

A Novel Antiinflammatory Role for Andrographolide in Asthma via Inhibition of the Nuclear Factor- κ B Pathway

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Rationale: Persistent activation of nuclear factor (NF)- κ B has been associated with the development of asthma. Andrographolide, the principal active component of the medicinal plant *Andrographis paniculata*, has been shown to inhibit NF- κ B activity.

Objectives: We hypothesized that andrographolide may attenuate allergic asthma via inhibition of the NF- κ B signaling pathway.

Methods: BALB/c mice sensitized and challenged with ovalbumin (OVA) developed airway inflammation. Bronchoalveolar lavage fluid was assessed for total and differential cell counts, and cytokine and chemokine levels. Serum IgE levels were also determined. Lung tissues were examined for cell infiltration and mucus hypersecretion, and the expression of inflammatory biomarkers. Airway hyperresponsiveness was monitored by direct airway resistance analysis.

Measurements and Main Results: Andrographolide dose-dependently inhibited OVA-induced increases in total cell count, eosinophil count, and IL-4, IL-5, and IL-13 levels recovered in bronchoalveolar lavage fluid, and reduced serum level of OVA-specific IgE. It attenuated OVA-induced lung tissue eosinophilia and airway mucus production, mRNA expression of E-selectin, chitinases, Muc5ac, and inducible nitric oxide synthase in lung tissues, and airway hyperresponsiveness to methacholine. In normal human bronchial epithelial cells, andrographolide blocked tumor necrosis factor- α -induced phosphorylation of inhibitory κ B kinase- β , and downstream inhibitory κ B α degradation, p65 subunit of NF- κ B phosphorylation, and p65 nuclear translocation and DNA-binding activity. Similarly, andrographolide blocked p65 nuclear translocation and DNA-binding activity in the nuclear extracts from lung tissues of OVA-challenged mice.

Conclusions: Our findings implicate a potential therapeutic value of andrographolide in the treatment of asthma and it may act by inhibiting the NF- κ B pathway at the level of inhibitory κ B kinase- β activation.

Keywords: ovalbumin; inhibitory κ B kinase- β ; inhibitory κ B α ; inducible nitric oxide synthase; chitinase; lung epithelium

Allergic asthma is a chronic airway disorder characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR) (1). Cumulative evidence revealed that these inflammatory responses are mediated by T-helper type 2 (Th2) cells together with mast cells, B cells, and eosinophils, and a number of inflammatory cytokines and chemokines (1, 2). IL-4 is imperative for B cell isotype switching for the synthesis of IgE. Allergen-induced crosslinking of IgE-bound high affinity

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Persistent activation of nuclear factor (NF)- κ B is associated with asthma. Andrographolide, a principal active component of the medicinal plant *Andrographis paniculata*, has been shown to inhibit NF- κ B activity.

What This Study Adds to the Field

This study shows the antiinflammatory role of andrographolide in ovalbumin-induced allergic lung disease and human bronchial epithelial cells by inhibiting the NF- κ B activity at the level of inhibitory κ B kinase- β activation.

IgE receptors (Fc ϵ RI) on the surface of mast cells leads to degranulation and activation of mast cells, and the release of inflammatory mediators, such as histamine, leukotrienes, and cytokines, and immediate bronchoconstriction (3, 4). IL-5 is vital for the growth, differentiation, recruitment, and survival of eosinophils, which contribute to inflammation and even airway remodeling in asthma (5). IL-13 plays a pivotal role in the effector phase of Th2 responses, such as eosinophilic inflammation, mucus hypersecretion, AHR, and airway remodeling (6). In addition, chemokines such as RANTES (regulated on activation, normal T cells expressed and secreted) and eotaxin are crucial to the delivery of eosinophils to the airways (7). Airway eosinophilia, together with Th2 cytokines IL-4, IL-5, and IL-13, may ultimately contribute to AHR in asthma (8).

Andrographolide is a labdane diterpene lactone isolated from the leaves of the *Andrographis paniculata* plant (9). *A. paniculata* has long been used as herbal medicine for the prevention and treatment of upper respiratory tract infection in Asian countries and in Scandinavia (10, 11). Andrographolide has also been shown to possess anticancer (12, 13), hepatocyte-protective (14), and antiinflammatory (15–17) activities. Andrographolide has been reported to suppress IL-2 production and T-cell proliferation in a mixed lymphocyte reaction and to inhibit dendritic cell maturation and antigen presentation (15). In addition, andrographolide inhibited lipopolysaccharide-induced nitric oxide (NO) production and inducible NO synthase (iNOS) expression in murine macrophage-like cell line RAW 264.7 (18) and in primary rat microglia cells (19). Furthermore, andrographolide reduced E-selectin expression in the neointima of injured mouse carotid arteries (16). The exact molecular mechanism that mediates these antiinflammatory effects of andrographolide has not been unequivocally determined. Nevertheless, there is evidence pointing to the inhibition of nuclear factor (NF)- κ B transcriptional activity by andrographolide, and more specifically, andrographolide may covalently bind to the

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reduced cysteine 62 of the p50 subunit of NF- κ B to prevent binding to κ B DNA motif and activation (17, 20).

Because asthma is associated with persistent NF- κ B activation (21–23), we investigated the effects of andrographolide on various aspects of ovalbumin (OVA)-induced Th2-mediated allergic airway inflammation in an *in vivo* mouse asthma model. Our results clearly indicate that andrographolide attenuates allergic airway inflammation by potentially inhibiting the NF- κ B pathway at the level of inhibitory κ B (I κ B) kinase- β (IKK β) activation.

METHODS

Animals

Female BALB/c mice, 6 to 8 weeks old (Interfauna, East Yorkshire, UK), were sensitized and challenged with OVA as described (24). Andrographolide (0.1, 0.5, and 1 mg/kg; Sigma, St. Louis, MO) or vehicle (2% dimethyl sulfoxide [DMSO]) in 0.1 ml saline was given by intraperitoneal injections 1 hour before and 11 hours after each OVA aerosol challenge. Saline aerosol was used as a negative control. Animal experiments were performed according to the Institutional Guidelines for Animal Care and Use Committee of the National University of Singapore.

Bronchoalveolar Lavage Fluid and Serum Analysis

Mice were anesthetized 24 hours after the last aerosol challenge and bronchoalveolar lavage (BAL) was performed as described (24). BAL fluid total and differential cell counts, and cytokine and chemokine levels were determined as described (24, 25). Blood was collected by cardiac puncture, and serum levels of total IgE and OVA-specific IgE, IgG1, and IgG2a levels were determined as described (25).

Histologic Analysis

Lungs were fixed in 10% neutral formalin, paraffinized, cut into 6- μ m sections, and stained with hematoxylin and eosin for examining cell infiltration and with periodic acid-Schiff stain for measuring mucus production. Quantitative analysis was performed blinded as described (24).

Measurements of Airway Hyperresponsiveness

Mice were anesthetized and tracheotomy was performed as described (24). The internal jugular vein was cannulated and connected to a microsyringe for intravenous methacholine administration. Airway resistance (RI) and dynamic compliance (Cdyn) in response to increasing concentrations of methacholine were recorded using a whole-body plethysmograph chamber (Buxco, Sharon, CT) as described (24). Results are expressed as a percentage of the respective basal values in response to phosphate-buffered saline.

Cell Cultures

To determine the effects of andrographolide on OVA-specific immune responses in lymphocytes, thoracic lymph node cells were prepared as

described (26). Cells were exposed to 200 μ g/ml OVA for 72 hours. Concanavalin A (Con A, 10 μ g/ml) was used as a positive control. Supernatants from parallel triplicate cultures were analyzed for cytokine levels by ELISA. Normal human bronchial epithelial cells were cultured in optimized bronchial epithelial bulletkit medium with supplements (Lonza, Basel, Switzerland). Cells were pretreated with 30 μ M andrographolide or vehicle (0.01% DMSO) 4 hours before stimulation with 10 ng/ml tumor necrosis factor (TNF)- α . Total and nuclear proteins and mRNA were extracted from cells at specified time intervals.

Immunoblotting, mRNA Expression and Nuclear Factor- κ B DNA-binding

Lung and cell culture protein lysates (10 mg per lane) were separated by 10% SDS-PAGE and immunoblots were developed as described (24). Immunoblots were probed with anti-IKK β , anti-phospho-IKK β (Ser¹⁸⁰), anti-I κ B α , anti-phospho-I κ B α (Ser^{32/36}), anti-p65, anti-phospho-p65 (Ser⁵³⁶), anti- β -actin antibodies (all from Cell Signaling Technology, Beverly, MA), and anti-TATA binding protein (TBP, Abcam, Cambridge, UK). Primers for inflammatory biomarkers are shown in Table 1 and mRNA expression was analyzed as described (24). Nuclear proteins were analyzed for NF- κ B DNA-binding activity using the TransAM NF- κ B p65 transcription factor assay kit (Active Motif, Carlsbad, CA).

Statistical Analysis

Data are presented as means $\pm$ SEM. One-way analysis of variance followed by Dunnett test was used to determine significant differences between treatment groups. Significant levels were set at $P < 0.05$.

RESULTS

Andrographolide Suppresses Ovalbumin-induced Inflammatory Cell Recruitment and Mucus Production

BAL fluid was collected 24 hours after the last OVA or saline aerosol challenge, and total and differential cell counts were performed. OVA inhalation markedly increased total cell and eosinophil counts, but slightly yet significantly ($P < 0.05$) increased macrophage, lymphocyte, and neutrophil counts, as compared with saline aerosol control. Andrographolide (0.1, 0.5, and 1 mg/kg) drastically decreased the total cell and eosinophil counts in BAL fluid in a dose-dependent manner as compared with the DMSO vehicle control (Figure 1A). At high dose (1 mg/kg), andrographolide also reduced macrophage, lymphocyte, and neutrophil counts. We have conducted flow cytometric analysis of peripheral blood leukocytes obtained from saline-challenged, OVA-challenged, vehicle control, and andrographolide-treated mice. Similar percentages of CD3⁺, CD4⁺, CD8⁺ T cells, B cells (B220), NK cells (NK 1.1), neutrophils, and monocytes were observed in all mice (data

TABLE 1. PRIMER SETS FOR REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION ANALYSIS

Targets	Sequences	
	Forward	Reverse
AMCase (mouse)	5'-TGGGTTCTGGGCCTACTATG-3'	5'-GCTTGACAATGCTGCTGGTA-3'
Ym1 (mouse)	5'-CTGGAATTGGTGCCCTACA-3'	5'-CAAGCATGGTGG TTTTACAGGA-3
Ym2 (mouse)	5'-CAGAACCCTGACACATTCATTA-3'	5'-ATGGTCCTTC CAGTAGG TAATA-3'
YKL-40 (mouse)	5'-GTACAAGCTGGTCTGCTACT-3'	5'-GTTGAGGCAATCTCGGAAA-3'
E-selectin (mouse)	5'-AACGCCAGAACAAATTCC-3'	5'-TGAATTGCCACCAGATGTGT-3'
Muc5ac (mouse)	5'-GAGTGACATTGCAGGAAGCA-3'	5'-CAGAGGACAGGAAGGTGAGC-3'
iNOS (mouse)	5'-GTCAACTGCAAGAGAACGGAGAC-3'	5'-GAGCTCCTCCAGACGGGTAGGCTTG -3'
IL-6 (human)	5'-CAGGAGAAGATTCCAAAGAT-3'	5'-ACTGTTCTGTGCTCGCAGC-3'
IL-8 (human)	5'-ATGACTTCCAAGCTGGCCGTGGCT-3'	5'-TCTCAGCCCTCTTCAAACTTCTC-3'
RANTES (human)	5'-ATGAAGGTCTCCGCGCAGCGCT-3'	5'-CTAGCTCATCTCCAAGAGTTG-3'
β -Actin (both)	5'-TCATGAAGTGTGACGTTGACATCCGT-3'	5'-CCTAGAAGCATTGCGGTGCACGATG-3'

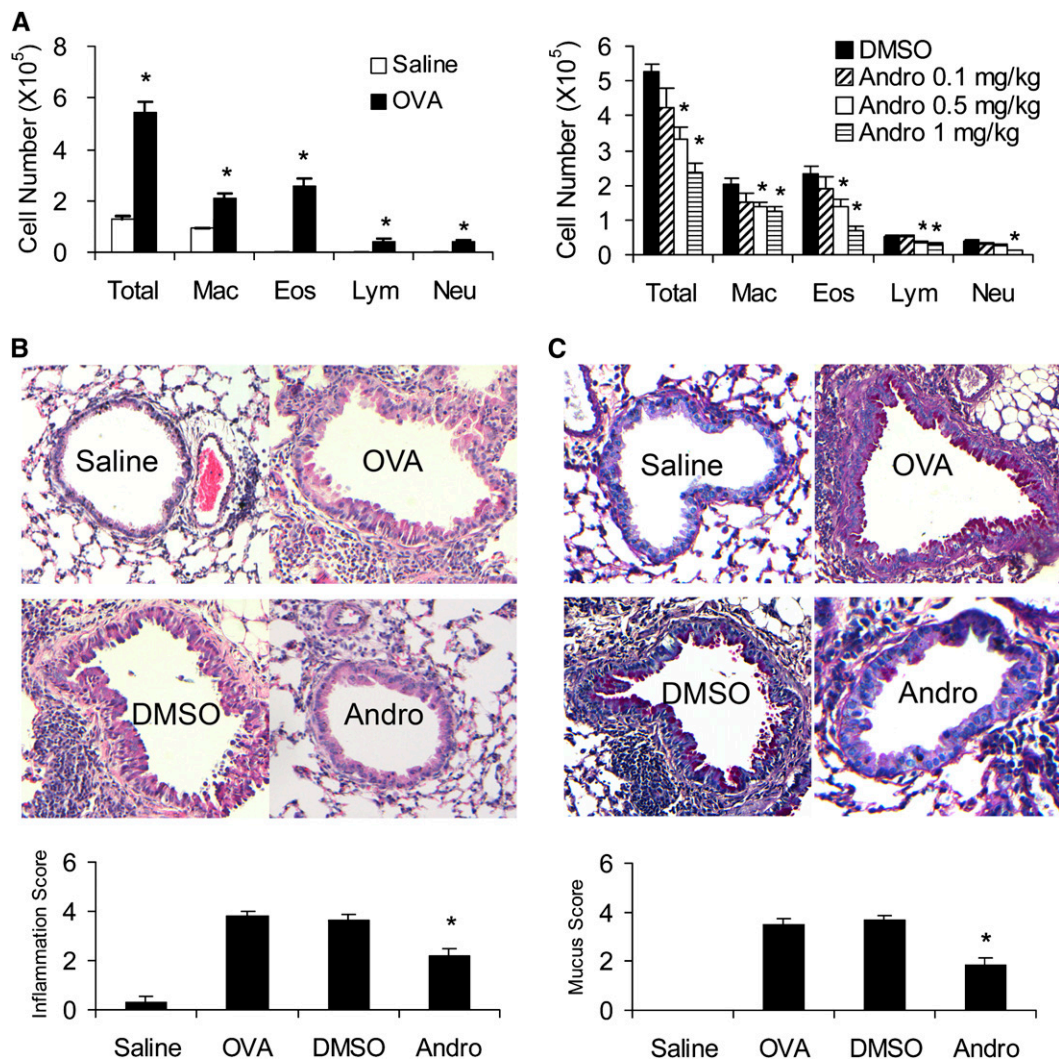


Figure 1. Effects of andrographolide on ovalbumin (OVA)-induced inflammatory cell recruitment and mucus hypersecretion. (A) Inflammatory cell counts in bronchoalveolar lavage (BAL) fluid obtained from sensitized mice 24 hours after the last saline aerosol ($n = 6$ mice per group) or OVA aerosol ($n = 7$ mice per group) challenge. Andrographolide dose-dependently reduced OVA-induced inflammatory cell counts in BAL fluid from sensitized mice 24 hours after the last OVA aerosol challenge (dimethyl sulfoxide [DMSO], $n = 7$; 0.1 mg/kg, $n = 7$; 0.5 mg/kg, $n = 10$; and 1 mg/kg, $n = 9$ mice per group). Differential cell counts were performed on a minimum of 500 cells to identify eosinophil (Eos), macrophage (Mac), neutrophil (Neu), and lymphocyte (Lym). Histological examination of lung tissue eosinophilia (B, magnification $\times 200$) and mucus secretion (C, magnification $\times 200$) 24 hours after the last challenge of saline aerosol, OVA aerosol, OVA aerosol plus DMSO, or OVA aerosol plus 1 mg/kg andrographolide. Quantitative analyses of inflammatory cell infiltration and mucus production in lung sections were performed as previously described

(24). Briefly, to determine the severity of inflammatory cell infiltration, peribronchial cell counts were performed blind based on a 5-point scoring system: 0, no cells; 1, a few cells; 2, a ring of cells one cell layer deep; 3, a ring of cells two to four cells deep; 4, a ring of cells of more than four cells deep. To determine the extent of mucus production, goblet cell hyperplasia in the airway epithelium was quantified blind using a 5-point grading system: 0, no goblet cells; 1, $<25\%$; 2, 25 to 50%; 3, 50 to 75%; 4, $>75\%$. Scoring of inflammatory cells and goblet cells was performed in at least three different fields for each lung section. Mean scores were obtained from four animals. *Significant difference from DMSO control, $P < 0.05$.

not shown). Hence, andrographolide-induced reduction of eosinophil and lymphocyte pulmonary recruitment is unlikely due to any potential nonspecific cytotoxic effects of the drug.

Lung tissue was also collected 24 hours after the last OVA or saline aerosol challenge. OVA aerosol challenge induced marked infiltration of inflammatory cells into the peribronchial and perivascular connective tissues as compared with saline aerosol challenge. Andrographolide (1 mg/kg) markedly diminished the eosinophil-rich leukocyte infiltration as compared with DMSO control (Figure 1B). On the other hand, OVA-challenged mice, but not saline-challenged mice, developed marked goblet cell hyperplasia and mucus hypersecretion in the bronchi. OVA-induced mucus hypersecretion was significantly halted by andrographolide (1 mg/kg) (Figure 1C).

Andrographolide Reduces Ovalbumin-induced Bronchoalveolar Lavage Fluid T Helper Type 2 Cytokine Levels and Serum Immunoglobulin Production

OVA inhalation in sensitized mice caused a notable increase in IL-4, IL-5, IL-13, and eotaxin levels into BAL fluid as compared

with saline aerosol control (Figure 2A). In contrast, BAL fluid level of IFN- γ , a Th1 cytokine, dropped slightly in OVA-challenged mice. Andrographolide significantly ($P < 0.05$) reduced IL-4, IL-5, and IL-13 and, to a lesser extent, eotaxin levels in BAL fluid in a dose-dependent manner as compared with DMSO control (Figure 2A). Noticeably, andrographolide at 1 mg/kg markedly upregulated IFN- γ level in BAL fluid. This finding implies that andrographolide is able to modify the Th2-predominant immune activity in our OVA-induced mouse asthma model.

To further evaluate whether andrographolide could modify an ongoing OVA-specific Th2 response *in vivo*, serum levels of total IgE and OVA-specific IgE, IgG1, and IgG2a were determined using ELISA. Marked elevation in serum total IgE, OVA-specific IgE, and OVA-specific IgG1 levels, but not OVA-specific IgG2a level, were observed in OVA-challenged mice as compared with saline-challenged mice (Figure 2B). Andrographolide strongly suppressed OVA-specific IgE levels even at the lowest dose (0.1 mg/kg), and, to a lesser extent, the serum level of total IgE and OVA-specific IgG1 with significant effects at higher doses (Figure 2B). Andrographolide had

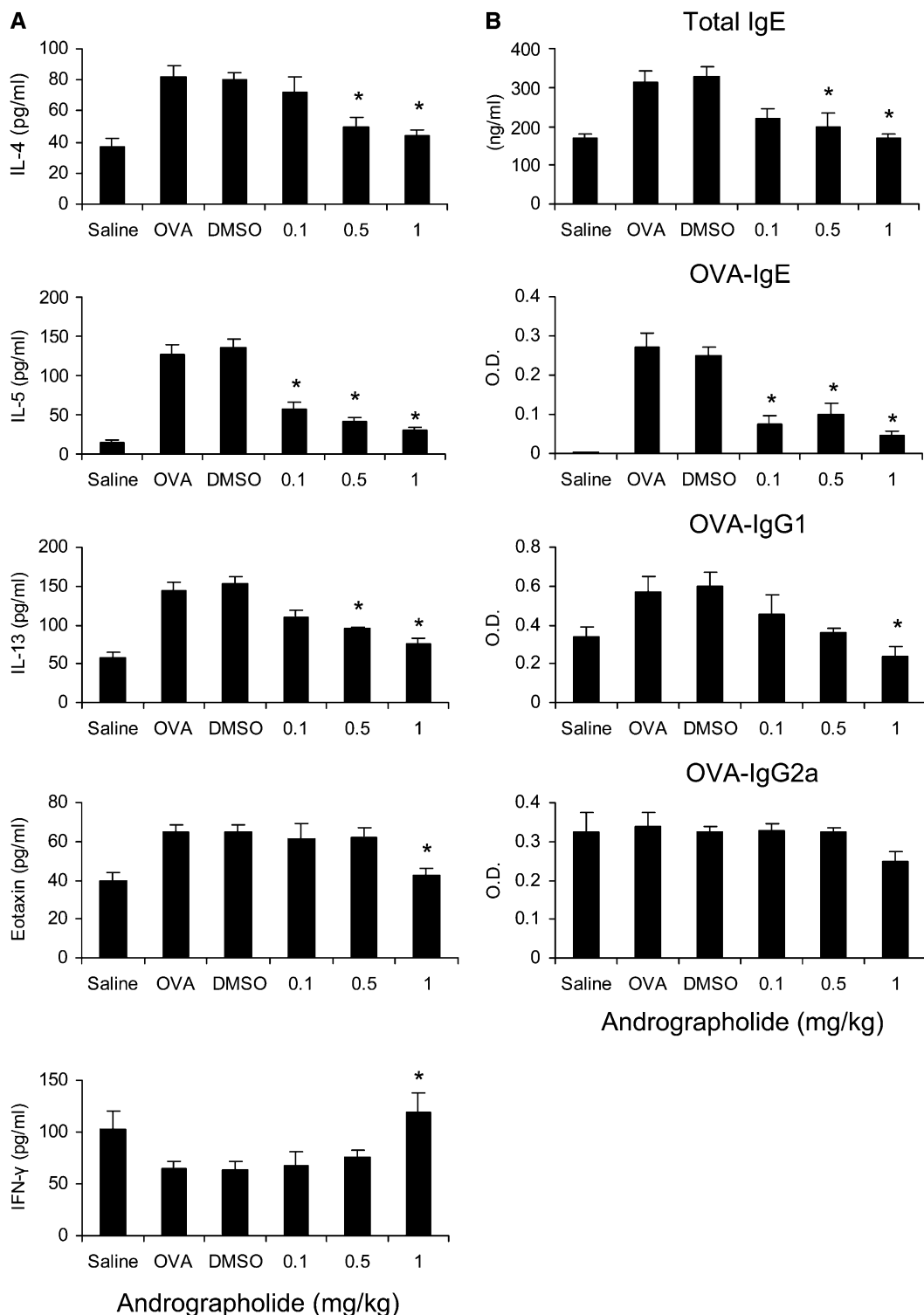


Figure 2. Effects of andrographolide on ovalbumin (OVA)-induced bronchoalveolar lavage (BAL) fluid cytokine and chemokine levels and serum Ig production. (A) BAL fluids were collected 24 hours after the last OVA aerosol challenge. Levels of IL-4, IL-5, IL-13, eotaxin, and IFN- γ were analyzed using ELISA ($n = 6$ to 9 mice per group). Lower limits of detection were as follows: IL-4 and IL-5 at 4 pg/ml, IL-13 and IFN- γ at 15.6 pg/ml, and eotaxin at 2 pg/ml. (B) Mouse serum was collected 24 hours after the last OVA aerosol challenge. The levels of total IgE, OVA-specific IgE, OVA-specific IgG1, and OVA-specific IgG2a were analyzed using ELISA ($n = 6$ to 9 mice per group). Values shown are the mean $\pm$ SEM. DMSO = dimethyl sulfoxide. *Significant difference from dimethyl sulfoxide control, $P < 0.05$.

no effects on the serum level of OVA-specific IgG2a, indicating a specific inhibition of the Th2 response by andrographolide.

Andrographolide Suppresses Ovalbumin-specific Lymphocyte Responses *In Vitro*

To assess whether andrographolide treatment could directly influence lymphocyte function, we examined OVA-specific immune responses in thoracic lymph node cultures. The *in vitro*

OVA-specific production of IL-4 and IL-5 was markedly higher in lymphocytes isolated from OVA-challenged mice than those from saline-challenged mice (Figure 3). Andrographolide (1 mg/kg) pretreatment significantly ($P < 0.05$) lowered the levels of IL-4 and IL-5. In contrast, *in vitro* OVA-specific IFN- γ production was found to be elevated in mice treated with andrographolide (1 mg/kg). The observed immune modulation by andrographolide *in vitro* was OVA specific because Con A-induced production of IL-4, IL-5, and IFN- γ in parallel cultures was not affected (data not shown).

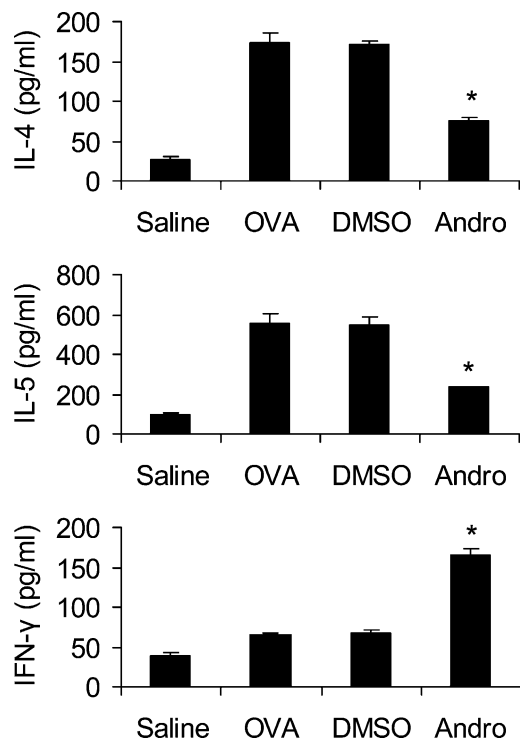


Figure 3. Effects of andrographolide on ovalbumin (OVA)-specific response *in vitro*. Thoracic lymph node cells ($n = 3$ mice per group) were harvested from mice 24 hours after the last OVA or saline aerosol challenge, and cultured for 72 hours with medium alone or OVA (200 $\mu\text{g/ml}$). The levels of IL-4, IL-5, and IFN- γ in culture supernatant were determined using ELISA. Values shown are the mean $\pm$ SEM of triplicate cultures of pooled lymph node cell suspensions. Andro = Andrographolide; DMSO = dimethyl sulfoxide. *Significant difference from dimethyl sulfoxide control, $P < 0.05$.

Andrographolide Reduces Ovalbumin-induced Airway Hyperresponsiveness in Mice

To investigate the effect of andrographolide on AHR in response to increasing concentrations of methacholine, we measured both RI and Cdyn in mechanically ventilated mice. RI is defined as the pressure driving respiration divided by flow. Cdyn refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. OVA-challenged mice developed AHR, which is typically reflected by high RI and low Cdyn (Figure 4). Andrographolide (1 mg/kg) dramatically reduced RI and restored Cdyn in OVA-challenged mice in response to methacholine, suggesting that immune-mediated airway pathology *in vivo* was modified.

Andrographolide Inhibits Ovalbumin-induced Nuclear Factor- κB Function and Inflammatory Gene Expression in Allergic Airway Inflammation

OVA aerosol challenge markedly upregulated lung mRNA levels of adhesion molecule E-selectin, which is pivotal for pulmonary recruitment of inflammatory cells, such as eosinophils and lymphocytes (7, 27); chitinase family members, including acidic mammalian chitinase (AMCase), Ym1, Ym2, and YKL-40, which have recently been shown to play critical roles in airway inflammation and remodeling (28–30); Muc5ac, which is essential for mucus hypersecretion (31); and iNOS, the enzyme responsible for NO production in allergic airway inflammation (32). Pretreatment with andrographolide (1 mg/kg)

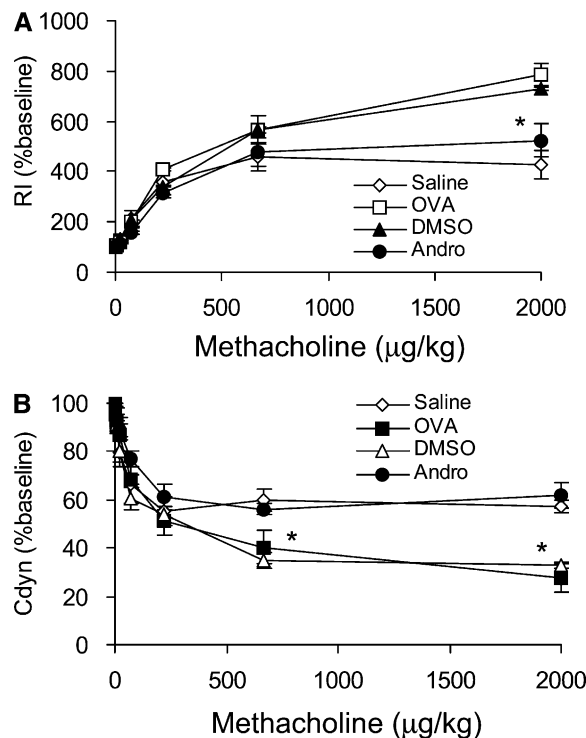


Figure 4. Effects of andrographolide on ovalbumin (OVA)-induced airway hyperresponsiveness. Airway responsiveness of mechanically ventilated mice in response to intravenous methacholine was measured 24 hours after the last saline aerosol or OVA aerosol with pretreatment of either dimethyl sulfoxide (DMSO) or 1 mg/kg andrographolide. Airway hyperresponsiveness is expressed as percentage change from the baseline level of (A) lung resistance (RI, $n = 5$ mice per treatment group) and (B) dynamic compliance (Cdyn, $n = 5$ mice per treatment group). RI is defined as the pressure driving respiration divided by flow. Cdyn refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. Andro = Andrographolide. *Significant difference from DMSO control, $P < 0.05$.

demonstrated strong suppression of E-selectin, AMCase, Ym-2, YKL-40, Muc5ac, and iNOS in the allergic airways (Figure 5A).

To verify that the antiinflammatory effects of andrographolide in OVA-challenged mice were mediated by the inhibition of NF- κB , we examined nuclear translocation of the p65 subunit of NF- κB and p65 DNA-binding activity in lung tissues obtained 24 hours after the last OVA or saline aerosol challenge. OVA challenge markedly raised the level of p65 subunit in the nuclear extract of the lung tissue and promoted nuclear p65 DNA-binding activity as compared with saline aerosol control (Figures 5B and 5C). Andrographolide (1 mg/kg) significantly ($P < 0.05$) reduced both nuclear p65 amount and DNA-binding activity to the basal levels, suggesting that andrographolide may exert its antiinflammatory actions via inhibition of NF- κB activity.

Andrographolide Inhibits Tumor Necrosis Factor- α -induced Nuclear Factor- κB Activation in Normal Human Bronchial Epithelial Cells

To further explore antiinflammatory mechanisms of action of andrographolide in a relevant airway cell type, we studied the effects of andrographolide on TNF- α -induced activation of NF- κB and cytokine mRNA expression in normal primary human bronchial epithelial cells. TNF- α plays a critical role in

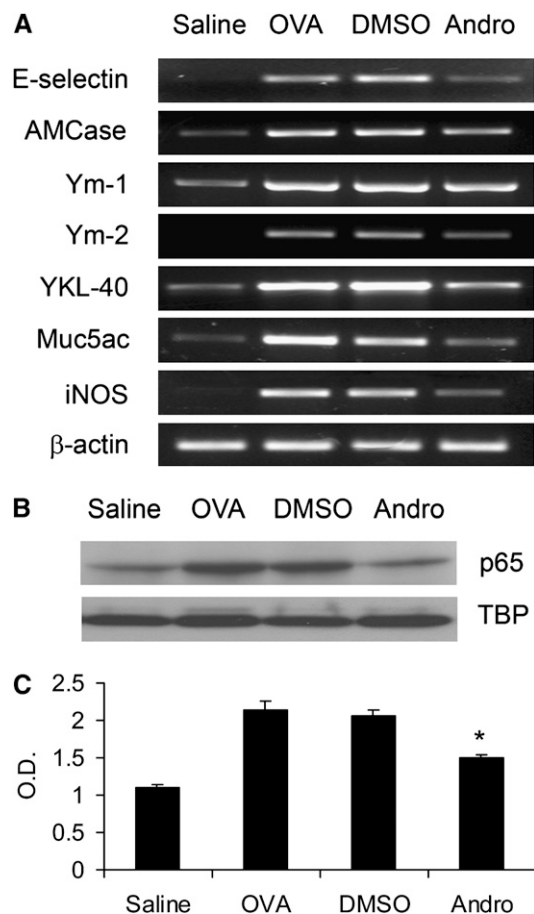


Figure 5. Effects of andrographolide on ovalbumin (OVA)-induced NF- κ B activity and inflammatory gene expression in allergic airway inflammation. (A) Lung tissues were collected 24 hours after the last OVA aerosol challenge. Total mRNA was extracted using TriZol reagent and the polymerase chain reaction products were separated in a 2% agarose gel visualized under ultraviolet light. β -actin was used as an internal control. The experiments were repeated three times ($n = 3$ mice per group) with similar pattern of results. (B) Immunoblotting of p65 NF- κ B in nuclear extract of lung tissues isolated from mice 24 hours after the last saline aerosol or OVA aerosol challenge pretreated with either dimethyl sulfoxide (DMSO) or 1 mg/kg andrographolide. Nuclear proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, probed with anti-p65 or anti-TATA binding protein (TBP) antibody, and developed by enhanced chemiluminescence reagent. TBP nuclear protein was used as an internal control. The experiments were repeated three times ($n = 3$ mice per group) with similar pattern of results. (C) Nuclear p65 DNA-binding activity was determined using a TransAM p65 transcription factor ELISA kit. Values shown are the mean $\pm$ SEM of three separate experiments. Andro = Andrographolide. *Significant difference from DMSO control, $P < 0.05$.

asthma (33, 34) and is a potent stimulator of human airway epithelial cells (35). TNF- α induced a rapid phosphorylation of IKK β , I κ B α , and p65 subunit of NF- κ B within 5 minutes, leading to an instant degradation of I κ B α (Figure 6A). This was followed by the observation of a sharp increase in nuclear p65 level and in p65 DNA-binding activity (Figures 6B and 6C). Andrographolide markedly inhibited the TNF- α -induced phosphorylation of IKK β , I κ B α , and p65, delayed the degradation of I κ B α , and abated p65 nuclear translocation and DNA binding (Figures 6A–6C). Furthermore, andrographolide noticeably blocked TNF- α -induced upregulation of IL-6, IL-8,

and RANTES mRNA expression in normal human bronchial epithelial cells (Figure 6D).

DISCUSSION

Persistent NF- κ B activation has been observed in allergic airway inflammation both in human and in animal models of asthma (21–23, 36, 37). Antigen receptor activation in T and B lymphocytes and mast cells culminates in NF- κ B activation (38, 39). In addition, TNF- α stimulation of airway epithelial cells triggers NF- κ B-dependent gene expression (35). The activity of the NF- κ B pathway is tightly controlled by the IKK complex, which consists of NF- κ B essential modifier (NEMO), IKK α , and IKK β , together with its downstream substrate I κ B α . On stimulation, the activated phospho-IKK β , the predominant player of I κ B kinases, phosphorylates I κ B α and the p65 subunit of NF- κ B. The phosphorylated I κ B α then undergoes a rapid ubiquitin-induced degradation by 26S proteasome, leading to enhanced NF- κ B nuclear translocation and κ B oligonucleotide sequence binding and transcriptional activation (40). Various therapeutic strategies targeted at the NF- κ B signaling pathway, such as NF- κ B-specific decoy oligonucleotide (41), p65-specific antisense oligonucleotide (42), and IKK β -selective small molecule inhibitor (43) have demonstrated beneficial effects in experimental asthma models. Our findings reveal a significant inhibition of p65 nuclear translocation and κ B DNA-binding activity by andrographolide in OVA-challenged lungs *in vivo*. To be more specific, our immunoblotting analysis of TNF- α -stimulated normal human bronchial epithelial cells *in vitro* shows that andrographolide inhibited phosphorylation of IKK β and of its downstream substrates I κ B α and p65, delayed degradation of phospho-I κ B α , reduced nuclear translocation of p65, and diminished p65 κ B oligonucleotide binding. Taken together, we have established that andrographolide, the principal active component of the medicinal plant *A. paniculata*, can effectively suppress various aspects of OVA-induced Th2-mediated allergic airway inflammation in mice potentially via inhibition of NF- κ B activity at the level of IKK β activation.

Th2 cytokines play an essential role in the pathogenesis of the allergic airway inflammation (1, 2), and NF- κ B is a critical transcription factor for Th2 cell differentiation (44). IL-4, IL-5, and IL-13 can be produced by various lung resident cells, such as bronchial epithelial cells, tissue mast cells, and alveolar macrophages, as well as infiltrated inflammatory cells, such as lymphocytes and eosinophils. Our present results show that andrographolide significantly reduced the levels of IL-4, IL-5, IL-13, and eotaxin in BAL fluids from OVA-challenged mice. Similar findings were observed in OVA-challenged mice with disrupted NF- κ B function via conditional knockout of IKK β or transgenic I κ B α mutant expression selectively in airway epithelium (36, 37). In addition, repression of NF- κ B signaling pathway has been shown to block IL-13-induced eotaxin production in cultured human airway smooth muscle cells (43). Therefore, the observed reduction of IL-4, IL-5, IL-13, and eotaxin levels in BAL fluid from andrographolide-treated mice may be due to inhibition of NF- κ B activation in the inflammatory and airway resident cells. Furthermore, our *in vitro* study using direct OVA stimulation of lymph node cells isolated from andrographolide-treated mice produced markedly lower level of IL-4 and IL-5 together with higher IFN- γ level as compared with DMSO-treated mice. These data show that the anti-inflammatory effect of andrographolide is at least in part mediated through a suppressive action on T lymphocytes.

Eosinophils play a central role in the pathogenesis of allergic inflammation (5, 7). Our present findings showed that andrographolide prevented inflammatory cell infiltration into the air-

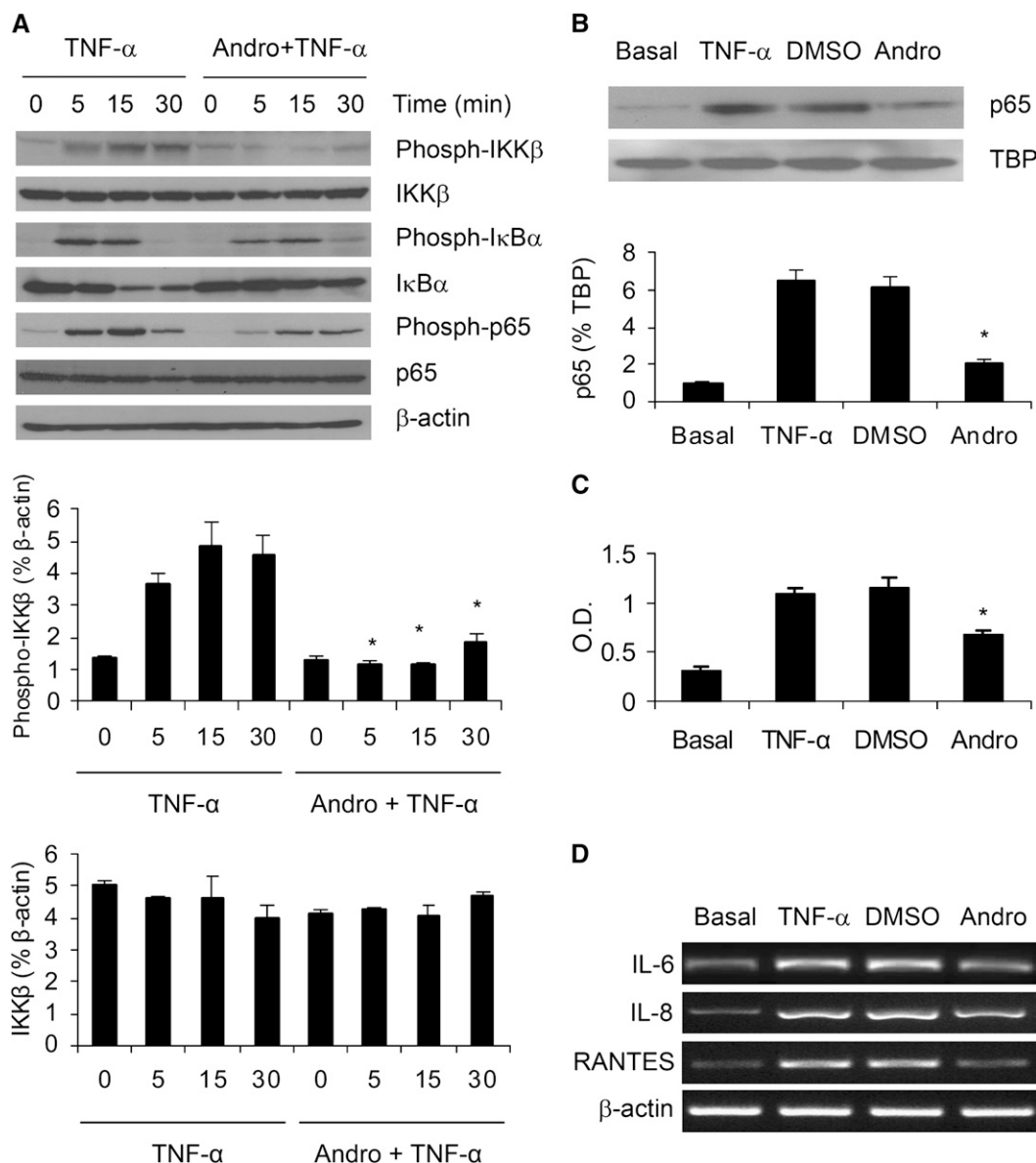


Figure 6. Effects of andrographolide on tumor necrosis factor (TNF)- α stimulation of normal human bronchial epithelial cells. (A) Epithelial cells were stimulated with 10 ng/ml TNF- α in the presence and absence of 30 μ M andrographolide for 5, 15, and 30 minutes before total proteins were extracted for subsequent immunoblotting analysis. Immunoblots were probed with anti-inhibitory κ B kinase- β (IKK β), anti-phospho-IKK β (Ser¹⁸⁰), anti-I κ B α , anti-phospho-I κ B α (Ser^{32/36}), anti-p65, anti-phospho-p65 (Ser⁵³⁶), or anti- β -actin antibody, and developed by enhanced chemiluminescence reagent. Protein bands of IKK β and phospho-IKK β were quantitated using Gel-Pro imaging software (Media Cybernetics, Silver Spring, MD). β -actin was used as an internal control. (B) Immunoblotting of p65 level in nuclear extracts of epithelial cells stimulated with TNF- α for 30 minutes in the presence and absence of 30 μ M andrographolide. Nuclear proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, probed with anti-p65 or anti-TATA binding protein (TBP) antibody, and quantitated using Gel-Pro imaging software. TBP nuclear protein was used as an internal control. (C) DNA-binding activity of p65 nuclear factor- κ B in nuclear extracts of epithelial cells stimulated with TNF- α for

30 minutes in the presence and absence of 30 μ M andrographolide was determined using a TransAM p65 transcription factor ELISA kit. (D) Epithelial cells were stimulated with 10 ng/ml TNF- α in the presence and absence of 30 μ M andrographolide for 12 hours before total mRNA was extracted using TriZol reagent. Polymerase chain reaction products were separated in a 2% agarose gel and visualized under ultraviolet light. β -actin was used as an internal control. This is a representative gel from three separate experiments with similar pattern of results. Values shown are the mean $\pm$ SEM of three separate experiments. Andro = Andrographolide; DMSO = dimethyl sulfoxide. *Significant difference from dimethyl sulfoxide control, $P < 0.05$.

ways as shown by a significant decrease in total cell counts and eosinophil and lymphocyte counts in BAL fluid, and in tissue eosinophilia in lung sections. Leukocyte transmigration into the airways is orchestrated by cytokines, such as IL-4, IL-5, and IL-13, and coordinated by specific chemokines, such as eotaxin and RANTES, in combination with adhesion molecules, such as VCAM-1 and E-selectin (7, 27). IL-13 is by far the most potent inducer of eotaxin expression in airway epithelial cells (45). We have demonstrated that andrographolide strongly suppressed E-selectin mRNA expression and eotaxin production in OVA-challenged lungs, and RANTES mRNA expression in TNF- α -stimulated normal human bronchial epithelial cells. These findings are likely to be due to andrographolide-mediated NF- κ B inhibition, as the genes for E-selectin, eotaxin, and RANTES contain the κ B site for NF- κ B within their promoters (46). Taken together, the observed reduction in airway eosin-

ophilia by andrographolide may be a result of combined inhibitory effects on IL-13, eotaxin, and RANTES production, and on E-selectin expression, secondary to inhibition of NF- κ B activation.

We have also demonstrated a dramatic reduction in airway mucus production in andrographolide-treated mice as compared with DMSO control. Cumulative evidence indicates that IL-4, IL-5, and IL-13 play a critical role in goblet cell hyperplasia and mucin Muc5ac gene and protein expression in mice (31, 47). Interestingly, Muc5ac gene expression depends on the transcriptional activity of NF- κ B (31, 46, 48). We also observed a substantial drop in Muc5ac mRNA expression by andrographolide in OVA-challenged lungs. Selective ablation of NF- κ B function in airway epithelium has been shown to reduce OVA-induced mucus production in mice (36, 37). As such, the marked decrease in mucus production in the lungs of andrographolide-

treated mice may be attributable to a significant reduction of IL-4, IL-5, and IL-13 levels, and a direct inhibitory action on NF- κ B in airway epithelium.

Elevated serum IgE levels are a hallmark of the Th2 immune response. Our data showed that serum levels of total IgE and OVA-specific IgE were substantially reduced by andrographolide in OVA-challenged mice. This is consistent with a recent report showing that andrographolide is able to downregulate both antigen presentation by dendritic cells to T cells, and B cell antibody response (15). In addition, NF- κ B plays a crucial role in B-cell proliferation and development (38, 49), and IL-4 and IL-13 are important in directing B-cell growth, differentiation, and secretion of IgE (3, 6). The biological activities of IgE are mediated through its interaction with the Fc ϵ RI on mast cells and basophils. Cross-linking of Fc ϵ RI initiates multiple signaling cascades leading to NF- κ B activation and production of lipid mediators, cytokines, and chemokines (4, 39). Therefore, the observed reduction in serum total IgE and OVA-specific IgE by andrographolide in our asthma model may be contributed by its inhibitory effect on B-cell activation via inhibition of NF- κ B activation, and on IL-4- and IL-13-mediated class switching to IgE.

A family of chitinase proteins, including AMCase, Ym1, Ym2, and YKL-40, has recently been found to be markedly elevated in allergic airway inflammation in human and in mouse asthma models (28–30). They are mainly expressed in airway epithelium and alveolar macrophages. AMCase level is increased in a mouse asthma model and in subjects with asthma in an IL-13-dependent manner (28). When given intratracheally, IL-13 elevates Ym1 and Ym2 levels in BAL fluid from mice *in vivo* (50). YKL-40 serum level also correlates positively with asthma severity, airway remodeling, and deterioration of pulmonary function in subjects with asthma (30). Overall, chitinases may play a role in airway inflammation and remodeling. Our data show that andrographolide markedly downregulated AMCase, Ym2, and YKL-40 mRNA expression in the lungs of OVA-challenged mice. These may be a consequence of the major drop in IL-4 and IL-13 levels in the airways with andrographolide treatment and may contribute to the diminished pulmonary eosinophilia.

Patients with bronchial asthma produce higher level of exhaled NO as compared with healthy controls, and the NO level may reflect the severity of asthma (51). It appears that increased exhaled NO is associated with increased iNOS expression in the lung epithelium of patients with asthma (52). IL-13 has been shown to induce iNOS expression in normal human bronchial epithelial cells leading to elevated NO production (32). In addition, iNOS gene expression is regulated by the NF- κ B pathway (46). Our results show that andrographolide markedly suppressed the OVA-induced iNOS expression in the lungs, which may be due to the direct inhibition of NF- κ B pathway and the reduced level of IL-13 in the allergic airways.

It is believed that inflammatory mediators released during the allergic inflammation play a critical role in AHR development (8). We report here that andrographolide significantly inhibited OVA-induced AHR to increasing concentrations of methacholine. It has been established that IL-5 plays a critical role in AHR by mobilizing and activating eosinophils, leading to the release of proinflammatory products, such as major basic protein and cysteinyl-leukotrienes, which are closely associated with AHR (5, 7). In addition, IL-4 and IL-13 have been shown to induce AHR in mouse asthma models in which cysteinyl-leukotrienes have been implicated in AHR (6, 53, 54). Moreover, IgE-mediated mast cell activation may contribute to AHR by producing a wide array of inflammatory mediators and cytokines (4, 39). Thus, the observed reduction of AHR by

andrographolide may be associated with the reduction in Th2 cytokine production, tissue eosinophilia and serum IgE level.

Allergic airway inflammation and AHR development involve multiple inflammatory cells and a wide array of mediators. We report here for the first time that andrographolide effectively reduced OVA-induced inflammatory cell recruitment into BAL fluid, IL-4, IL-5, IL-13, and eotaxin production, serum IgE synthesis, pulmonary eosinophilia, mucus hypersecretion, and AHR in a mouse asthma model, potentially via inhibition of NF- κ B activity at the level of IKK β activation. These findings support a therapeutic value for andrographolide in the treatment of asthma.

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