The Journal of Immunology

Anti-Inflammatory Effects of Mitogen-Activated Protein Kinase Kinase Inhibitor U0126 in an Asthma Mouse Model

Wei Duan, Jasmine H. P. Chan, Chui Hong Wong, Bernard P. Leung and W. S. Fred Wong

J Immunol 2004; 172:7053-7059; ;
doi: 10.4049/jimmunol.172.11.7053
http://www.jimmunol.org/content/172/11/7053

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Anti-Inflammatory Effects of Mitogen-Activated Protein Kinase Kinase Inhibitor U0126 in an Asthma Mouse Model[1]

Wei Duan,* Jasmine H. P. Chan,* Chui Hong Wong,* Bernard P. Leung,† and W. S. Fred Wong[2]*

Mitogen-activated protein kinase (MAPK) signaling cascade plays a pivotal role in the activation of inflammatory cells. Recent findings revealed that the activity of p42/44 MAPK (also known as extracellular signal-regulated kinase (ERK)) in the lungs was significantly higher in asthmatic mice than in normal controls. We hypothesized that inhibition of ERK activity may have anti-inflammatory effects in allergic asthma. BALB/c mice were sensitized with OVA and, upon OVA aerosol challenge, developed airway eosinophilia, mucus hypersecretion, elevation in cytokine and chemokine levels, up-regulation of VCAM-1 expression, and airway hyperresponsiveness. Intraperitoneal administration of U0126, a specific MAPK/ERK kinase inhibitor, significantly ($p < 0.05$) inhibited OVA-induced increases in total cell counts, eosinophil counts, and IL-4, IL-5, IL-13, and eotaxin levels recovered in bronchoalveolar lavage fluid in a dose-dependent manner. U0126 also substantially ($p < 0.05$) reduced the serum levels of total IgE and OVA-specific IgE and IgG1. Histological studies show that U0126 dramatically inhibited OVA-induced lung tissue eosinophilia, airway mucus production, and expression of VCAM-1 in lung tissues. In addition, U0126 significantly ($p < 0.05$) suppressed OVA-induced airway hyperresponsiveness to inhaled methacholine in a dose-dependent manner. Western blot analysis of whole lung lysates shows that U0126 markedly attenuated OVA-induced tyrosine phosphorylation of ERK1/2. Taken together, our findings implicate that inhibition of ERK signaling pathway may have therapeutic potential for the treatment of allergic airway inflammation. ***The Journal of Immunology*****, 2004, 172: 7053–7059.**

Allergic asthma is a chronic airway disorder characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR)[3] (1). Cumulative evidence showed that these inflammatory responses are contributed by Th2 cells, together with other inflammatory cells such as mast cells, B cells and eosinophils, and inflammatory cytokines and chemokines (1, 2). Upon activation, Th2 cells produce cytokines such as IL-4, IL-5, and IL-13. IL-4 is essential for B cell maturation and IgE synthesis, and plays an important role in the initiation of Th2 inflammatory responses. IL-5 is pivotal for the growth, differentiation, recruitment, and survival of eosinophils. IL-13 plays a prominent role in the effector phase of Th2 responses, such as eosinophilic inflammation, mucus secretion, and AHR (2, 3). In contrast, chemokines such as RANTES and eotaxin are central to the delivery of eosinophils to the airways. The specific transendothelial migration of eosinophils is regulated by the interaction of adhesion molecules such as VLA-4 and its ligand VCAM-1 (4). Airway eosinophilia together with effector cytokines such as IL-13 may ultimately contribute to AHR in asthma (5).

Mitogen-activated protein kinase (MAPK) signaling cascade has been shown to be important in the activation of various immune cells (6). It is activated by a three-tiered sequential phosphorylation of MAPK kinase kinase, MAPK/ERK kinase (MEK), and MAPK. There are three major groups of MAPK in mammalian cells, including extracellular signal-regulated protein kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase. ERK1 and 2 are activated by their direct upstream kinases MEK1 and MEK2, respectively. ERK signaling pathway is activated upon ligation of TCR in T cells, B cell receptor in B cells, and FcεRI in mast cells, leading to proliferation, differentiation, cytokine production, and degranulation (7–10). It has also been shown that ERK pathway is critically important for the activation of IL-5R and eotaxin receptor in eosinophils, resulting in survival, activation, degranulation, and chemotaxis of eosinophils (11, 12).

A recent study showed that ERK activity in the lungs of asthmatic mice was significantly higher as compared with normal mice (13). Our recent in vitro study revealed that inhibition of the ERK signaling pathway markedly abated OVA-induced anaphylactic bronchial contraction and release of peptido-leukotrienes from sensitized guinea pig lung fragments (14). These findings implicate that ERK pathway can be a pharmacological target for the treatment of allergic asthma. We propose that inhibition of ERK pathway may have anti-inflammatory effects in allergic asthma. In this study, we investigated the potential anti-inflammatory effects of U0126, a potent and selective MEK1/2 inhibitor (15), in an in vivo mouse asthma model. U0126 significantly reduced OVA-induced increases in total cell counts, eosinophil counts, and IL-4, IL-5, IL-13, and eotaxin levels in bronchoalveolar lavage (BAL) fluid. U0126 also inhibited OVA-induced IgE elevation in serum, VCAM-1 expression in the lung, and mucus production in the airways. In addition, U0126 markedly attenuated OVA-induced AHR in sensitized mice. Our findings clearly indicate that regulation of ERK signaling pathway could modulate allergic airway inflammation.

*Department of Pharmacology, National University of Singapore, Singapore; and †Department of Rheumatology, Allergy, and Immunology, Tan Tock Seng Hospital, Singapore

Received for publication October 23, 2003. Accepted for publication March 22, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Medical Research Council of Singapore Grant NMRC/0433/2000.

[2] Address correspondence and reprint requests to Dr. W. S. Fred Wong, Department of Pharmacology, Faculty of Medicine, National University of Singapore, MD2 18 Medical Drive, Singapore 117597. E-mail address: phcwongf@nus.edu.sg

[3] Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; ERK, extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PEG, polyethylene glycol.

0022-1767/04/$02.00

Materials and Methods

Animals

Male BALB/c mice 6–8 wk of age (Interfauna, East Yorkshire, U.K.) were sensitized by i.p. injections of 20 μg of OVA and 4 mg of $Al(OH)_3$ suspended in 0.1 ml of saline on days 0 and 14. On days 18, 19, and 20, animals were challenged with 1% OVA aerosol for 20 min. U0126 (7.5, 15, and 30 mg/kg; a generous gift from J. Trzaskos of DuPont Pharmaceuticals, Wilmington, DE), U0124 (30 mg/kg; EMD Biosciences, San Diego, CA), or vehicle (polyethylene glycol (PEG); Sigma-Aldrich, St. Louis, MO) was given i.p. 2 h before and 6 h after each OVA aerosol challenge. Saline aerosol was used as a negative control.

BAL and serum collection

BAL was performed 24 h after the last OVA challenge. Mice were anesthetized by an i.p. injection of 100 μl of anesthetic mixture (hypnorm: dormicum:H_20 = 1:1:2; Roche, Basel, Switzerland). Tracheotomy was performed, and a cannula was inserted into the trachea. Ice-cold PBS (0.8 ml × 3) was instilled into the lungs, and BAL fluid was collected. Total cell counts were performed using a hemocytometer. For cytological examination, cytospin preparations were prepared using a Cytospin (Thermo Shandon, Pittsburgh, PA), fixed, and stained in a modified Wright stain. Differential cell count was then performed on at least 500 cells in each cytospin slide. Blood was collected by cardiac puncture and allowed to clot, then centrifuged, and aliquots of serum were stored at −70°C before ELISA for serum Igs.

ELISA

Murine cytokines and chemokine in BAL fluid were assayed by ELISA using paired Abs, according to the manufacturers' recommendations. IL-4 and IL-5 ELISA were obtained from BD PharMingen (San Diego, CA). Eotaxin, IL-13, and IFN-γ ELISA were purchased from R&D Systems (Minneapolis, MN). 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. Total serum IgE was measured using purified IgE as standard (BD PharMingen). Serum OVA-specific IgG1, IgG2a, and IgE were determined by ELISA, as previously described (16).

Histologic examination

The lungs were infused via trachea with 1 ml of 10% neutral formalin and immersed in the same fixative for at least 24 h. Tissues were paraffinized, and 6-μm sections were cut and stained with H&E for examining cell infiltration and with periodic acid-Schiff stain (both from Sigma-Aldrich) for measuring mucus production under a light microscope. To determine the severity of inflammatory cell infiltration, peribronchial cell counts were performed blind based on a 5-point scoring system described by Myou et al. (17). Briefly, the scoring system was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells >4 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 described by Tanaka et al. (18). The adopted grading system was: 0, no goblet cells; 1, <25%; 2, 25–50%; 3, 50–75%; 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 three to four animals.

Immunohistochemistry

Lung tissues were harvested 24 h after the last OVA challenge and frozen in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) in liquid nitrogen. Sections of 7-μm thickness were cut and placed onto silanized glass slides, and air dried before being fixed in cold acetone for 10 min. Sections were then rehydrated in PBS for 10 min and blocked in PBS containing 10% mouse serum for 30 min in a humidified chamber. VCAM-1 was probed with FITC-conjugated rat anti-mouse CD106 mAb (BD PharMingen) and visualized using confocal microscopy (Olympus Fluoview 500, Tokyo, Japan) with excitation wavelength at 488 nm and emission range at 510–550 nm. FITC-conjugated nonspecific rat IgG isotype control was used as a negative control.

Measurement of AHR

On day 21, mouse airway responsiveness to increasing concentrations of methacholine (5–80 mg/ml; Sigma-Aldrich) was measured using a single-chamber whole-body plethysmograph (Buxco, Sharon, CT) based on the method of Hamelmann et al. (19). Mice were challenged with aerosolized methacholine for 3 min, and bronchoconstriction was recorded for an additional 5 min for each increasing dose of methacholine. The highest Penh value obtained during each methacholine challenge was expressed as percentage of basal Penh value in response to PBS challenge.

Western blot analysis

Western blot analysis for phospho-ERK was conducted in lung tissues freshly isolated from mice with and without U0126 pretreatment. The lung fragments (0.05 g) were homogenized in ice-cold lysis buffer. Proteins were separated by 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was probed with anti-pan ERK and anti-phospho-ERK (Thr^{202}/Tyr^{204}) Abs (Cell Signaling Technology, Beverly, MA), followed by alkaline phosphatase-conjugated secondary Ab, and visualized using chromogenic substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Life Technologies, Gaithersburg, MD). Protein band intensity was quantitated using Gel-Pro imaging software (Media Cybernetics, Silver Spring, MD).

Statistical analysis

Data are presented as means ± SEM. One-way ANOVA followed by Dennett test was used to determine significant differences between treatment groups. The critical level for significance was set at $p < 0.05$.

Results

Effects of U0126 on OVA-induced eosinophil recruitment in BAL fluid

BAL fluid was collected 24 h after the last OVA aerosol challenge, and total and differential cell counts were performed. OVA inhalation significantly ($p < 0.05$) increased total cell and eosinophil counts as compared with saline control (Fig. 1*A*). In contrast, the numbers of neutrophils, macrophages, and lymphocytes were unchanged. U0126 (7.5, 15, and 30 mg/kg) substantially reduced the total cell number recovered in BAL fluid as compared with PEG control, which was mainly due to a significant ($p < 0.05$) reduction in eosinophils in the U0126-treated mice in a dose-dependent manner (Fig. 1*B*). U0126 did not show any inhibitory effects on BAL fluid cell counts from sensitized mice challenged with saline aerosol (Fig. 1*C*). In addition, 30 mg/kg U0124, a compound structurally similar to U0126, but devoid of MEK1/2-inhibitory effect (15), failed to influence OVA-induced increase in BAL fluid cell counts in asthmatic mice (Fig. 1*D*). The numbers of neutrophils, macrophages, and lymphocytes were not affected by U0126. These results clearly suggest that ERK pathway may be specific in eosinophil recruitment during the allergic inflammation.

Effects of U0126 on OVA-induced eosinophil infiltration and mucus production

Lung tissue was collected 24 h after the last OVA challenge. OVA aerosol challenge induced marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues as compared with saline challenge (Fig. 2, *A* and *B*). The majority of the infiltrated inflammatory cells were eosinophils. U0126 (30 mg/kg) markedly attenuated the eosinophil-rich leukocyte infiltration as compared with PEG control (Fig. 2, *C*, *D*, and *I*). In contrast, OVA-challenged mice, but not saline-challenged mice, developed marked goblet cell hyperplasia and mucus hypersecretion within the bronchi in the lung (Fig. 2, *E* and *F*). Fig. 2*F* also showed the formation of mucus plug in the bronchial lumen. The OVA-induced mucus secretion was significantly abated by U0126 (30 mg/kg) as compared with the PEG control (Fig. 2, *G*, *H*, and *J*).

Effects of U0126 on cytokine levels in BAL fluid

To determine the levels of cytokines in vivo, BAL fluid samples were collected 2 h after the last OVA challenge. IL-4, IL-5, IL-13, eotaxin, and IFN-γ levels were measured using ELISA. As shown in Fig. 3, OVA inhalation in sensitized mice induced substantial cytokine release into BAL fluid as compared with untreated mice. U0126 significantly ($p < 0.05$) reduced IL-4, IL-5, IL-13, and

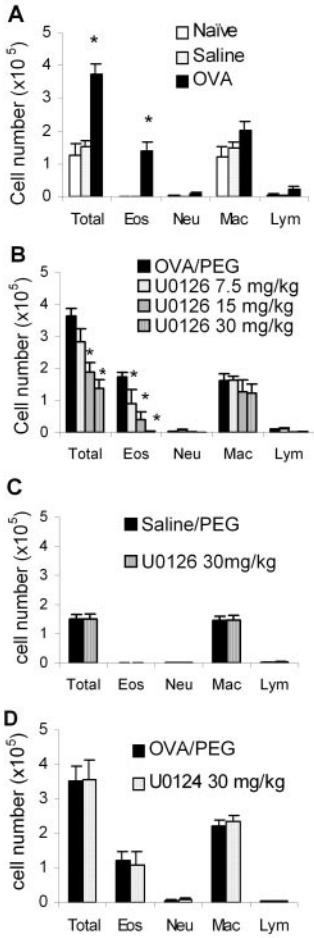

FIGURE 1. Effects of U0126 on BAL fluid cell infiltration. BAL fluids were collected 24 h after the last OVA challenge. *A*, Inflammatory cell counts in BAL fluid obtained from sensitized mice 24 h after the last saline aerosol or 10 mg/ml OVA aerosol challenge (naive, $n = 4$; saline, $n = 4$; OVA, $n = 10$). *B*, Effects of U0126 on OVA-induced inflammatory cell counts in BAL fluid from sensitized mice 24 h after the last 10 mg/ml OVA challenge (OVA, $n = 8$; 7.5 mg/kg, $n = 4$; 15 mg/kg, $n = 3$; 30 mg/kg, $n = 5$). *C*, Effects of U0126 on inflammatory cell counts in BAL fluid from sensitized mice challenged with saline aerosol (saline, $n = 5$; U0126, 30 mg/kg, $n = 6$). *D*, Effects of U0124 on OVA-induced inflammatory cell counts in BAL fluid from sensitized mice 24 h after the last 10 mg/ml OVA challenge (OVA, $n = 8$; U0124, 30 mg/kg, $n = 3$). Differential cell count was performed on a minimum of 500 cells to identify eosinophil (Eos), neutrophil (Neu), macrophage (Mac), and lymphocyte (Lym). ∗, Significant difference from PEG control, $p < 0.05$.

eotaxin levels in BAL fluid as compared with PEG control (Fig. 3, *A–D*). In contrast, U0126 did not show any significant effects on IFN-γ level (Fig. 3*E*).

Effects of U0126 on serum Ig levels

We next determined whether U0126 could modify an ongoing OVA-specific Th2 response in vivo by analyzing Ab subtypes. Serum was collected 24 h after the last OVA challenge. Levels of total IgE, and OVA-specific IgE, IgG1, and IgG2a were determined using ELISA. Substantial elevation in total IgE, and OVA-specific IgE, IgG1, and IgG2a was observed in serum from OVA-sensitized and -challenged mice as compared with untreated mice (Fig. 4). U0126 significantly ($p < 0.05$) lowered total IgE levels in a dose-dependent manner (Fig. 4*A*). In agreement with the inhibitory effect on total IgE level, U0126 at 15 and 30 mg/kg significantly reduced OVA-specific IgE levels by 78 and 91%, respectively (Fig. 4*B*). Moreover, U0126 significantly inhibited OVA-specific IgG1 levels by 54% at 30 mg/kg (Fig. 4*C*), whereas IgG2a levels were unaltered (Fig. 4*D*), indicating a specific inhibition on the Th2 response by U0126.

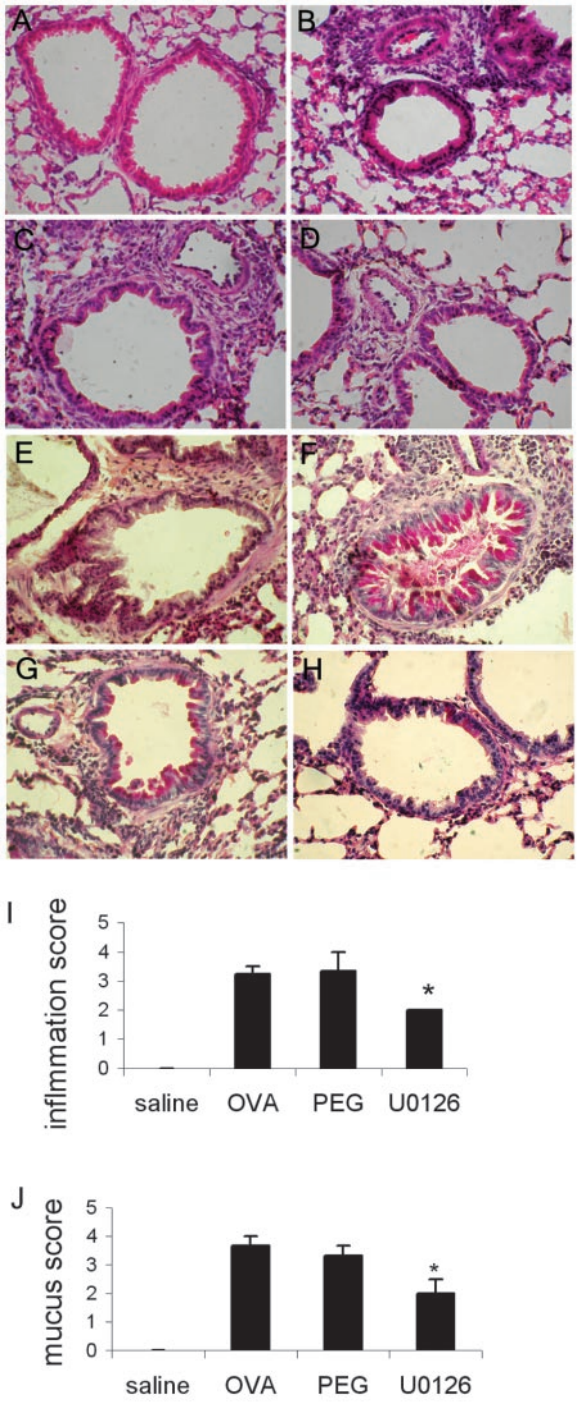

FIGURE 2. Effects of U0126 on lung tissue eosinophilia and mucus production. Histological examination of lung tissue eosinophilia (*A–D*, magnification ×400) and mucus secretion (*E–H*, magnification × 400) 24 h after the last challenge of saline aerosol (*A* and *E*), OVA aerosol (*B* and *F*), OVA aerosol plus PEG (*C* and *G*), or OVA aerosol plus 30 mg/kg U0126 (*D* and *H*). Quantitative analyses of inflammatory cell infiltration (*I*) and mucus production (*J*) in lung sections were performed based on the methods of Myou et al. (17) and Tanaka et al. (18), respectively. Lung tissues were fixed, sectioned at 7 μm thickness, and stained with H&E for tissue eosinophilia or periodic acid-Schiff for mucus production. ∗, Significant difference from PEG control, $p < 0.05$.

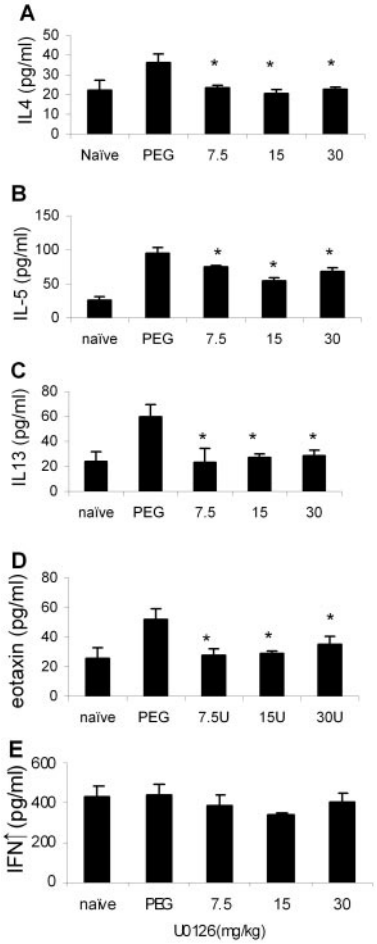

FIGURE 3. Effects of U0126 on cytokine and chemokine levels in BAL fluid. BAL fluids were collected 2 h after the last OVA challenge. Levels of IL-4 (*A*), IL-5 (*B*), IL-13 (*C*), eotaxin (*D*), and IFN-γ (*E*) were analyzed using ELISA ($n = 5$). Values shown are the mean ± SEM. ∗, Significant difference from PEG control, $p < 0.05$.

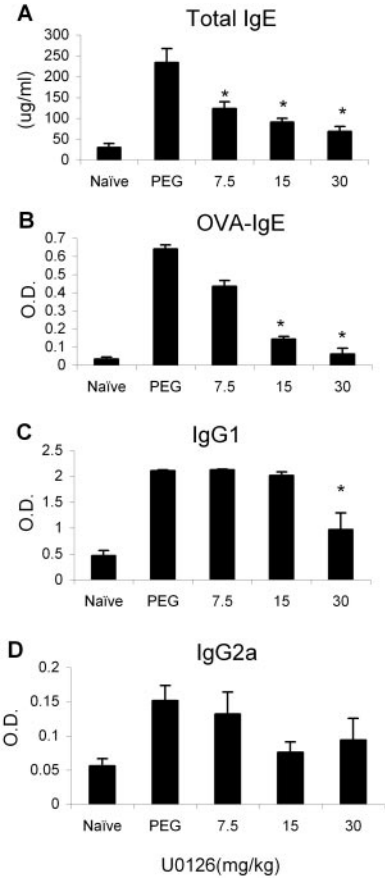

FIGURE 4. Effects of U0126 on serum Ig production. Mouse serum was collected 24 h after the last OVA challenge. The levels of total IgE (*A*), OVA-specific IgE (*B*), OVA-specific IgG1 (*C*), and OVA-specific IgG2a (*D*) were analyzed using ELISA ($n = 4$). Values shown are the mean ± SEM. ∗, Significant difference from PEG control, $p < 0.05$.

Effects of U0126 on the expression of VCAM-1 in lung tissues

The expression of VCAM-1 was up-regulated in the lungs from OVA-treated mice as compared with naive mice (data not shown). The increased expression of VCAM-1 occurred predominantly in vascular endothelium. Treatment with U0126 (30 mg/kg) substantially reduced VCAM-1 up-regulation (Fig. 5).

Effects of U0126 on AHR

We next investigated the effect of U0126 on the development of AHR in BALB/c mice. Sensitized animals challenged with 1% OVA aerosol for 20 min daily for 3 consecutive days developed AHR to inhaled methacholine. Airway responsiveness was determined by Penh (19), and was substantially increased in OVA-challenged group in response to methacholine inhalation as compared with the saline-challenged group (Fig. 6*A*). U0126 dramatically prevented AHR to inhaled methacholine in a dose-dependent manner, as shown in Fig. 6*B*, suggesting that immune-mediated pathology in vivo was modified. U0126 did not show any inhibitory effects on airway responsiveness in sensitized mice challenged with saline aerosol (Fig. 1*C*). In contrast, U0124 (30 mg/kg) failed to influence OVA-induced AHR in asthmatic mice (Fig. 1*D*).

Phospho-ERK immunoblot analysis

To verify that the inhibitory effects of U0126 on the mouse asthma model were mediated by MEK inhibition, we examined the effects of U0126 on OVA-induced ERK1/2 phosphorylation in lungs isolated from mice treated with PEG or 30 mg/kg U0126. Fig. 7 shows ERK1/2 phosphorylation was up-regulated in lungs from OVA-challenged mice and U0126 effectively blocked the OVA-induced ERK1/2 phosphorylation.

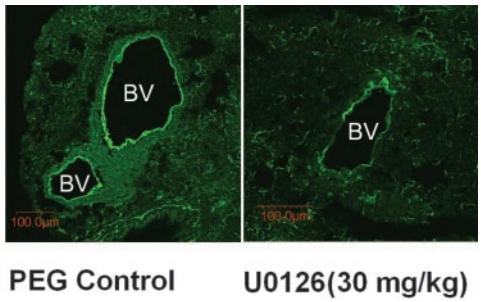

FIGURE 5. Immunohistochemistry of VCAM-1 expression in lung tissue. Lung tissues were harvested 24 h after the last OVA challenge from mice treated with PEG or 30 mg/kg U0126. Lung tissues were frozen in liquid nitrogen. Sections of 7 μm thickness were cut and probed with FITC-conjugated rat anti-mouse CD106 Ab to detect VCAM-1 expression. Labeled VCAM-1 proteins were visualized using confocal microscopy (Olympus Fluoview 500). BV, blood vessel.

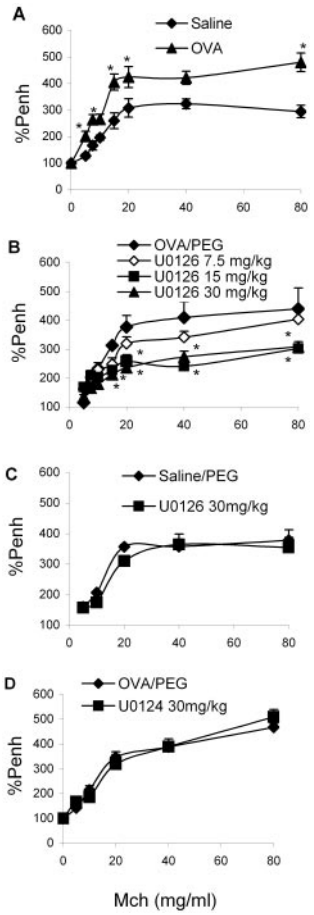

FIGURE 6. Effects of U0126 on AHR. *A*, Airway responsiveness of sensitized mice challenged with aerosolized saline or 10 mg/ml OVA for 3 consecutive days to increasing doses of inhaled methacholine (saline, $n = 4$; OVA, $n = 6$). *B*, Effects of U0126 on inhaled methacholine-induced AHR (PEG, $n = 4$; 7.5 mg/kg, $n = 7$; 15 mg/kg, $n = 7$; 30 mg/kg, $n = 10$). *C*, Effects of U0126 on methacholine-induced AHR in sensitized mice challenged with saline aerosol (saline, $n = 4$; U0126, 30 mg/kg, $n = 4$). *D*, Effects of U0124 on methacholine-induced AHR in sensitized mice 24 h after the last 10 mg/ml OVA challenge (OVA, $n = 8$; U0124, 30 mg/kg, $n = 3$). ∗, Significant difference from control groups, $p < 0.05$.

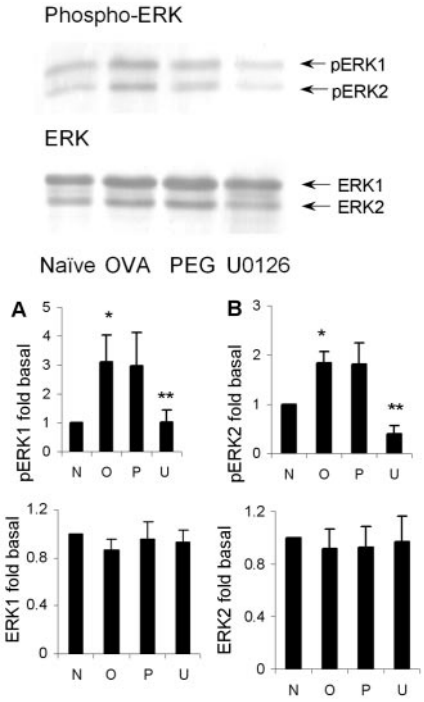

FIGURE 7. Effects of U0126 on ERK1/2 phosphorylation in lung tissue. Western blot analysis of OVA-induced ERK1/2 tyrosine phosphorylation in lung fragments isolated from mice treated with PEG and 30 mg/kg U0126. Lung fragments were homogenized in ice-cold lysis buffer. Proteins (20 μg/lane) were separated by SDS-PAGE and probed with anti-pan ERK and anti-phospho-ERK Abs. The blot was developed by nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, and analyzed using Gel-Pro imaging software. Results are expressed as fold increase of basal ERK level in naive mice (*A* and *B*). Basal ERK level is given a value of 1. Values shown are the mean ± SEM of three separate experiments. ∗, Significant difference from naive, and ∗∗, significant difference from PEG control, $p < 0.05$. N, naive; O, OVA; P, PEG; U, U0126.

Discussion

U0126, a potent and selective MEK inhibitor, has been shown to inhibit MEK1 and MEK2 with negligible effect against other protein kinases such as ERK, p38 MAPK, and c-Jun N-terminal kinase. The inhibition is noncompetitive with respect to ERK and ATP (15). Our present findings reveal that inhibition of MEK/ERK pathway by U0126 could attenuate OVA-induced pulmonary inflammation, release of Th2 cytokines and chemokine into the airway, airway mucus production, serum Ig levels, and AHR in sensitized mice.

There is now clear evidence that Th2 cells play an essential role in the pathogenesis of the allergic airway inflammation (2, 20). Th2 cytokines can be produced by various 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 data show that U0126 significantly reduced the levels of IL-4, IL-5, and IL-13, and of eotaxin in BAL fluids. In contrast, the level of IFN-γ, a Th1 cytokine, was not affected by U0126. ERK signaling pathway has been shown to be involved in the cytokine production from a variety of cell types. TCR engagement in naive $CD4^+$ T cells has been shown to produce IL-4 via activation of ERK (21). U0126 has been shown to inhibit the IL-13 synthesis from 12-*O*-tetradecanoylphorbol-13-acetate/ionomycin-activated human T cells via ERK inhibition (22). It has been reported that by lowering the ratio of ERK to p38 MAPK activation, dendritic cells could be raised with Th1-polarizing capacity, indicating that ERK signaling pathway in the APCs may promote the initial commitment of naive Th cells toward Th2 phenotype (23). Airway smooth muscle is another source of proinflammatory cytokines during the airway inflammation (24). Enhanced ERK tyrosine phosphorylation has been observed in human airway smooth muscle cells upon the stimulation with IL-4 and IL-13 (25). Additional studies showed that ERK signaling pathway is critical for eotaxin production from airway smooth muscle cells upon stimulation from IL-4 and IL-13 (26, 27). In addition, ERK signaling pathway has been implicated in cytokine production in bronchial epithelial cells (28) and mast cells (29) as well as in chemokine release from murine fibroblasts (30). As such, the observed reduction of Th2 cytokine levels in BAL fluid from U0126-treated mice may be due to inhibition of ERK signaling pathway in those inflammatory and airway resident cells. Indeed, the present study also verified specific ERK inhibition by U0126 in the mouse asthma model by blocking phosphorylation (Thr^{202}/Tyr^{204}) of ERK in lung tissue from U0126-treated mice as compared with vehicle controls. This finding is in agreement with a recent report showing increased ERK activity in lung tissues from asthmatic mice than in normal mice (13). ERK activation has been shown to regulate gene expression via stimulation of transcription factors

such as AP-1 (6, 7, 13, 29). It is likely that U0126-induced down-regulation of cytokine production observed in the present study was mediated by inhibition of ERK and of its downstream AP-1.

Eosinophil is the principal effector cell for the pathogenesis of allergic inflammation. Our present findings showed that U0126 prevented eosinophil infiltration into the airways, as shown by a significant drop in total cell counts and eosinophil counts in BAL fluid. Similarly, tissue eosinophilia was also inhibited, as revealed by a significant reduction of inflammatory cell infiltration in histological examination. Eosinophil transmigration into the airways is a multistep process that is orchestrated by Th2 cytokines such as IL-4, IL-5, and IL-13, and coordinated by specific chemokines such as eotaxin in combination with adhesion molecules such as VCAM-1 and VLA-4 (4, 31). IL-13 has been shown by far the most potent inducer of eotaxin expression by airway epithelial cells (32). Our result showed that eotaxin level in BAL fluid from U0126-treated mice was substantially reduced, which may be associated with the significant drop in IL-13 level in BAL fluid produced by U0126. In addition, ERK pathway has been shown to be involved in the intrinsic mechanism of eotaxin-induced eosinophil rolling and migration by regulating actin polymerization (12, 33). Furthermore, detection of VCAM-1 by FITC-conjugated Ab revealed that U0126 down-regulated the expression of VCAM-1 from endothelium in lung tissue. Taken together, the observed reduction in airway eosinophilia by U0126 may be a result of composite effects of reduction in Th2 cytokine production, eotaxin formation, VCAM-1 expression, and eosinophilic cytoskeletal rearrangement via ERK pathway inhibition.

Our findings demonstrated a dramatic reduction in mucus production with less goblet cell hyperplasia in U0126-treated mice as compared with untreated control. In addition, mucus plug was not detectable within the bronchial tissue in lungs of U0126-treated mice, whereas it is common in lungs from OVA-challenged group. Studies using undifferentiated tracheobronchial epithelial cells or cancer cell lines showed that Th2 cells and cytokines (6, 34–36) are linked to mucus hypersecretion by their effects on mucin gene expression in the airway epithelium. In addition, a recent study showed that IL-13-induced goblet cell hyperplasia and mucin MUC5AC protein expression in human bronchial epithelial cell cultures were mediated by ERK signaling pathway and could be inhibited by U0126 (37). As such, the observed decrease in mucus production in U0126-treated lung tissue may be attributed to the substantial drop in Th2 cytokines in asthmatic mice treated with U0126 as well as a direct ERK inhibition by U0126 on IL-13-induced mucus hypersecretion in airway epithelium.

Elevated serum IgE and IgG1 are the hallmarks of a Th2 immune response. Our data showed that serum levels of total IgE and OVA-specific IgE and IgG1 were substantially reduced by U0126, whereas no significant inhibition was observed in IgG2a production, an indicator of Th1 response. ERK signaling pathway has been shown to play a crucial role in B cell receptor-induced proliferation of mature B cells (8, 9, 38). In addition, IL-4 and IL-13 are important in directing B cell growth, differentiation, and secretion of IgE and IgG1 (39). The biological activities of IgE are mediated through its interaction with the high affinity IgE receptor (FcϵRI) on mast cells and basophils. Cross-linking of FcϵRI initiates multiple signaling cascades leading to cellular degranulation and activation (10, 40). It has been shown that ERK signaling pathway is critical for IgE-mediated release of cysteinyl-leukotrienes and cytokines (10, 14, 29, 40). Therefore, the observed reduction in serum IgE and IgG1 in our asthma model by U0126 may be contributed by its inhibitory effects on B cell activation and on Th2 cytokine production from inflammatory cells such as mast cells via ERK pathway inhibition.

It is believed that inflammatory mediators released during the allergic inflammation play a critical role in AHR development (1, 5, 41). Our data showed that U0126 significantly inhibited OVA-induced AHR to inhaled methacholine in a dose-dependent manner. It has been established that IL-5 plays a critical role in AHR by mobilizing and activating esoinophils, leading to the release of proinflammatory products such as major basic protein and cysteinyl-leukotrienes, which are closely associated with AHR (42–44). In addition, IL-4 and IL-13 have been shown to induce AHR in mouse asthma models in which cysteinyl-leukotrienes have been implicated to play a major role in AHR (44–46). Moreover, IgE-mediated mast cell activation may contribute to AHR by producing a wide array of inflammatory mediators and cytokines (10, 29). As such, the observed reduction of AHR by U0126 may be associated with reduction in Th2 cytokine production, tissue eosinophilia, and serum IgE level by U0126.

Allergic airway inflammation and AHR development involve multiple inflammatory cells and a wide array of mediators. We report in this work for the first time that MEK inhibition effectively reduced OVA-induced Th2 cytokine production, pulmonary eosinophilia, serum IgE and IgG1 synthesis, mucus hypersecretion, and AHR in a mouse asthma model. These findings support a potential role for MEK inhibitor in the treatment of asthma.

References

1. Busse, W. W., and R. F. Lemanske, Jr. 2001. Asthma. *N. Engl. J. Med. 344:350.*
2. Herrick, C. A., and K. Bottomly. 2003. To respond or not to respond: T cells in allergic asthma. *Nat. Rev. Immunol. 3:1.*
3. Wynn, T. A. 2003. IL-13 effector functions. *Annu. Rev. Immunol. 21:425.*
4. Lukacs, N. W. 2001. Role of chemokines in the pathogenesis of asthma. *Nat. Rev. Immunol. 1:108.*
5. Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol. 17:255.*
6. Chang, L., and M. Karin. 2001. Mammalian MAP kinase signaling cascades. *Nature 410:37.*
7. Nel, A. E. 2002. T-cell activation through the antigen receptor. 1. Signaling components, signaling pathways, and signal integration at the T-cell antigen receptor synapse. *J. Allergy Clin. Immunol. 109:758.*
8. Jacob, A., D. Cooney, M. Pradhan, and K. M. Coggeshall. 2002. Convergence of signaling pathways on the activation of ERK in B cells. *J. Biol. Chem. 277:23420.*
9. Gauld, S. B., J. M. Dal Porto, and J. C. Cambier. 2002. B cell antigen receptor signaling: roles in cell development and disease. *Science 296:1641.*
10. Nadler, M. J., S. A. Matthews, H. Turner, and J. P. Kinet. 2000. Signal transduction by the high-affinity immunoglobulin E receptor FcϵRI: coupling form to function. *Adv. Immunol. 76:325.*
11. Pazdrak, K., B. Olszewska-Pazdrak, S. Stafford, R. P. Garofalo, and R. Alam. 1998. Lyn, Jak2, and Raf-1 kinases are critical for the antiapoptotic effect of interleukin 5, whereas only Raf-1 kinase is essential for eosinophil activation and degranulation. *J. Exp. Med. 188:421.*
12. Boehme, S. B., S. K. Sullivan, P. D. Crowe, M. Santos, P. J. Conlon, P. Sriramarao, and K. B. Bacon. 1999. Activation of mitogen-activated protein kinase regulates eotaxin-induced eosinophil migration. *J. Immunol. 163:1611.*
13. Kumar, A., S. Lnu, R. Malya, D. Barron, J. Moore, D. B. Corry, and A. M. Boriek. 2003. Mechanical stretch activates nuclear factor-κB, activator protein-1, and mitogen-activated protein kinases in lung parenchyma: implications in asthma. *FASEB J. 17:1800.*
14. Tsang, F., A. H. M. Koh, W. L. Ting, P. T. H. Wong, and W. S. F. Wong. 1998. Effects of mitogen-activated protein kinase kinase inhibitor PD 098059 on antigen challenge of guinea-pig airways in vitro. *Br. J. Pharmacol. 125:61.*
15. Favata, M. F., K. Y. Horiuchi, E. J. Manos, A. J. Daulerio, D. A. Stradley, W. S. Feeser, D. E. Van Dyk, W. J. Pitts, R. A. Earl, F. Hobbs, et al. 1998. Identification of a novel inhibitor of mitogen-activated protein kianse kinase. *J. Biol. Chem. 273:18623.*
16. Garside, P., M. Steel, E. A. Worthey, A. Satoskar, J. Alexander, H. Bluethmann, F. Y. Liew, and A. M. Mowat. 1995. T helper 2 cells are subject to high dose oral tolerance and are not essential for its induction. *J. Immunol. 154:5649.*
17. Myou, S., A. R. Leff, S. Myo, E. Boetticher, J. Tong, A. Y. Meliton, J. Liu, N. M. Munoz, and X. Zhu. 2003. Blockade of inflammation and airway hyperresponsiveness in immune-sensitized mice by dominant-negative phosphoinositide 3-kinase-TAT. *J. Exp. Med. 198:1573.*
18. Tanaka, H., T. Masuda, S. Tokuoka, M. Komai, K. Nagao, Y. Takahashi, and H. Nagai. 2001. The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. *Inflamm. Res. 50:616.*
19. Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med. 156:766.*

20. Larche, M., D. S. Robinson, and A. B. Kay. 2002. The role of T lymphocytes in the pathogenesis of asthma. *J. Allergy Clin. Immunol. 111:450*.
21. Jorritsma, P. J., J. L. Brogdon, and K. Bottomly. 2003. Role of TCR-induced extracellular signal-regulated kinase activation in the regulation of early IL-4 expression in naive $CD4^+$ T cells. *J. Immunol. 170:2427*.
22. Pahl, A., M. Zhang, H. Kuss, I. Szelenyi, and K. Brune. 2002. Regulation of IL-13 synthesis in human lymphocytes: implications for asthma therapy. *Br. J. Pharmacol. 135:1915*.
23. Puig-Kroger, A., M. Relloso, O. Fernandez-Capetillo, A. Zubiaga, A. Silva, C. Bernabeu, and A. L. Corbi. 2001. Extracellular signal-regulated protein kinase signaling pathway negatively regulates the phenotypic and functional maturation of monocyte-derived human dendritic cells. *Blood 98:2175*.
24. Reynold, A. A., Jr. 2002. Airway smooth muscle: an immunomodulatory cell. *J. Allergy Clin. Immunol. 110:S269*.
25. Laporte, J. C., P. E. Moore, S. Baraldo, M.-H. Jouvin, T. L. Church, I. N. Schwartzman, R. A. Panettieri, Jr., J. P. Kinet, and S. A. Shore. 2001. Direct effects of interleukin-13 on signaling pathways for physiological responses in cultured human airway smooth muscle cells. *Am. J. Respir. Crit. Care Med. 164:141*.
26. Hirst, S. J., M. P. Hallsworth, Q. Peng, and T. H. Lee. 2002. Selective induction of eotaxin release by interleukin-13 or interleukin-4 in human airway smooth muscle cells is synergistic with interleukin-1β and is mediated by the interleukin-4 receptor α-chain. *Am. J. Respir. Crit. Care Med. 165:1161*.
27. Moore, P. E., T. L. Church, D. D. Chism, R. A. Panettieri, Jr., and S. A. Shore. 2002. IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK. *Am. J. Physiol. Lung Cell Mol. Physiol. 282:L847*.
28. Cui, C. H., T. Adachi, H. Oyamada, Y. Kamada, T. Kuwasaki, Y. Yamada, N. Saito, H. Kayaba, and J. Chihara. 2002. The role of mitogen-activated protein kinases in eotaxin-induced cytokine production from bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol. 26:329*.
29. Lorentz, A., I. Klopp, T. Gebhardt, M. P. Manns, and S. C. Bischoff. 2003. Role of activator protein 1, nuclear factor-κB, and nuclear factor of activated T cells in IgE receptor-mediated cytokine expression in mature human mast cells. *J. Allergy Clin. Immunol. 111:1062*.
30. Langdon, C., C. Kerr, L. Tong, and C. D. Richards. 2003. Oncostatin M regulates eotaxin expression in fibroblasts and eosinophilic inflammation in C57BL/6 mice. *J. Immunol. 170:548*.
31. Jia, G.-Q., J. A. Gonzalo, A. Hidalgo, D. Wagner, M. Cybulsky, and J. C. Gutierrez-Ramos. 1999. Selective eosinophil transendothelial migration triggered by eotaxin via modulation of Mac-1/ICAM-1 and VLA-4/VCAM-1 interactions. *Int. Immunol. 11:1*.
32. Li, L., Y. Xia, A. Nguyen, Y. H. Lai, L. Feng, T. R. Mosmann, and D. Lo. 1999. Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J. Immunol. 162:2477*.
33. Kampen, G. T., S. Stafford, T. Adachi, T. Jinquan, S. Quan, J. A. Grant, P. S. Skov, L. K. Poulsen, and R. Alam. 2000. Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. *Blood 95:1911*.
34. Justice, J. P., J. Crosby, M. T. Borchers, A. Tomkinson, J. J. Lee, and N. A. Lee. 2002. $CD4^+$ T cell-dependent airway mucus production occurs in response to IL-5 expression in lung. *Am. J. Physiol. Lung Cell Mol. Physiol. 282:L1066*.
35. Cohn, L., R. J. Homer, A. Marinov, J. Rankin, and K. Bottomly. 1997. Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J. Exp. Med. 186:1737*.
36. Zhu, Z., R. J. Homer, Z. Wang, Q. Chen, G. P. Geba, J. Wang, Y. Zhang, and J. A. Elias. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J. Clin. Invest. 103:779*.
37. Atherton, H. C., G. Jones, and H. Danahay. 2003. IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. *Am. J. Physiol. Lung Cell Mol. Physiol. 285:L730*.
38. Richards, J. D., S. H. Dave, C. H. G. Chou, A. A. Mamchak, and A. L. DeFranco. 2001. Inhibition of the MEK/ERK signaling pathway blocks a subset of B cell responses to antigen. *J. Immunol. 166:3855*.
39. Emson, C. L., S. E. Bell, A. Jones, W. Wisden, and A. N. J. McKenzie. 1998. Interleukin (IL)-4-independent induction of immunoglobulin (Ig)E, and perturbation of T cell development in transgenic mice expressing IL-13. *J. Exp. Med. 188:399*.
40. Kawakami, T., and S. J. Galli. 2002. Regulation of mast-cell and basophil function and survival by IgE. *Nat. Rev. Immunol. 2:773*.
41. Fernandes, D. J., R. W. Mitchell, O. Lakser, M. Dowell, A. G. Stewart, and J. Solway. 2003. Do inflammatory mediators influence the contribution of airway smooth muscle contraction to airway hyperresponsiveness in asthma? *J. Appl. Physiol. 95:844*.
42. Gleich, G. J. 2000. Mechanisms of eosinophil-associate inflammation. *J. Allergy Clin. Immunol. 105:651*.
43. Foster, P. S., S. P. Hogan, A. J. Ramsay, K. I. Matthaei, and I. G. Young. 1996. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J. Exp. Med. 183:195*.
44. Vargaftig, B. B., and M. Singer. 2003. Leukotrienes mediate murine bronchopulmonary hyperreactivity, inflammation, and part of mucosal metaplasia and tissue injury induced by recombinant murine interleukin-13. *Am. J. Respir. Cell Mol. Biol. 28:410*.
45. To, Y., M. Dohi, R. Tanaka, A. Sato, K. Nakagome, and K. Yamamoto. 2001. Early interleukin 4-dependent response can induce airway hyperreactivity before development of airway inflammation in a mouse model of asthma. *Lab. Invest. 81:1385*.
46. Taube, C., C. Duez, Z. H. Cui, K. Takeda, Y. H. Rha, J. W. Park, A. Balhorn, D. D. Donaldson, A. Dakhama, and E. W. Gelfand. 2002. The role of IL-13 in established allergic airway disease. *J. Immunol. 169:6482*.