

Dendritic cells modulated by cytokine-expressing adenoviruses alleviate eosinophilia and airway hyperresponsiveness in an animal model of asthma

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Background: It has been found that T_H1-related cytokines can decrease the accumulation of eosinophils in lung tissue and relieve airway constriction.

Objective: Dendritic cells (DCs) have been found to prime naive T-helper cells efficiently. In this study, DCs infected with T_H1 cytokine-expressing adenovirus can be used to induce antigen-specific T_H1 cells for treatment in an animal model of asthma.

Methods: Cytokine gene-modulated DCs pulsed with ovalbumin antigen (OVA) were injected intravenously into naive mice 1 week before sensitization with OVA antigen. The mice were then monitored for OVA-specific IgE, airway inflammatory cell infiltration, and airway hyperresponsiveness in the study.

Results: Significant levels of IL-12 or IL-18 were expressed by Ad-IL-12 or Ad-IL-18 infected, bone marrow-derived DCs. Ad-IL-12 and Ad-IL-18 co-infected DCs effectively, decreasing sera anti-OVA IgE antibody levels, lung eosinophilia, and airway hyperresponsiveness.

Conclusion: We concluded that DCs modulated by T_H1-prone cytokine-expressing adenoviruses can alleviate T_H2-type airway inflammation in a murine model and can provide possible therapeutic application for DCs in asthma. (J Allergy Clin Immunol 2004;114:88-96.)

Key words: Asthma animal model, cytokine expressing adenovirus, dendritic cell

There is strong evidence regarding the therapeutic effect of T_H1 cytokine administration. T_H1-related cytokine proteins,¹⁻³ cytokine plasmids,⁴⁻⁹ and adenovirus vectors expressing cytokine genes^{10,11} have been applied to decrease airway inflammation in allergen presensitized

Abbreviations used

AHR: Airway hyperresponsiveness
BAL: Bronchoalveolar lavage
DCs: Dendritic cells
GFP: Green fluorescence protein
MOI: Multiplicity of infection
OVA: Ovalbumin

mice. The therapeutic effect of microbial products on asthmatic inflammation also appears to be mediated through the induction of T_H1 responses.¹²⁻¹⁵ In our previous study we found that direct administration of IL-12 suppressed allergen-induced eosinophil infiltration. However, it did not completely suppress the development of T_H2 cells after multiple antigen challenge in the absence of IL-12.¹⁶ Because of the short half-life of the cytokine proteins and the non-antigen-specific treatment, it failed to induce a memory response in the T helper cells. For these reasons, more powerful strategies for priming allergen-specific type 1 T helper cells should be developed.

Dendritic cells (DCs) are noted for their distinct ability to initiate T-cell responses. In the application of DC lineage-specific suicide gene thymidine kinase transgenic mice, airway inflammation, including eosinophilia and goblet cell hyperplasia, abrogates after treatment with ganciclovir.¹⁷ Furthermore, the cytokine profile secreted by DCs can influence the differentiation of T cells. Many studies have demonstrated the ability of DCs to produce IL-12 after stimulation of CD40 ligand, LPS, oligo CpG nucleotides,¹⁸ or double-stranded RNA.¹⁹ In contrast, prostaglandin E₂, β_2 -agonist, TGF- β , IL-1, TNF- α , or Fas engagement leads to the development of IL-12 non-secreting DCs.²⁰ The heterogeneity of DC itself or different end after stimulation made the manipulation of DCs for therapeutic purposes more difficult.

Although IL-18 can enhance basophil secretion of IL-13 and IL-4 in the presence of IL-3,²¹ eotaxin expression is also enhanced by intratracheal injection with IL-18.²² It has been known for its T_H1-promoting effect when accompanied with IL-12.³ We aim to develop more effective strategies for priming allergen-specific type 1 T

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helper cells. With this goal in mind, mice were treated with DCs that express IL-12 and/or IL-18 by adenoviral vector infection and monitored for airway eosinophilia. The data suggested that IL-12 and IL-18 co-expressing DCs can decrease allergen-specific IgE and airway eosinophilia in murine model of asthma.

METHODS

Mice

BALB/c mice between 6 to 8 weeks of age were obtained from and maintained in the Animal Center of the College of Medicine of National Taiwan University. Animal care and handling conformed to the NIH Guide for the Care and Use of Laboratory Animals.

Cytokine-expressing adenovirus vectors

Cytokine- and green fluorescence protein (GFP)—expressing adenovirus vectors were from Dr Tao's laboratory (Academia Sinica, Taipei). Construction of the single-chain IL-12 gene was performed as previously described.^{9,23} The p40 and p35 subunits of the murine IL-12 gene were connected by a linker and generated the single-chain IL-12 gene. Mouse IL-12 (Ad-IL-12) gene or IL-18 gene (Ad-IL-18) was inserted into the pAdhPGK vector. As a control, Ad-Mock carried no cytokine transgene.

Preparation of bone marrow—derived DCs

Tibial and femoral bone marrow from 5- to 6-week-old BALB/c mice was isolated. The bone marrow cells were cultured in RPMI-1640 and 5% FBS complete medium with IL-4 (1000 U/mL) and GM-CSF (750 U/mL) at day 0. On day 2, the fresh medium containing IL-4 (3000 U/mL) and GM-CSF (750 U/mL) was resupplemented. Suspended cells were collected by pipette and changed into new culture plates on day 4. At the same time, ovalbumin (OVA) (500 µg/mL) was added into each well. Different cytokine-expressing adenoviruses (multiplicity of infection [MOI], 3000) were used to infect the DCs on day 6.

Establishment of the animal model of airway inflammation

Briefly, the mice were sensitized by intraperitoneal injections of 20 µg OVA (Sigma, St Louis, Mo) mixed with 2 mg of alum (Pierce, Rockford, Ill) as an adjuvant on day 0. On day 14, the mice were boosted with 50 µg OVA mixed with 2 mg of alum and exposed to aerosolized OVA solution (5% in normal saline). DCs modulated by cytokine-expressing adenoviruses without pulsing OVA underwent the same procedure to examine the preventive effects of engineered DCs. The mice given intraperitoneal injection of 1×PBS in each immunization were regarded as the negative control group. On day 28, mice were exposed to aerosolized 5% OVA for a 20-minute period by placing them in a chamber at a time on each day for 3 days. The output of the nebulizer was 0.3 mL/min, and the particles produced were 0.5 to 5 µm in size. The concentration of OVA in the ultrasonic nebulizer was 5 % (wt/vol, in normal saline; DeVilbiss, Somerset, Pa).

Serum level of anti-OVA antibody in sensitized mice

Sera anti-OVA IgE and IgG2a antibody titers were determined by ELISA. Briefly, 96-well microtiter plates were coated with 10 µg/mL OVA. Serum samples were added to each well for an overnight period. Then either biotin-conjugated anti-mouse IgE (1 µg/mL; The Binding

Site, Birmingham, United Kingdom) or IgG2a (1 µg/mL; PharMingen, San Diego, Calif) was added for 1 hour at 37°C. Streptavidin-conjugated alkaline phosphatase (1:2000; Sigma) was added for an additional 2 hours at room temperature. Finally, the reaction was developed by 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid), and the absorbance was determined at 420 nm in a microplate reader (Anthos reader 2010; Anthos Labtec Inc, Salzburg, Austria).

Cytokine profile of CD4⁺ T cells

Cells that were isolated from spleens were stained with anti-CD4—coated magnetic microbeads (Miltenyi Biotec, Sunnyvale, Calif) and isolated by magnetic-activated cell sorter methods. The splenocytes from naive mice were also prepared and irradiated with 3000 rads by a γ -irradiator to be used as antigen-presenting cells. Then 1.5×10^5 CD4⁺ cells were co-cultured with 1.5×10^5 irradiated naive splenocytes and 20 µg/mL OVA in serum free lymphocyte AIM-V medium (Gibco, Invitrogen Corporation, Carlsbad, Calif) containing 2% serum replacement TCM (Celox Laboratories, Inc, Hopkins, Minn) for 48 hours in 96-well culture plates. The cultured supernatant was collected and stored at -20°C for cytokine ELISA assay.

Bronchoalveolar lavage and cellular differential counts

The mice were bled from the retro-orbital venous plexus and then killed. With a cannula, their lungs were immediately lavaged through the trachea. The lavaged fluid was centrifuged at 400g for 10 minutes at 4°C. After washing, the cells were resuspended, and the total cells were cytocentrifuged and then stained with Liu's stain for differential cell counts. On the basis of standard morphologic criteria, a minimum of 200 cells were counted and classified as macrophages, lymphocytes, neutrophils, or eosinophils.

Airway hyperresponsiveness

Buxco system (Biosystem XA; Buxco Electronics Inc, Troy, Conn) was used to evaluate the extent of airway constriction in different groups of mice following the protocol described previously.²⁴ Penh is equal to $\text{Pause} \times \text{PIF}/\text{PEF}$, where $\text{Pause} = (\text{Te} - \text{Tr})/\text{Tr}$ (PIF, peak inspiratory flow; PEF, peak expiratory flow; Te, expiratory time; Tr, relaxation time). In this experiment the mice were challenged 3 times with 5% OVA in normal saline. At 24 hours after final inhalation, the mice were given aerosolized normal saline, 6.25 mg/mL, 12.5 mg/mL, 25 mg/mL, or 50 mg/mL methacholine (Sigma) serially. The average Penh value was divided by the Penh of normal saline and was presented as the relative percentage increase of Penh.

Histopathologic study of lung tissue

The lungs were immediately removed after bronchoalveolar lavage (BAL) and fixed in a solution of 3% vol/vol formalin (in 0.01 mol/L phosphate buffer, pH 7.2). The tissues were subsequently embedded in paraffin and cut into 5-µm thick sections. These sections were stained with hematoxylin-eosin and examined by light microscopy for histopathologic changes.

Measurement of cytokine or chemokine expression

The levels of cytokines or chemokines in the culture supernatants or BAL fluid were assayed by sandwich ELISA kit (R&D Systems, Minneapolis, Minn) according to the manufacturer's instructions. The sensitivity of this assay was 15.6 pg/mL for IL-4, 31.3 pg/mL for

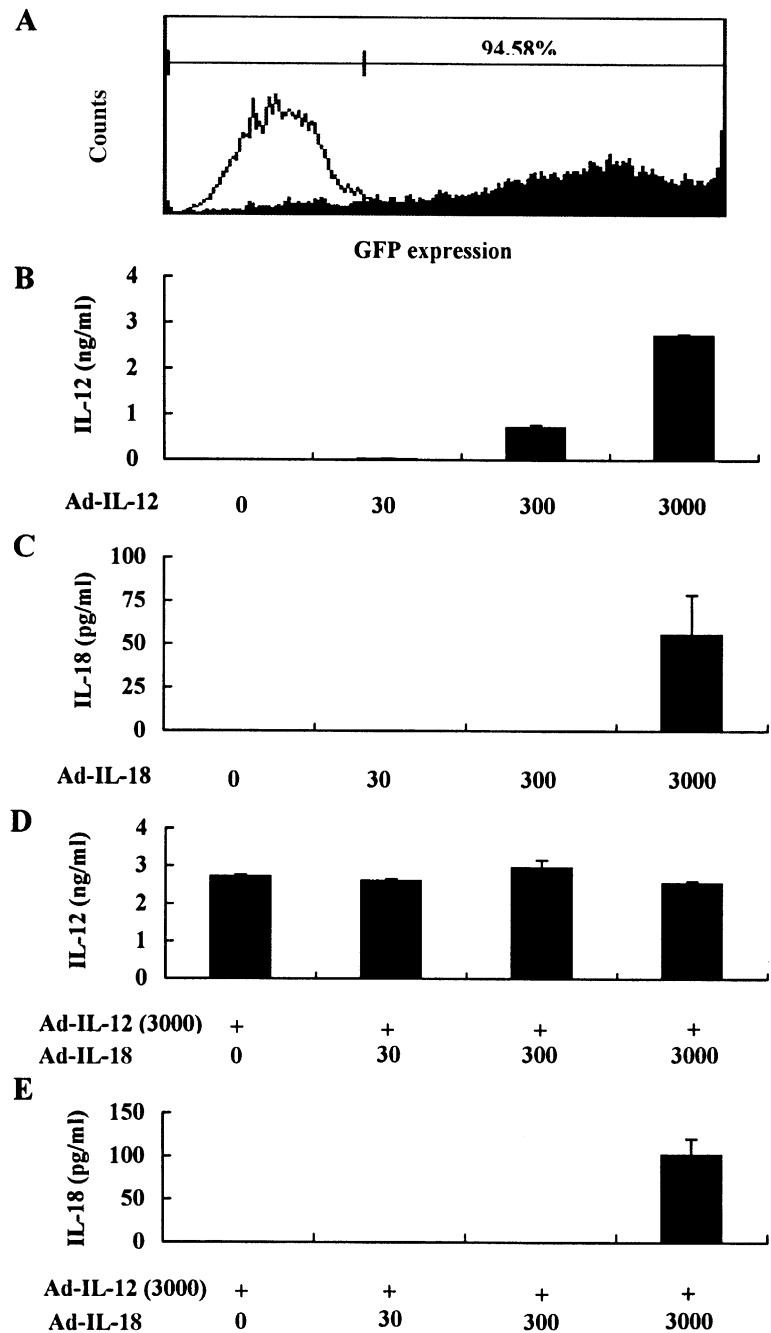


FIG 1. The expression efficiency of adenovirus-infected DCs. The *bold area* represents the GFP expression after Ad-GL-1 infection, and the *filled line* represents DCs without Ad-GL-1 infection (**A**). Different MOI of Ad-IL-12 (**B**) or Ad-IL-18 (**C**) or 3000 MOI of Ad-IL-12 combined with different MOI of Ad-IL-18 infected DCs (**D**, **E**) were analyzed by ELISA assay. Data represent the means $\pm$ SEMs.

IFN- γ , 23.4 pg/mL for IL-12 p70, 62.5 pg/mL for IL-18, 39.1 pg/mL for IL-13, 1.9 pg/mL for eotaxin, and 2.0 pg/mL for KC, respectively.

Statistical analysis

Individual experimental values were compared with the Student *t* test. Differences between the 2 differently treated groups of mice were considered statistically significant at *P* values less than .05 or .01.

RESULTS

Cytokine expression from Ad-IL-12 or Ad-IL-18 infected DCs

To evaluate the infection efficiency of pAdhPGK adenovirus vectors, the fluorescence expression level derived from DCs infected with Ad-GL-1 was assayed (Fig 1, A). The high percentage of DCs after Ad-GL-1

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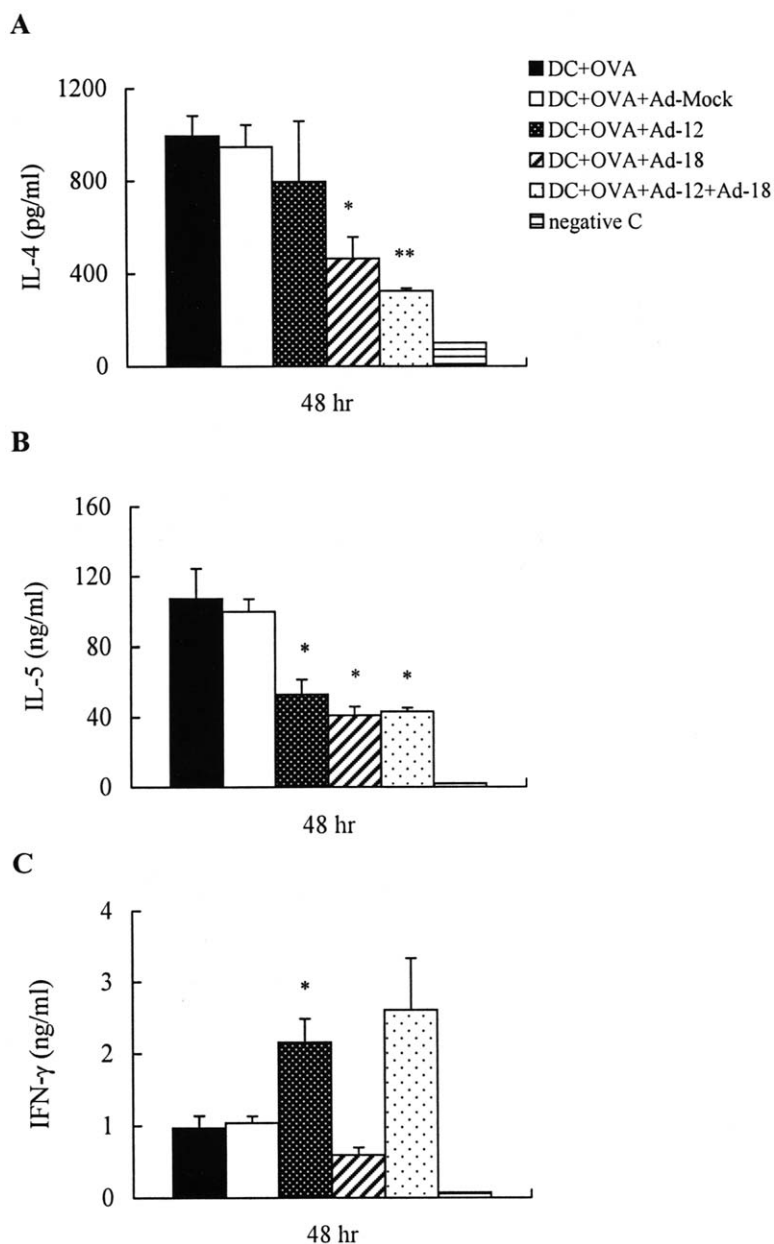


FIG 2. The cytokine levels of CD4⁺ positive splenocytes from cytokine gene-modulated DC treated mice. CD4⁺ positive cells were co-cultured with OVA and irradiated splenocytes for 48 h in 96-well culture plates and then the cultured supernatant was collected for IL-4 (**A**), IL-5 secretion (**B**), or IFN-γ (**C**) cytokine assay. * $P < .05$; ** $P < .001$; each adenovirus infected group vs DCs pulsed with OVA group.

infection expressed green fluorescence (94.58%). The DC viability was only impaired slightly by adenovirus infection (DC plus OVA only, 90.1%; Ad-Mock infected DC, 89.8%; Ad-IL-12 infected DC, 81.8%; Ad-IL-18 infected DC, 86.8%; co-infected DC, 72.7%). IL-12 and IL-18 expression by each infected DC was measured by ELISA. Ad-IL-12 infected DCs secreted IL-12 in a dose-dependent pattern (Fig 1, B). The IL-18 protein expression that was detected from the MOI titer of Ad-IL-18 virus was only 3000 (Fig 1, C). Co-infection with Ad-IL-12 and Ad-IL-18 did not influence the expression level of IL-12

(Fig 1, D) or IL-18 (Fig 1, E). Neither IL-12 nor IL-18 was detectable in the supernatant of DCs pulsed with OVA only (data not shown).

Cytokine-expressing adenovirus-modulated DCs can influence the levels of cytokine and different isotypes of anti-OVA antibody in an animal model of asthma

To study the effect of T_H1 or T_H1-related cytokine-modulated DCs in vivo, naive mice received

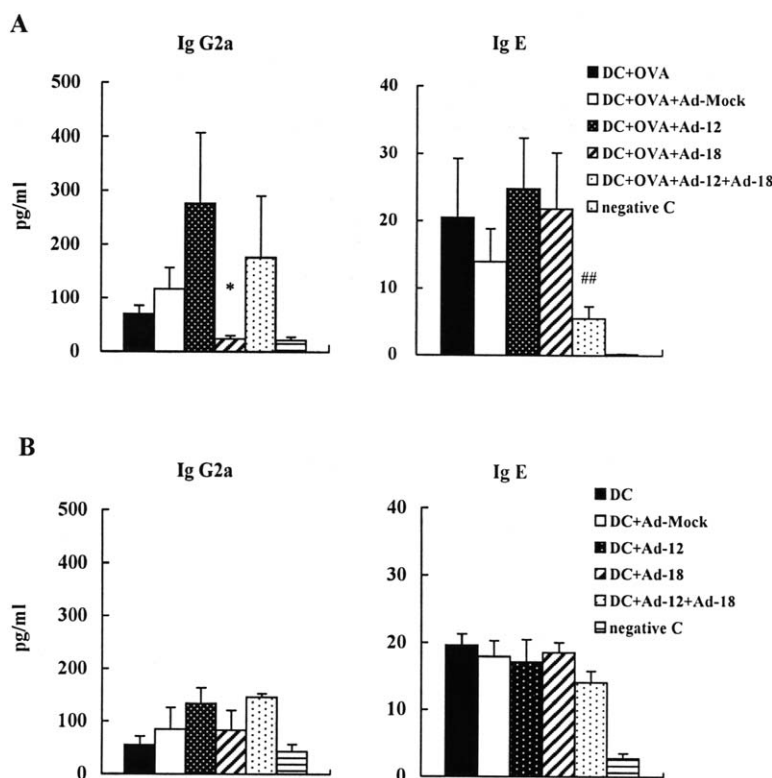


FIG 3. Anti-OVA antibody expression levels of DC-treated mice. The IgG2a and IgE anti-OVA antibody expression from mice that received DCs pulsed with OVA (**A**) or without OVA (**B**) was measured by ELISA and shown as pg/mL. * $P < .05$ compared with the DC pulsed OVA group; ## $P < .01$ compared with the Ad-IL-12 infected group.

intravenous injection with Ad-IL-12, Ad-IL-18, or co-infected DCs 7 days before the first immunization. After OVA immunization on day 0 and day 14, the cytokine profile of $CD4^+$ T cells for each group of mice was measured. IL-4 secretion by $CD4^+$ T cells from Ad-IL-18 and co-infected DC treated mice was inhibited (Fig 2, A). $CD4^+$ T cells from mice that received OVA-pulsed DCs released more IL-4 and IL-5 than negative control mice, which received no DCs at all. Although IL-4 cytokine secretion from Ad-IL-12 treated mice was not decreased, IL-5 cytokine expression was inhibited in the same manner as the Ad-IL-18 and Ad-IL-18 co-infected DC treated mice (Fig 2, B). Only Ad-IL-12 and co-infected DC treated mice induced significant IFN- γ secretion (Fig 2, C).

The IgG2a and IgE anti-OVA antibody expression from mice that received DCs pulsed with OVA (Fig 3, A) or without OVA (Fig 3, B) was measured by ELISA. Ad-IL-18 infected DCs inhibited the IgG2a antibody secretion on day 31. Although the Ad-IL-12 or co-infected DCs enhanced IgG2a secretion, only Ad-IL-12 and Ad-IL-18 co-infected DCs efficiently inhibited IgE secretion. Mice that received Ad-IL-12 or co-infected DCs that were not pulsed with OVA did not induce IgG2a expression and suppress IgE production.

Ad-IL-12 and Ad-IL-18 coinfecting DCs decrease the airway eosinophilia and hyperresponsiveness

Cytokine-modulated DC treated mice were challenged with aerosol OVA in a chamber by using an ultrasonic nebulizer on days 28 to 30. Co-infected DCs alleviated the airway hyperresponsiveness (AHR) compared with other groups in mice stimulated with 50 mg/mL methacholine (compared with DCs pulsed with OVA only; $P < .01$) (Fig 4, A). The eosinophils in BAL fluid decreased in both Ad-IL-12 and co-infected DC treated mice (Fig 4, B). Mice that received Ad-IL-12 or co-infected DCs without pulsed OVA did not alleviate eosinophilia when compared with DC-treated mice (data not shown). The result suggested that antigen-specific Ad-IL-12 and Ad-IL-18 co-infected DCs were necessary for this preventive effect.

Inflammatory mediators such as eotaxin, KC, and IL-13 were also analyzed. Correlatively, the eotaxin expression in BAL fluid decreased in both Ad-IL-12 and co-infected DC treated mice (Fig 5, A). KC is a functional homologue of human IL-8 protein and could induce inflammation in transgenic mice²⁵ or mice that received intratracheal KC administration.²⁶ On the other hand, IL-13 plays an

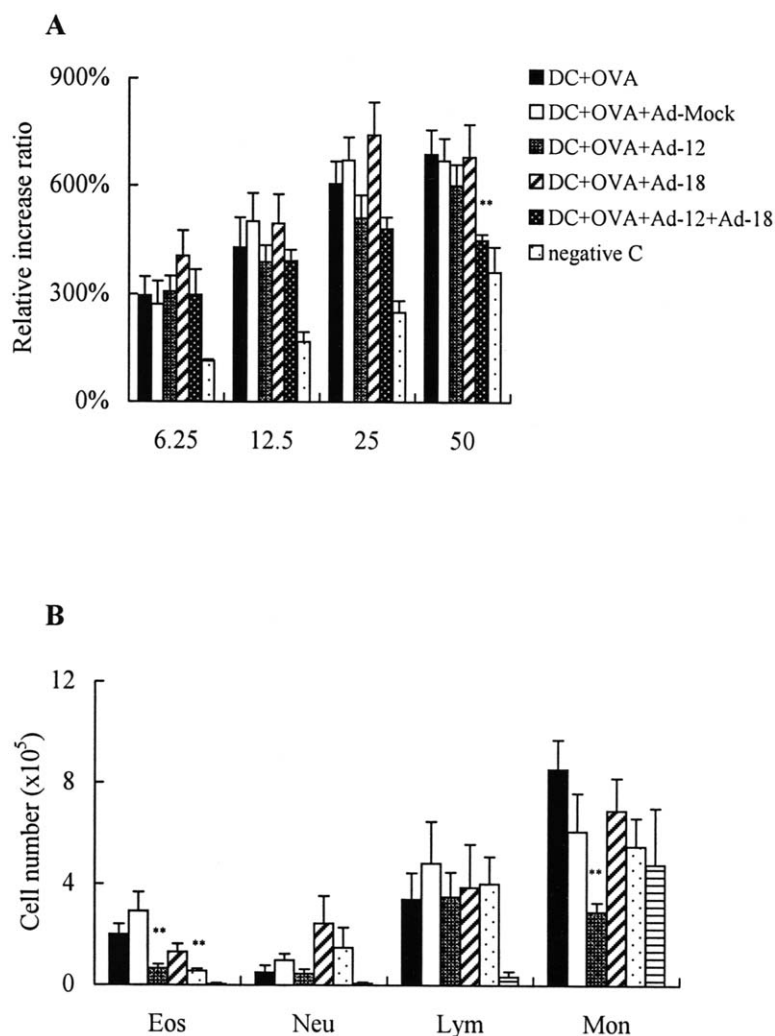


FIG 4. Preventive effect of modified DCs on AHR. The value of AHR (**A**) was measured after the challenge, and then BAL fluid samples were collected. Cells were counted and classified as monocytes (*Mon*), lymphocytes (*Lym*), neutrophils (*Neu*), or eosinophils (*Eos*) (**B**). Data represent the means $\pm$ SEMs of 7-8 mice per group. ** $P < .01$, compared with DCs pulsed with OVA group.

important role in the pathogenesis of allergic inflammation. It can cause an inflammatory phenotype and increase airway resistance and AHR.²⁷ In this study, the level of KC protein (Fig 5, B) and IL-13 (Fig 5, C) in BAL fluid decreased in co-infected DC-treated mice.

Effect of cytokine gene-modulated DCs on histopathologic change of lung tissue

The data showed extensive cellular infiltration of the periairway region in the group of mice treated with DCs pulsed with OVA only or infected with Ad-Mock or Ad-IL-18. In contrast, lung tissue from Ad-IL-12 or co-infected DC treated mice demonstrated a less severe case of histologic inflammation.

DISCUSSION

The applications of T_H1 cytokine for treatment of asthmatic inflammation have been studied profoundly. However, the short half-life of cytokine protein and the non-antigen-specific nature of treatment does not easily induce the memory responses of T helper cells for purposes of long-term prevention.¹⁶ That limitation makes it difficult to improve on current application for clinical therapeutic purposes.

DCs are the most important antigen-presenting cells for priming naive T helper cells.²⁸ In addition to priming naive T helper cells, DCs can determine the cytokine profile of T cells together with other factors in the lung environment. In different sites, such as mucosal lymphoid

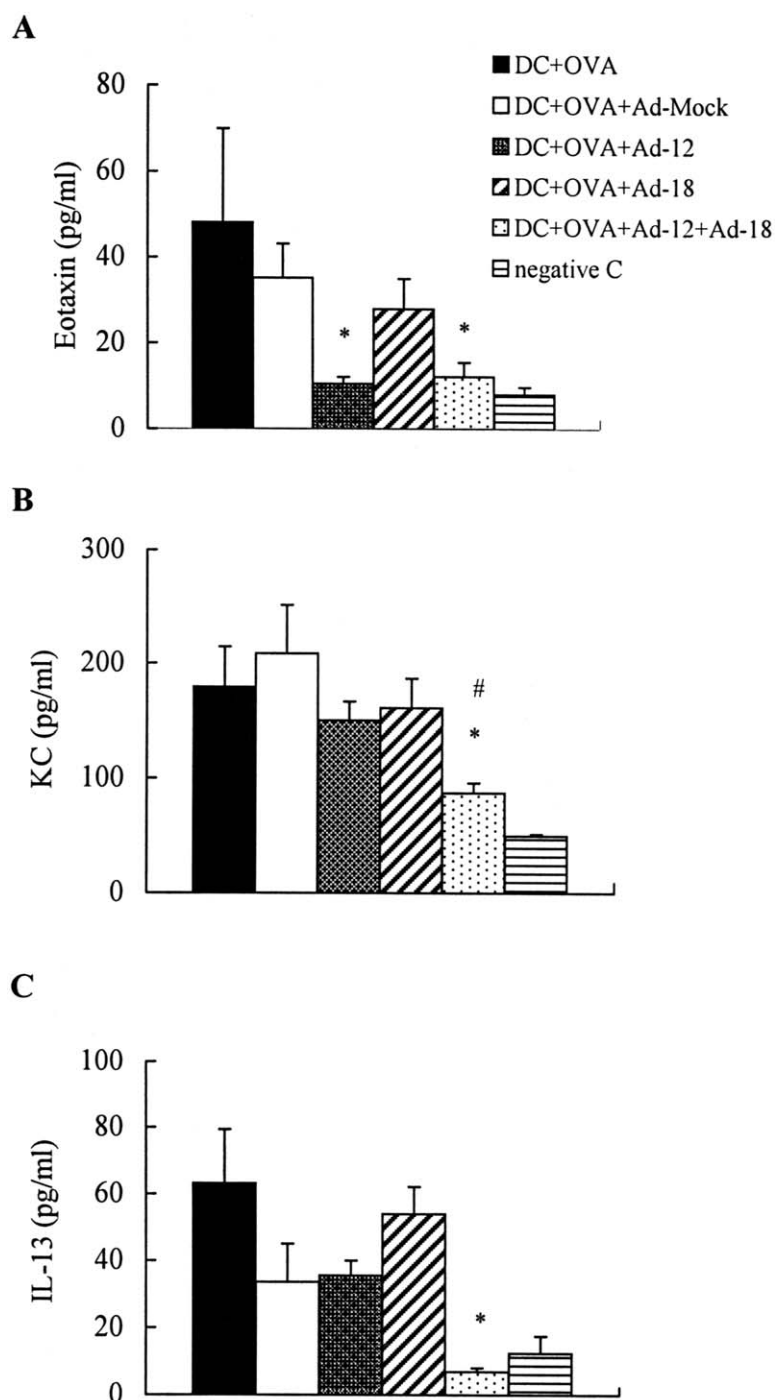


FIG 5. Inflammatory mediator levels were analyzed in BAL fluid. BAL fluid samples were collected after the challenge. Eotaxin (A), KC (B), and IL-13 (C) levels in BAL fluid from mice were measured by ELISA assay. Data represent the means $\pm$ SEMs of 5-7 mice per group. * $P < .05$, compared with DC+OVA+Ad-mock group; # $P < .05$, compared with DC+OVA+Ad-IL-12 group.

tissue, freshly isolated gut-associated DCs²⁹ or resting respiratory tract DCs³⁰ preferentially induce T_H2 immune response. Furthermore, it is reported that mature DCs cannot express IL-12 as much as immature DCs because of the down-regulation of the IFN- γ receptor.³¹ The heterogeneity of DCs as a result of different precursors and

manipulation makes it difficult to control for therapeutic purposes.

The most recent data suggest that allergen-pulsed DCs modulated by ribavirin or CpG deoxyoligonucleotide can decrease IgE level and airway inflammation.³² In this study we modified the DCs for inducing cytokine protein

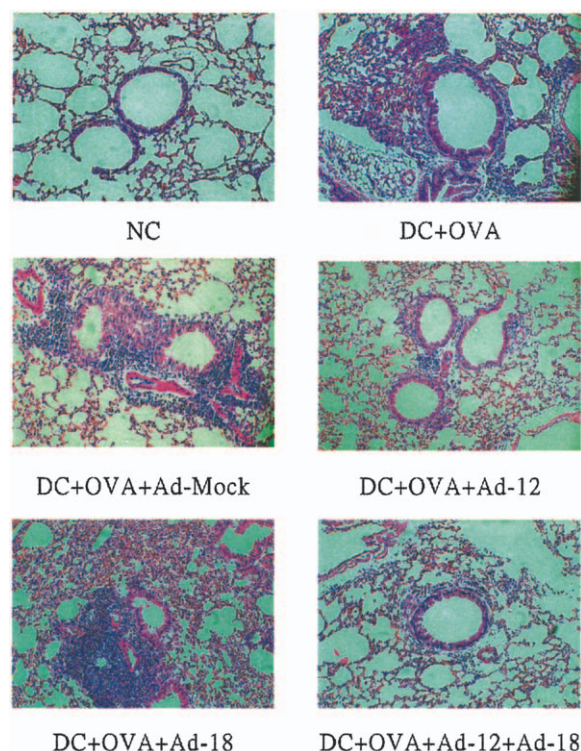


FIG 6. Histopathologic study of lung tissue in mice that received the cytokine-modulated DCs. Histopathologic changes were examined by light microscopy for DC pulsed with OVA group mice (DC+OVA), Ad-Mock (DC+OVA+Ad-mock), Ad-IL-18 (DC+OVA+Ad-18), Ad-IL-12 (DC+OVA+Ad-12), and co-infected (DC+OVA+Ad-12+Ad-18) treated mice. Data showed different extent of cellular infiltration of the periairway region. Original magnification, $\times 200$.

expression with adenovirus inserted with the IL-12 or IL-18 gene according to the IFN- γ inducing ability of IL-18. First, Ad-GL-1 infected DCs were analyzed, and the high percentage of positive expression proved which is good for modulating protein expression by DCs. The same phenomena could be seen in the expression of IL-12 or IL-18 from the DC culture supernatant after Ad-IL-12 or Ad-IL-18 infection in *in vitro* assay. It has been reported that DCs have lower Coxsackie-adenovirus receptor on the DC cell surface.³³ This is the reason why the high MOI titer was needed in the study in contrast to other reports without using DCs as target cells.

Many studies^{34,35} including ours have demonstrated that adenovirus infection can enhance the expression of surface activation markers such as MHC II, B7-1, and B7-2. Mouse bone marrow-derived DCs with adenovirus vectors can enhance the ability to migrate to regional lymph nodes *in vivo* and to activate T-cell hybridoma at lower DC:T-cell ratios.³⁶ Although IL-5 secretion from Ad-IL-12, Ad-IL-18, and co-infected DC-treated mice all decreased, the CD4⁺ T cells from Ad-IL-12-infected DC-treated mice could not suppress the secretion of IL-4. That might be the reason why the titers of anti-OVA IgE of Ad-IL-12-infected DC treated mice remained high, and only the eosinophilia but not AHR was suppressed. Nevertheless, Ad-IL-12 and Ad-IL-18 co-infected DCs

can prevent the development of T_H2-type immune response including IgE antibody expression, eosinophilia, and AHR.

According to our data, IL-18 and IL-12 co-infected DCs can promote T_H1 immune response and efficiently alleviate the airway eosinophilia in an animal model of asthma. The role of IL-18^{11,23,37,38} in an animal model of asthma is still complicated and controversial. It might be influenced by different time points³⁹ and routes of administration, such as intratracheal or intraperitoneal injection.³⁸ In our study, IL-18-expressing DCs could not induce CD4⁺ T cells to secrete IFN- γ , possibly because of the fact that IL-18-expressing DCs would first prime naive T cells to either T_H1 or T_H2 cells.

Eotaxin can chemoattract CCR3 positive cells, including eosinophils and T_H2 cells.^{39,40} KC is a member of the mouse alpha chemokine family. KC and MIP-2 are the functional homologues of IL-8 and might function as the major proinflammatory alpha chemokine in mice.⁴¹ IL-13 has been reported to account for airway inflammation and AHR. Transgenic mice that selectively express IL-13 in lung organ contained an inflammatory phenotype and increased airway resistance and AHR.²⁸ Actually, Ad-IL-12 and Ad-IL-18 co-infected DCs suppressed the eotaxin, KC, and IL-13 expression in BAL fluid. That leads to the alleviation of airway inflammation.

Although there is no obvious T_H1 pathology in human studies, the therapeutic effect of T_H1-type treatment in asthmatic disease is still controversial.⁴² DCs pulsed with OVA and infected with adenovirus could induce immune responses. Histopathologically, the damage and infiltrative cells were less serious in the Ad-IL-12 and co-infected groups. In contrast, many cells accumulated around the bronchial and lung alveoli in the other groups. We have reported that intravenous injection with IL-12 DNA plasmid into Der p1 sensitized mice can subsequently alleviate eosinophilia and eotaxin expression from BAL fluid.⁴³ DCs infected with adenovirus without pulsed OVA did not efficiently alleviate airway inflammation (data not shown). That indicated the prior contact with the antigen was needed for therapeutic effect of IL-12 and IL-18 co-secreting DCs. We believe this cytokine gene-modulated DC treatment can alleviate the T_H2-type airway inflammation and also enhance the long-term development of T_H1 cells.

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