitment, growth, and differentiation of eosinophils; and directly causing airway hyper-responsiveness (AHR) [2]. Inhaled adrenergic agonists and steroid treatments are effective, but are costly, require daily dosing, and depend on patient compliance and coordination. Currently, the potential of cytokine-based therapies to treat asthma, such as IL-10, interferon (IFN)- γ , and IL-12, are under intense investigation [3–6].

The cytokine, IL-12, has an essential role in Th1 development and has therefore been considered a potential therapy for asthma. IL-12 is a heterodimeric cytokine, produced by activated monocytes, macrophages, dendritic cells, and neutrophils. In general, IL-12 up-regulates the IFN- γ production of T-cells and natural killer cells, promotes Th1 responses, and suppresses the expression of Th2 cytokines [7][8]. These immunoregulatory properties of IL-12 have led to its therapeutic use in allergic asthma. Indeed, several studies have demonstrated that the systemic administration of the IL-12 protein can decrease eosinophil infiltration and airway inflammation in murine models of

asthma [4][5][9-11]. However, high doses of IL-12 treatment do not simply down-regulate allergic Th2 inflammation but also induce another type of acute pulmonary inflammation [12]. Thus, the ability to transfer the IL-12 gene into the lungs may localize cytokine expression and potentially reduce any associated deleterious effects in treating pulmonary allergic diseases.

Many studies have indicated that regulating asthma and allergic diseases might not be reflected in a simple, dichotomous balance of polarized cells, as suggested by the Th1/Th2 paradigm [13-15]. IL-10 is a cytokine that has well-documented anti-inflammatory and immunoregulatory activities. In addition, IL-10 is produced by a wide variety of cell types, including monocytes, macrophages, B cells, mast cells, and eosinophils [16]. IL-10 was originally described in mice as a factor inhibiting cytokine production from murine Th1 clones [17]. Subsequent studies showed that IL-10 can also down-regulate Th2 clones and their production of IL-4 and IL-5 [18]. In addition, IL-10 inhibits the synthesis of pro-inflammatory cytokines and chemokines by monocytes/macrophages, neutrophils, and eosinophils [19-22]. *In vitro*, IL-10 inhibits antigen-specific proliferation of peripheral blood T cells and CD4+ T cells regardless of the subset (Th1, Th2, or Th0) [23]. Those findings led to the hypothesis that the expression of IL-10 in the airway environment may inhibit the development of airway inflammation. However, the regulatory role of endogenous IL-10 in asthma remains unknown. A few studies found reduced IL-10 mRNA expression in both peripheral blood mononuclear cells and bronchoalveolar lavage (BAL) lymphocytes of asthmatic patients, whereas other studies demonstrated elevated levels in asthmatics [18][24][25]. In a mouse model of asthma, studies on C57BL/6 IL-10^{-/-} mice demonstrated that IL-10 is necessary for the expression of AHR but not pulmonary inflammation after allergic sensitization [26]. By contrast, in a similar mouse model of allergy, Tournoy *et al.* [27] found that IL-10^{-/-} mice developed significantly increased ovalbumin (OVA)-induced AHR [27]. These data further suggest the complexity of the role of IL-10 in the pathogenesis of asthma.

Recently, Oh *et al.* [28] showed that IL-10-producing T cells could abolish AHR and airway inflammation in a murine model of asthma. Thus, not only Th1-related cytokines, but also anti-inflammatory cytokines can regulate airway inflammation. Because the anti-allergic effects of IL-12 are only partially dependent on IFN- γ , and IL-12 is a potent inducer of the anti-inflammatory cytokine, IL-10 [29][30], we therefore hypothesized that IL-10 participates in the development of beneficial effects in addition to IL-12. Thus, the ability to transfer both IL-12 and IL-10 genes into the lungs, at doses lower than those used via the systemic route, may localize cytokine expression and potentially reduce IL-12-induced inflammation for treating pulmonary allergic diseases. To date, the consequences of combining IL-10 and IL-12 in allergic airway inflammation have not been clearly defined. In the present study, we elucidated whether treatment with IL-10- and IL-12-expressing adenoviruses could achieve a synergistic effect to alleviate the development of airway symptoms in a mouse model of allergic asthma. The experiment protocol was performed in an established murine model of OVA-induced asthma. These treated mice were then evaluated for changes in AHR, airway eosinophilia, histology of lung tissues, and cytokine production.

Materials and methods



Animals

Female BALB/c mice aged 6-8 weeks old were obtained from the Animal Center of National Taiwan University and maintained in the Animal Center of Taipei Medical University. Animals were age-matched within each experiment. Animal care and handling protocols were approved by the Animal Committee of the College of Medicine, Taipei Medical University.

Construction and preparation of adenoviral vectors

Construction of a single-chain IL-12 gene was performed as described previously [31]. The p40 and p35 subunits of the murine IL-12 gene were connected by a linker and were used to generate a single-chain IL-12 gene. The mouse single-chain IL-12 or IL-10 gene was inserted into a recombinant adenoviral shuttle vector (pAdhPGK) containing the human phosphoglycerate kinase (hPGK) gene promoter. Next, Ad-IL-12 and Ad-IL-10 recombinant adenoviruses were generated by homologous recombination with an adenoviral backbone plasmid (pAdEasy-1) and amplified in the AD293 adenovirus packaging cell line. As a control, Ad-mock was made from the pAdhPGK vector, which did not carry the cytokine transgene. After propagation in AD293 cells, the recombinant viruses were purified from infected cells 24-36 h after infection by three freeze-thaw cycles followed by successive banding on cesium chloride density-gradient centrifugation. The purified viruses were dialysed and stored at -70 °C until the experiment. Viral titers were measured by a standard endpoint dilution assay using AD293 cells. Furthermore, expressions of IL-10 and IL-12 were confirmed by measuring culture supernatants of infected AD293 cells.

Sensitization, treatment, and challenge of mice

Groups of mice were sensitized by an intraperitoneal injection of 30 µg of OVA (grade V; Sigma Chemical, St Louis, MO, USA) emulsified in 2 mg of aluminium hydroxide (Pierce Chemical, Rockford, IL, USA) in a total volume of 200 µl on day 0. The mice were then boosted twice with 50 µg of OVA emulsified in 4 mg of aluminium hydroxide in a total volume of 200 µl on days 14 and 28. On days 37 and 38, the mice were challenged with nebulized OVA for 30 min/day. Aerosols were generated in a chamber using an ultrasonic nebulizer, and the concentration of OVA in the nebulizer was 5% (w/v).

To avoid serious airway inflammation by the vectors and to induce adequate cytokine transgene expression, groups ($n = 5-8$ per group) of OVA-sensitized mice received various doses [1×10^8 , 2×10^8 , and 5×10^8 plaque-forming units (pfu)] of a single administration of Ad-IL-10 or Ad-IL-12 alone on day 35. Control mice were administered phosphate-buffered saline (PBS) or Ad-mock (5×10^8 pfu). Then mice were subsequently challenged on two consecutive days (days 37 and 38) with OVA inhalation, and AHR was measured 1 day after the last challenge. BAL fluid (BALF) was collected (on day 40) to determine cytokine and chemokine production by an enzyme-linked immunosorbent assay (ELISA).

After determining the optimal dose of vector for gene therapy, we further examined the therapeutic effects of combined treatment with Ad-IL-10 and Ad-IL-12. Groups ($n = 5-8$ per group) of OVA-sensitized mice received single intratracheal deliveries of Ad-IL-10 (1×10^8 pfu/mouse) alone, Ad-IL-12 (1×10^8 pfu/mouse) alone, or Ad-IL-10 (1×10^8 pfu/mouse) plus Ad-IL-12 (1×10^8 pfu/mouse) 2 days before the first challenge with OVA. Control mice were administered PBS or Ad-mock (2×10^8 pfu/mouse). AHR was assessed 1 day after the last challenge. On day 40, BALF was obtained, and the lungs were fixed.

Measurement of cytokine and chemokine expressions

Quantities of IL-10, IL-12, IFN- γ , tumor necrosis factor (TNF)- α , IL-4, IL-5, eotaxin and Keratinocyte-derived chemokine (KC) in BALF supernatants were evaluated by commercially available ELISA kits (DuoSet; R&D, Minneapolis, MN, USA). Cytokine levels were calculated by linear regression analysis based on the values obtained from a standard curve.

Measurement of AHR

Airway responsiveness was measured in unrestrained animals by barometric whole-body plethysmography (Buxco Electronics, Sharon, CT, USA). Briefly, mice were placed in the main chamber, and baseline readings were taken and averaged for 3 min. Aerosolized normal saline or methacholine (acetyl- β -methycholine chloride; Sigma) in increasing concentrations (6.25–100 mg/ml) was administered by nebulization through an inlet of the main chamber for 3 min. Recordings were taken and averaged for 3 min after each nebulization. Airway reactivity was expressed as the mean enhanced pause [$Penh = \text{pause} \times (\text{peak expiratory box flow} / \text{peak inspiratory box flow})$], and data are expressed as the ratio of $Penh_{MCh}$ values compared to $Penh_{NaCl}$ from three independent experiments.

Analysis of the cellular composition of BALF

One day after measuring the pulmonary function parameters, mice were sacrificed, and their tracheas were immediately lavaged three times via a tracheal cannula with 1 ml of Hank's balanced salt solution (HBSS), which was free of ionized calcium and magnesium. The lavage fluid was cooled on ice and centrifuged (400 *g*) at 4 °C for 10 min. After washing, the supernatants were collected for the cytokine assay, and cell pellets were resuspended in 1 ml of HBSS. The total number of cells in BALF was counted with a standard hemocytometer. Differential cell counts were performed by counting at least 200 cells in the cytocentrifuged preparations, stained with Liu's staining solution (Chi I Pao, Taipei, Taiwan), and differentiated by standard morphological criteria.

Histological examination of lung tissue

After the lavage, the lungs were immediately removed and fixed in 10% v/v buffered formalin (in 0.01 M PBS, pH 7.2). Pulmonary tissues were subsequently sliced, embedded in paraffin, and cut into 5-µm thick sections. These frozen sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy for histopathological changes.

Statistical analysis

Results are expressed as the mean $\pm$ SEM. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's post-hoc test. $p < 0.05$ was considered statistically significant.

Results



Dose-dependent effects of intratracheal Ad-IL-10 and Ad-IL-12 alone on Th2 type cytokine production and AHR

Before using adenoviral vectors for gene therapy, we first investigated the relative efficacy of various doses of the adenovirus in alleviating serious airway inflammation and AHR. Groups of OVA-sensitized mice received a high (5×10^8 pfu Ad-IL-10 or 5×10^8 pfu Ad-IL-12), medium (2×10^8 pfu Ad-IL-10 or 2×10^8 pfu Ad-IL-12), or low dose of vector (1×10^8 pfu Ad-IL-10 or 1×10^8 pfu Ad-IL-12) (Figure 1). Control mice were treated with PBS or Ad-mock (5×10^8 pfu). Then these mice were subsequently challenged with two consecutive days of OVA inhalation. AHR was assessed 24 h after the last OVA-challenge and BALFs were collected on day 40. Levels of IL-10, IL-12, IL-4, IL-5, IFN- γ , and eotaxin in the BALF were evaluated by ELISA. We observed that both IL-10 and IL-12 expression levels in BALF showed dose-dependent responses (Figure 2).



Figure 1. Treatment regimen. Groups of mice were sensitized by an intraperitoneal (i.p.) injection of 30 μ g of OVA emulsified in 2 mg of aluminium hydroxide on day 0. Then the mice were boosted with 50 μ g of OVA emulsified in 4 mg of aluminium hydroxide on days 14 and 28. On day 35, these OVA-sensitized mice received an intratracheal (i.t.) injection of adenoviral vectors. On days 37 and 38, the mice were challenged with nebulized OVA for 30 min/day. AHR was measured 1 day after the last challenge. BALF was obtained, and lungs were fixed the following day (day 40)

[Normal View 6K | Magnified View 14K]

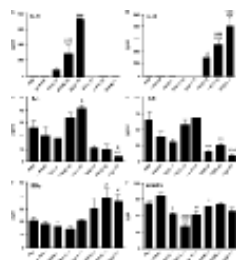


Figure 2. Dose-dependent effects of intratracheal Ad-IL-10 and Ad-IL-12 alone on BALF cytokine and chemokine profiles. Either Ad-IL-10 or Ad-IL-12 was administrated to OVA-sensitized and challenged BALB/c mice with different virus doses [Ad-IL-10 (1×10^8 pfu); 2Ad-IL-10 (2×10^8 pfu); 5Ad-IL-10 (5×10^8 pfu); Ad-IL-12 (1×10^8 pfu); 2Ad-IL-12 (2×10^8 pfu); and 5Ad-IL-12 (5×10^8 pfu)]. The protocol is described in Figure 1. The positive control (PBS) group consisted of mice that were immunized and challenged with OVA and treated with PBS instead of a vector. A mock virus (Ad-mock, 5×10^8 pfu) was also used as a control. BALF was performed 5 days after viral administration. The expression of (A) interleukin (IL)-10 (B) IL-12 (C) IL-4 (D) IL-5 (E) interferon (IFN)- γ , and (F) eotaxin in BALF were evaluated by ELISA. The results are expressed as the mean $\pm$ SEM of five to eight mice in each group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the PBS group. # $p < 0.05$ and ## $p < 0.01$ compared to the Ad-mock group

[Normal View 29K | Magnified View 73K]

We found that low-dose Ad-IL-10 treatment slightly decreased Th2-type cytokine secretion compared to medium- and high-dose Ad-IL-10 treatments. By contrast, the medium- and high-dose Ad-IL-10 treatments did not alter or even enhanced production of these cytokines. Furthermore, the data demonstrated that all of the various doses of Ad-IL-10 markedly decreased the eotaxin concentration relative to the PBS and mock groups. There were no apparent side-effects of Ad-IL-10 on body weight or hematology. However, although medium- and high-dose Ad-IL-12 treatments were better at reducing Th2-type cytokine production and inducing IFN- γ secretion than low-dose Ad-IL-12 treatment, exposure to high-dose levels of IL-12 in mice preferentially resulted in adverse effects, such as body weight loss or mortality. In addition, administration of all various doses of Ad-IL-12 notably alleviated the development of AHR compared to those of mice administered PBA or Ad-mock (Figure 3). Furthermore, low-dose but not medium- or high-dose Ad-IL-10 significantly reduced the severity of AHR. Taken together, under the consideration of therapeutic efficacy and safety, we suggest that co-administration of a low dose of Ad-IL-10 and Ad-IL-12 may be suitable to reduce

airway inflammation and AHR after allergen challenge.

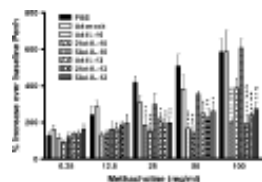


Figure 3. Effects of intratracheal Ad-IL-10 and Ad-IL-12 alone in OVA-induced AHR. The protocol is described in Figure 1. One day after the last OVA challenge, AHR was measured in response to increasing concentrations of methacholine (0–100 mg/ml) in conscious mice placed in a whole-body plethysmography. Data are reported as the percentage (%) increase in Penh over baseline values. The results are expressed as the mean $\pm$ SEM of five to eight mice in each group. Experiments were repeated two times with similar results. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the PBS group. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ compared to the Ad-mock group [Normal View 11K | Magnified View 31K]

Combined treatment with Ad-IL-10 and Ad-IL-12 decreased the severity of AHR

To assess the therapeutic effects of a low dose of combined Ad-IL-10 and Ad-IL-12 treatment on allergic asthma, both AHR and the accumulation of inflammatory cells in BALF were measured in an OVA-induced asthmatic animal model. As shown in Figure 1, OVA-sensitized mice were pretreated once intratracheally with 1×10^8 pfu/mouse of either Ad-IL-10, Ad-IL-12, or both, 2 days before the first OVA challenge. One day after the last OVA challenge, the airway responsiveness to aerosolized methacholine of each group of mice was measured (Figure 4). Mice sensitized and challenged with OVA but administered PBS or Ad-mock developed markedly increased airway responsiveness to methacholine inhalation compared to that of naïve mice, which were not sensitized but challenged with OVA. Although the treatment of mice with Ad-IL-10 slightly reduced the development of AHR, the difference was not significant. Administration of Ad-IL-12 alone partially decreased the severity of AHR. Furthermore, simultaneous administration of Ad-IL-10 and Ad-IL-12 resulted in a significant decrease in the development of AHR compared to mice given PBS or Ad-mock alone.

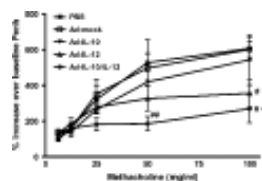


Figure 4. Co-administration of Ad-IL-10 and Ad-IL-12 suppressed methacholine-induced increases in airway hyperresponsiveness. Groups of OVA-sensitized mice received single intratracheal deliveries of mock virus (Ad-mock, 2×10^8 pfu/mouse), Ad-IL-10 (1×10^8 pfu/mouse) alone, Ad-IL-12 (1×10^8 pfu/mouse) alone, or Ad-IL-10 (1×10^8 pfu/mouse) plus Ad-IL-12 (1×10^8 pfu/mouse) 2 days before the first challenge with OVA. The protocol is described in Figure 1. Positive control mice were treated with PBS instead of a vector. One day after the last OVA challenge, AHR was measured by non-invasive whole-body plethysmography. Data are reported as the percentage (%) increase in Penh over baseline values. The results are expressed as the mean $\pm$ SEM of five to eight mice in each group. Experiments were repeated three times with similar results. * $p < 0.05$ compared to the PBS group. # $p < 0.05$ and ## $p < 0.01$ compared to the Ad-mock group [Normal View 8K | Magnified View 19K]

Combined treatment with Ad-IL-10 and Ad-IL-12 decreased eosinophil and neutrophil infiltration in BALF cells

The influx of inflammatory cells into lungs 48 h after the last OVA challenge was examined. In PBS-treated mice, exposure to aerosolized OVA induced a marked increase in the number of eosinophils and neutrophils in BALF (Figure 5). The delivery of Ad-mock did not decrease airway inflammation in this murine model of asthma. By contrast, the combination of Ad-IL-10 and Ad-IL-12 significantly reduced the increases in eosinophils and neutrophils. A similar result was also found in mice treated with Ad-IL-10 and Ad-IL-12 alone.

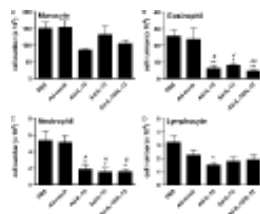


Figure 5. Co-administration of Ad-IL-10 and Ad-IL-12 alleviated allergen-induced airway inflammation. Groups of mice were treated as described in Figure 4. Two days after the last OVA challenge, mice were sacrificed and BALF was collected. Cell compositions of (A) monocyte, (B) eosinophil, (C) neutrophil and (D) lymphocyte in BALF were analysed and are expressed as the mean $\pm$ SEM of five to eight mice in each group. Experiments were repeated three times with similar results. * $p < 0.05$ and ** $p < 0.01$ compared to the PBS group. # $p < 0.05$ and ## $p < 0.01$ compared to the Ad-mock group

[Normal View 19K | Magnified View 45K]

Combined treatment with Ad-IL-10 and Ad-IL-12 alleviated the severity of peribronchial inflammation

A pathological examination of lung sections revealed that PBS and mock groups had greater degrees of inflammatory cell infiltration in the airways compared to naïve mice (Figure 6). The extent of damaged and infiltrated cells was less severe in the Ad-IL-12-treated group. After treatment with Ad-IL-10 or Ad-IL-10/Ad-IL-12, mice showed significant inhibitory effects on peribronchial inflammation compared to the PBS and mock groups. These results showed that co-administration of Ad-IL-10 and Ad-IL-12 efficiently inhibited the infiltration of cells and reduced the pathological damage within the lungs in this mouse model.

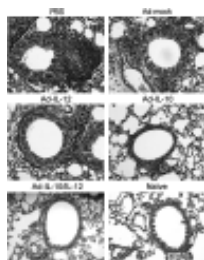


Figure 6. Histological examination of paraffin-embedded sections of lung tissues. Mice were treated as described in Figure 4. On day 40, lung sections were obtained from negative control (naïve) and virus-free (PBS) mice, and those pretreated with Ad-mock, Ad-IL-10 alone, Ad-IL-12 alone, or Ad-IL-10 plus Ad-IL-12. Sections, 5 μ m thick, were prepared and stained with H&E for the morphological analysis. Tissues were examined by light microscopy (original magnification: $\times 400$)

[Normal View 84K | Magnified View 146K]

Combined treatment with Ad-IL-10 and Ad-IL-12 decreased eotaxin, KC, IL-4, and IL-5 levels in BALF

Eotaxin and KC are the most potent chemokines for, respectively, recruiting eosinophils and neutrophils. Thus, evaluation of these chemokine levels in BALF showed that the administration of Ad-IL-10 combined with Ad-IL-12 significantly decreased both eotaxin and KC production (Figure 7). These results indicated that the co-administration of Ad-IL-10 and Ad-IL-12 reduced the secretion of eotaxin and KC, which then subsequently decreased the numbers of eosinophils and neutrophils recruited. Furthermore, we assessed the levels of two important Th2 cytokines, IL-4 and IL-5, in BALF in different groups of mice. Although levels of IL-4 and IL-5 decreased in both Ad-IL-12- and combined Ad-IL-10/IL-12-treated groups, the combined treatment exhibited a greater synergistic therapeutic effect than did Ad-IL-12 treatment alone compared to treatment with PBS or Ad-mock. In addition, we observed significant enhancement of BALF IFN- γ expression in mice treated with Ad-IL-12 alone or Ad-IL-12 plus Ad-IL-10. Notably, the TNF- α level in BALF obviously increased with the delivery of Ad-IL-12 in OVA-sensitized mice.

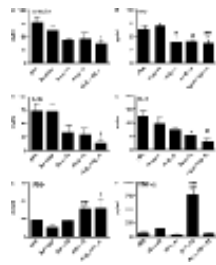


Figure 7. Levels of eotaxin, KC, IL-4, IL-5, and TNF- α decreased in BALF of mice receiving combined Ad-IL-10 and Ad-IL-12 treatment. Two days after the last OVA challenge, mice were sacrificed and BALF was collected. (A) Eotaxin, (B) KC, (C) IL-4, (D) IL-5, (E) IFN- γ and (F) TNF- α levels in the BALF of different groups of mice were measured by ELISA. The results are expressed as the mean $\pm$ SEM of five to eight mice in each group. Experiments were repeated two times with similar results. * p < 0.05, ** p < 0.01, and *** p < 0.001 compared to the PBS group. # p < 0.05, ## p < 0.01, and ### p < 0.001 compared to the Ad-mock group [Normal View 24K | Magnified View 57K]

Discussion



The cytokine, IL-12, has an essential role in Th1 development and has therefore also been considered to be a potential therapy for asthma. Studies have demonstrated that administration of IL-12 during allergen challenge inhibits airway eosinophilia and AHR but not immunoglobulin E production [4][9][10]. The exact mechanism responsible for the inhibitory effects of IL-12 is not well understood, and it is believed that IL-12 exerts its effect directly or requires the production of other cytokines, such as IFN- γ and IL-10, to mediate redirection of the allergen-specific Th2 response [30][32]. Because IL-10 is an important endogenous down-regulator of Th1 and Th2 cytokine synthesis, this cytokine should participate in the development of antiallergic effects observed after IL-12 administration. Indeed, neutralizing anti-IL-10 antibodies abolished the inhibition of airway eosinophilia in IL-12p35^{-/-} mice [33]. In addition, it was reported that Th type 1-like regulatory cells developed in the presence of IL-10 and IL-12 [34]. These Th1-type regulatory cells are able to suppress allergic airway inflammation in the absence of any Th1-related lung pathology. In the present study, we therefore tested whether IL-12 and IL-10 are both required to improve asthma and whether or not they conflict with one another in an established experimental model.

Although adenovirus-based gene therapy has demonstrated great promise for delivering therapeutic genes to the lung, dose-dependent acute inflammation and host local immune responses induced by vectors can shorten transgene expression. High doses of adenoviral vectors may lead to marked lung injury and speed up adenoviral clearance with lower transgene expression. To estimate the transgene expression and airway inflammation caused by adenoviral vectors, we delivered varying doses of Ad-IL-10 or Ad-IL-12 alone and determined that a low dose of these vectors could reach therapeutic levels and only induced very modest inflammatory responses. Furthermore, we used a low dose of combined Ad-IL-10 and Ad-IL-12 treatment in an OVA-sensitized murine asthma model. The results obtained clearly demonstrated that the single intratracheal co-administration of Ad-IL-10 plus Ad-IL-12 or Ad-IL-12 alone was capable of down-regulating allergic inflammation such as AHR and inflammatory cell recruitment in BALF. On the other hand, administration of Ad-IL-10 alone did not efficiently suppress the development of AHR, but it was able to reduce the severity of airway inflammation. There is evidence that IFN- γ plays a role in the development of AHR. In guinea pigs, IFN- γ enhanced β -adrenergic-mediated relaxation of airway smooth muscle [35] and, under this scenario, the reduction in IFN- γ secretion by IL-10 treatment may increase AHR. Alternatively, there is also evidence indicating that IFN- γ induces AHR in a murine model of allergic asthma [36]. Therefore, whether changes in IFN- γ levels can explain the IL-10-induced airway reactivity observed in the present study remains unclear.

The role of IL-10 in limiting AHR and airway inflammation is controversial. Some studies have indicated that IL-10^{-/-} mice exhibit exaggerated eosinophilic airway inflammation [27][37], whereas another study found that IL-10^{-/-} mice showed diminished airway inflammation [38]. In addition, Makela *et al.* [26] demonstrated that IL-10 enhanced AHR or was required for the development of AHR, although it reduced eosinophilia. However, the major problem with using IL-10^{-/-} mice as an asthma model is that an IL-10 deficiency may affect the entire process of allergic reactions, from the sensitization phase to the effector phase, as a result of recall challenge with an allergen. Recently, Matsumoto *et al.* [39] used an anti-IL-10 antibody to demonstrate that IL-10 plays a distinct role in the early and late phases of asthmatic responses. The data presented in the present study showed that IL-10 was equally effective in preventing both neutrophil and eosinophil infiltration in the airways of allergen-challenged mice. This suggests that IL-10 may modulate the local release of preformed and thus rapidly released cytokines, which are involved in the migration of these two cell types to the lung. In addition, the results obtained in the present study are consistent with other studies reporting

that IL-10 treatment increases airway reactivity [26][40]. By contrast, another study demonstrated that administration of adenovirus-expressing IL-10 into the lungs of granulocyte-macrophage colony-stimulating factor-treated mice inhibited AHR [6]. These conflicting results showing both pro-inflammatory and anti-inflammatory roles for IL-10 in asthma might reflect the pleiotropic nature of IL-10. On the other hand, differences in doses of IL-10 and the timing of IL-10 administration might also at least partially explain these differences.

It is known that the polarization of primed Th2 cells is hard to revert as a result of loss of IL-12 receptor expression by these cells [41]. Despite this, previous studies showed that local IL-12 gene transfer to the lung before allergen challenge can suppress antigen-induced eosinophilic airway inflammation [31]. It appears that the inhibitory effects of IL-12 are not directly mediated by T cell polarization. It was reported that high systemic levels of IL-12 can directly induce apoptosis of eosinophils in the lungs [42]. Nutku *et al.* [43] also demonstrated that IL-12 promotes *in vitro* human eosinophil apoptosis in a dose-dependent manner. Another possibility is the induction of IFN- γ production by IL-12, and it was shown that IFN- γ can prevent antigen-induced CD4⁺ T-cell infiltration into tissues, thereby reducing the recruitment of eosinophils [44].

Histological studies revealed that combined treatment with Ad-IL-10 and Ad-IL-12 was able to reduce OVA-induced peribronchial inflammation. However, mild mononuclear cell infiltration was detected in the lungs and BALF when Ad-IL-12 was given alone before allergen challenge. We propose that the increase in TNF- α modulates the inflammatory reaction observed after IL-12 therapy. The participation of TNF- α in the allergic reaction is linked to different observations, including its release by alveolar macrophages, mast cells, and basophils. High levels of TNF- α were found in monocytes and epithelial cells from asthmatics [45]. Thus, it is possible that treatment with IL-12 augments the production of TNF- α in the lungs. Once released into the airways, TNF- α directly stimulates the expression of adhesion molecules by endothelial cells. These molecules, which include intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 are responsible for the adhesion of leukocytes to the endothelium. This is followed by their transendothelial migration and subsequent recruitment into the inflamed area. It is noteworthy that IL-10 reduced TNF- α release [46], and macrophages are a widely described cell type sensitive to this cytokine. Accordingly, the results obtained in the present study showed that IL-10 suppressed the production of TNF- α and inhibited cellular recruitment into airways of OVA-sensitized mice.

It is generally accepted that T cells are the main source of IL-5 in murine models of allergen-induced airway inflammation. However, in contrast to IL-5, both IL-4 and TNF- α can be produced by mast cells, eosinophils, and monocytes/macrophages during asthmatic inflammation. Thus, we suggest that IL-10 can directly affect the ability of these cells to produce cytokines. In addition, it was also shown that enhancement of IFN- γ in antigen-challenged lungs is associated with significant decreases in IL-4 and IL-5 [31][47], and previous studies showed that treatment with IL-12 inhibits the Th2-cell response by IFN- γ production [4][31]. Indeed, the results obtained in the present study demonstrated that Ad-IL-10- and Ad-IL-12-treated mice showed significantly decreased levels of IL-4, IL-5, and TNF- α secretion in BALF. This leads to an alleviation of airway inflammation.

In summary, the present study demonstrates that supplementation with low doses of combined Ad-IL-10 and Ad-IL-12 gene therapy exerted synergistic and not antagonistic effects in modulating airway inflammation. It also possibly implies that the regulatory mechanisms of IL-10 and IL-12 in suppressing experimental asthma somewhat differ. However, the results obtained in the present study indicated that modulation of allergic asthma is complex and involves regulatory mechanisms not predicted by the Th1/Th2 paradigm. IL-10 may be one of the molecules involved in 'allergen control', and we suggest the concept of IL-10 administration in combination with IL-12 as an immunoregulatory strategy. A more complete understanding of the inhibitory roles of IL-10 and IL-12 is particularly important and is a prerequisite for developing therapies focused on immunomodulation of deleterious Th2-polarized responses in asthma.

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