

ORIGINAL ARTICLE

Oroxylin A Inhibits Allergic Airway Inflammation in Ovalbumin (OVA)-Induced Asthma Murine Model

De-Gang Zhou,¹ Bao-Zhong Diao,¹ Wen Zhou,^{2,4} and Jia-Long Feng³

Abstract—Oroxylin A, a natural flavonoid isolated from the medicinal herb *Scutellaria baicalensis Georgi*, has been reported to have anti-inflammatory property. In this study, we aimed to investigate the protective effects and mechanism of oroxylin A on allergic inflammation in OVA-induced asthma murine model. BALB/c mice were sensitized and airway-challenged with OVA to induce asthma. Oroxylin A (15, 30, and 60 mg/kg) was administered by oral gavage 1 h before the OVA treatment on day 21 to 23. The results showed that oroxylin A attenuated OVA-induced lung histopathologic changes, airway hyperresponsiveness, and the number of inflammatory cells. Oroxylin A also inhibited the levels of IL-4, IL-5, IL-13, and OVA-specific IgE in BALF. Furthermore, oroxylin A significantly inhibited OVA-induced NF- κ B activation. In conclusion, these results suggested that oroxylin A inhibited airway inflammation in OVA-induced asthma murine model by inhibiting NF- κ B activation. These results suggested that oroxylin A was a potential therapeutic drug for treating allergic asthma.

KEY WORDS: oroxylin A; OVA; asthma; NF- κ B.

INTRODUCTION

Allergic asthma, a chronic inflammatory disease of the lung, is characterized by infiltration of inflammatory cells and bronchial hyperreactivity [1, 2]. It is usually caused by the aberrant expansion in the lung of T helper cells that produce type 2 (Th2) cytokines [3, 4]. Previous studies showed that Th2-type cytokines such as IL-4, IL-5, and IL-13 played an important role in the development of allergic asthma [5, 6]. These cytokines could induce the release of IgE, inflammatory mediators and increase the infiltration of inflammatory cells into the airway [7]. The expression of these cytokines has been reported to be regulated by NF- κ B [8]. Activated

NF- κ B has been demonstrated in the airways of asthmatic patients [9]. Recent studies suggested that inhibition of NF- κ B had the ability to treat allergic asthma [10, 11].

Oroxylin A, a naturally occurring monoflavonoid isolated from *Scutellaria baicalensis Georgi*, has been reported to have anti-inflammatory effects [12]. Previous studies showed that oroxylin A inhibited LPS-induced iNOS and COX-2 expression in RAW264.7 cells [12]. Oroxylin A also inhibited LPS-induced migration and tube formation of human umbilical vein endothelial cells [13]. Moreover, oroxylin A has been reported to protect against LPS-induced acute lung injury [14] and LPS/D-galactosamine-induced liver injury in mice [15]. However, the anti-allergic effects of oroxylin A have not been reported. In the present study, we investigate the anti-allergic effects of oroxylin A in an OVA-induced asthma murine model.

MATERIALS AND METHODS

Reagents

Dimethyl sulfoxide (DMSO) and OVA were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

¹ Liaocheng People's Hospital and Liaocheng Clinical School of Taishan Medical University, Liaocheng 252000, Shandong, China

² The Second Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangdong Provincial Academy of Chinese Medical Sciences), Guangzhou 510006, Guangdong, China

³ Liaocheng City Food and Drug Inspection and Testing Center, Liaocheng 252000, Shandong, China

⁴ To whom correspondence should be addressed at The Second Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangdong Provincial Academy of Chinese Medical Sciences), Guangzhou 510006, Guangdong, China. E-mail: zhouwenyl@sina.com

Oroxylin A (purity > 98 %) was purchased from the Chinese Institute for Drug and Biological Product Control (Beijing, China). Mouse IL-4, IL-5, and IL-13 ELISA kits were obtained from Bender MedSystems (Vienna, Austria). Mouse IgE ELISA kit was obtained from R&D Systems (Minneapolis, MN). Antibodies against NF- κ B, I κ B α , and β -actin were purchased from Abcam (Cambridge, UK).

Animals

Female BALB/c mice (weight 18 to 22 g) were purchased from the Center of Experimental Animals of Shandong University (Shandong, China). The mice were housed in a temperature- and light-controlled room with free access to food and water. All animal experimental procedures were approved by the Health's Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.

Experimental Protocols

Sixty mice were randomly divided into five groups and each group contained 12 mice: normal control group, OVA group, and OVA + oroxylin A (15, 30, and 60 mg/kg) group. On day 1 and 14, the mice were sensitized with 20 μ g OVA with 1 mg aluminum hydroxide adsorbed in 200 μ l PBS by intraperitoneal injection. The mice were given 100 μ g OVA adsorbed in 20 μ l PBS by intranasal inhalations at day 21 to 23 once a day. Oroxylin A was dissolved in DMSO and further diluted in PBS. Oroxylin A (15, 30, and 60 mg/kg) was administered by oral gavage 1 h before the OVA treatment on day 21 to 23. The mice were sacrificed 24 h after the last challenge.

Histologic Analysis

Lung tissues were collected and fixed in 10 % neutral-buffered formalin, embedded in paraffin and sliced. Sections were stained with hematoxylin and eosin (H&E) using a standard protocol and analyzed by light microscopy.

Collection of BALF and Cell Counting

After mice were anesthetized, the tracheas were cannulated and lavaged with two 0.8-ml aliquots of cold PBS. The bronchoalveolar lavage fluid (BALF) samples were collected and immediately centrifuged. Neutrophils, eosinophils, macrophages, and lymphocytes in BALF were stained with the Kwik-Diff staining set (Thermo, USA) according to the manufacturer's instructions.

Measurement of Airway Hyperresponsiveness

Airway hyperresponsiveness (AHR) was detected using noninvasive whole-body plethysmography (Model PLY 3211; Buxco, Sharon, CT, USA) 24 h after the last OVA treatment as described previously.

Cytokines Assay

The levels of the cytokines, including IL-4, IL-5, and IL-13, in the BALF were analyzed using commercially ELISA kits (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. The level of IgE in serum was measured by using ELISA kit (R&D Systems, Minneapolis, MN)

Western Blot Analysis

Total proteins of lung tissues were extracted using T-PER Tissue Protein Extraction Reagent Kit (Thermo, USA). Equal amounts of protein were loaded into each well of a 12 % SDS-PAGE gel and transferred onto PVDF membranes. After blocking in 5 % skim milk for 2 h at room temperature, the membranes were incubated with primary antibodies: NF- κ B, p-NF- κ B, I κ B α , p-I κ B α , and β -actin at 4 °C overnight. Then the membranes were developed using enhanced chemiluminescence (ECL) kit (Thermo, USA).

Statistical Analysis

Results were analyzed by ANOVA followed by Bonferroni *post hoc*. All data were presented as mean \pm S.E.M. *P* values of 0.05 or less were considered statistically significant.

RESULTS

Effects of Oroxylin A on OVA-Mediated Lung Histopathologic Changes

To investigate the protective effects of oroxylin A on OVA-induced asthma, lung histological changes were detected. The results demonstrated that the OVA-challenged mice showed marked thickening of the alveolar wall and inflammatory cell infiltration in the peribronchial and perivascular areas. However, treatment of oroxylin A significantly inhibited OVA-induced thickening of the alveolar wall and inflammatory cell infiltration in the peribronchial and perivascular areas (Fig. 1).

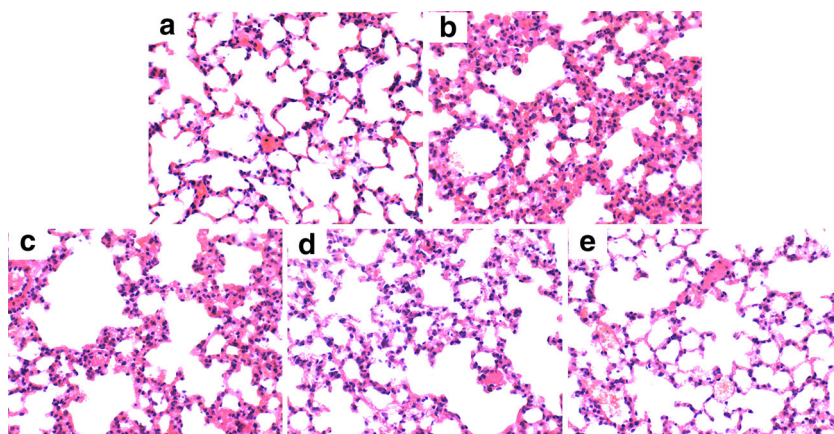


Fig. 1. Effects of oroxylin A on histopathological changes in lung tissues in OVA-induced asthma. Lungs tissues from each experimental group were processed for histological evaluation. Representative histological changes of lung obtained from mice of different groups. **a** Control group, **b** OVA group, **c** OVA+ oroxylin A (15 mg/kg) group, **d** OVA + oroxylin A (30 mg/kg) group, **e** OVA+ oroxylin A (60 mg/kg) group (Hematoxylin and eosin staining, magnification 200×).

Effects of Oroxylin A on Inflammatory Cells Infiltration in BALF

The effects of oroxylin A on OVA-induced inflammatory cells infiltration in BALF were detected in this study. As shown in Fig. 2, the infiltration of inflammatory cells (neutrophils, eosinophils, macrophages, and lymphocytes) in BALF increased significantly in OVA-challenged mice. However, treatment of oroxylin A significantly inhibited OVA-induced inflammatory cell infiltration in BALF (Fig. 2).

Effects of Oroxylin A on OVA-Specific Serum IgE Levels

The effects of oroxylin A on OVA-specific serum IgE levels were detected by ELISA. As shown in Fig. 3, serum

IgE level increased significantly in OVA-challenged mice. However, treatment of oroxylin A significantly inhibited OVA-induced serum IgE level (Fig. 3).

Effects of Oroxylin A on Th2 Cytokines in BALF

The effects of oroxylin A on Th2 cytokines production were detected by ELISA. As shown in Fig. 3, the levels of IL-4, IL-5, and IL-13 increased significantly in OVA-challenged mice. However, treatment of oroxylin A significantly inhibited OVA-induced IL-4, IL-5, and IL-13 production (Fig. 3).

Effects of Oroxylin A on Airway Hyperresponsiveness

The effects of oroxylin A on AHR was measured in this study. The results showed that the Penh value of OVA-

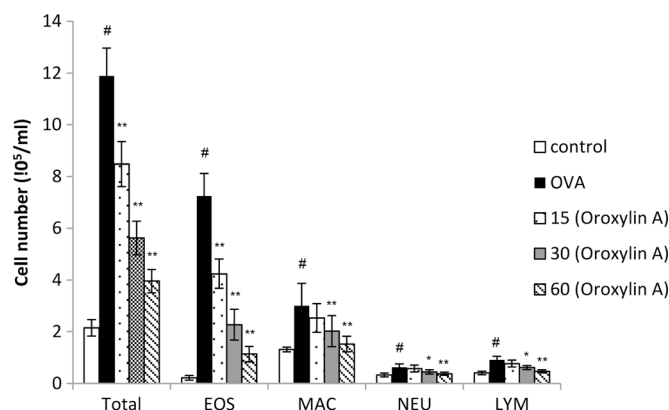


Fig. 2. Effects of oroxylin A on OVA-induced inflammatory cell count in the BALF. The values presented are the means ± SEM of three independent experiments. #*p* < 0.05 vs. control group; **p* < 0.05, ***p* < 0.01 vs. OVA group.

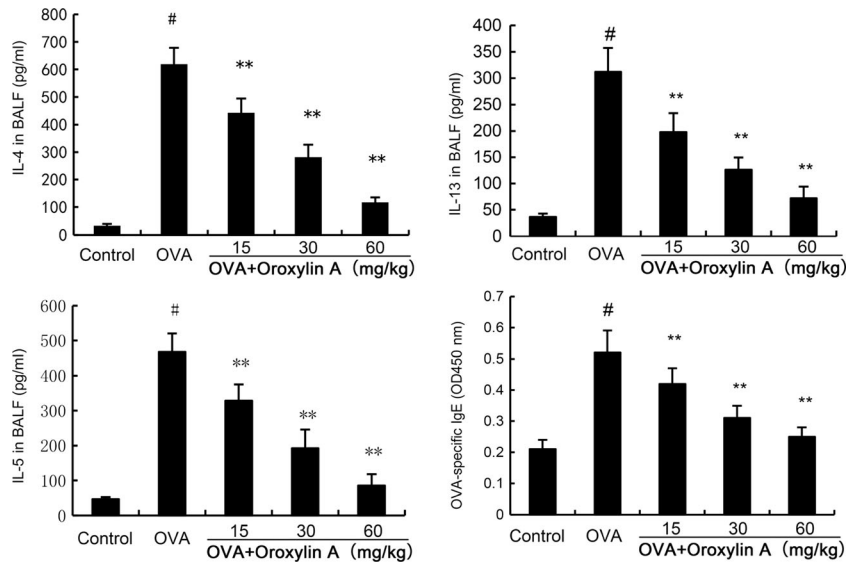


Fig. 3. Effects of oroxylin A on OVA-induced IL-4, IL-5, IL-13, and IgE productions in BALF were detected by ELISA. The values presented are the means \pm SEM of three independent experiments. [#] $p < 0.05$ vs. control group; * $p < 0.05$, ** $p < 0.01$ vs. OVA group.

challenged mice was higher than the control group. Treatment of oroxylin A significantly reduced AHR in OVA-challenged mice (Fig. 4).

Effects of Oroxylin A on OVA-Induced NF- κ B Activation

To investigate the protective mechanism of oroxylin A on OVA-induced asthma, the effects of oroxylin A on NF- κ B activation were detected in this study. The results showed that in OVA-challenged mice, the levels of p-I κ B and p-p65 were significantly increased. However, the increases were decreased by treatment of oroxylin A (Fig. 5).

DISCUSSION

Oroxylin A has been reported to have anti-inflammatory effects. In the present study, we investigated the protective effects of oroxylin A on OVA-induced asthma in mice. Our results showed that oroxylin A inhibited OVA-induced lung histopathologic changes, airway hyperresponsiveness, the levels of OVA-specific IgE in serum, and Th2 cytokines in BALF. Oroxylin A may be a useful agent for preventing OVA-induced asthma.

Previous studies showed that Th2 cytokines, including IL-4, IL-5, and IL-13, played an important role in the pathogenesis of allergic asthma [16, 17]. These cytokines

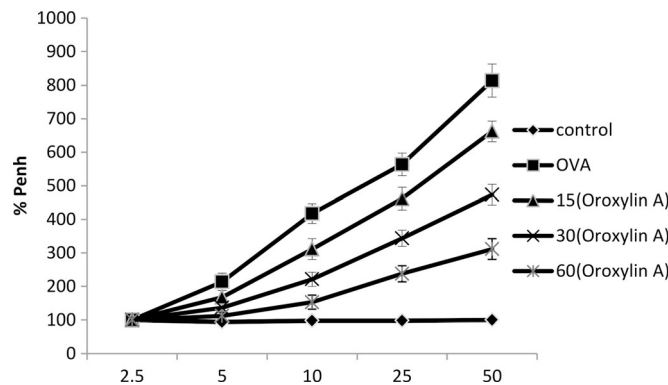


Fig. 4. Oroxylin A reduced OVA-induced airway hyperresponsiveness. The effects of oroxylin A on AHR were measured using noninvasive whole-body plethysmography. The values presented are the means \pm SEM of three independent experiments. [#] $p < 0.05$ vs. control group; * $p < 0.05$, ** $p < 0.01$ vs. OVA group.

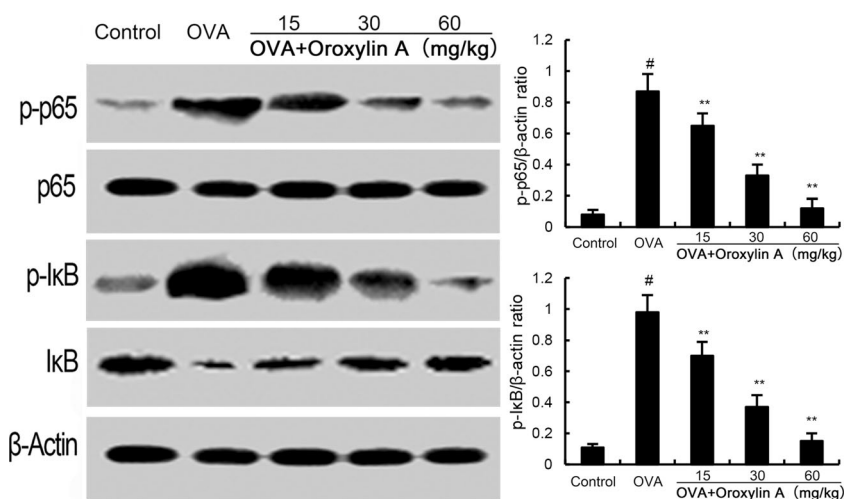


Fig. 5. Effects of oroxylin A on OVA-induced NF- κ B activation. The expression of NF- κ B and I κ B α were detected by western blotting. The values presented are the means \pm SEM of three independent experiments. [#] $p < 0.05$ vs. control group; * $p < 0.05$, ** $p < 0.01$ vs. OVA group.

are closely associated with the infiltration of inflammatory cells, IgE production, eosinophil activation, and AHR [18]. The infiltration of inflammatory cells into lungs can lead to lung injury by releasing cytotoxic granule proteins [19]. AHR is a marker of clinical symptom of asthma [20]. In this study, we found that OVA-induced asthma increased the level of IgE, the infiltration of inflammatory cells, and AHR development. However, oroxylin A treatment significantly inhibited these increase caused by OVA. Elevated IL-4, IL-5, and IL-13 were observed in patients or animal model of asthma [21]. Studies showed that inhibition of these cytokines could attenuate OVA-induced asthma [22]. In this study, our results showed that treatment of oroxylin A significantly inhibited OVA-induced Th2 cytokines. Furthermore, histological analysis showed that oroxylin A attenuated OVA-induced lung tissue injury. These results suggested that oroxylin A protected against OVA-induced asthma by inhibiting Th2 cytokines production.

NF- κ B is the main transcription molecular that plays critical roles in inflammation and immunity [23, 24]. Studies showed that NF- κ B played an important role in cytokines production [25]. It has been known NF- κ B signaling pathway played important roles in the development of inflammatory disease including sepsis and asthma [26, 27]. During asthma, NF- κ B regulates the production of Th2 cytokines such as IL-4, IL-5, and IL-13 [28]. To explore the protective mechanism of oroxylin A, the effects of oroxylin A on OVA-induced NF- κ B activation were measured. Our results demonstrated that oroxylin A significantly inhibited OVA-induced NF- κ B activation. These results suggested that the inhibition of Th2 cytokines by

oroxylin A may be related to the suppression of NF- κ B activation.

In conclusion, our results demonstrated that oroxylin A had a protective effect on OVA-induced asthma in mice. The possible mechanism is involved in inhibiting NF- κ B signaling pathway. These evidences suggest that oroxylin A may have potential for treating asthma.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no competing interests.

REFERENCES

- Barnes, P.J. 2008. Immunology of asthma and chronic obstructive pulmonary disease. *Nature Reviews Immunology* 8: 183–192.
- Postma, D., and H. Kerstjens. 1998. Characteristics of airway hyper-responsiveness in asthma and chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine* 158: S187–S192.
- Romagnani, S. 1994. Regulation of the development of type 2 T-helper cells in allergy. *Current Opinion in Immunology* 6: 838–846.
- Halim, T.Y., R.H. Krauß, A.C. Sun, and F. Takei. 2012. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* 36: 451–463.
- Jeffery, P.K. 2004. Remodeling and inflammation of bronchi in asthma and chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society* 1: 176–183.
- Chung, K. 2001. Cytokines in chronic obstructive pulmonary disease. *European Respiratory Journal* 18: 50s–59s.
- Bradding, P., J. Roberts, K. Britten, S. Montefort, R. Djukanovic, R. Mueller, C. Heusser, P. Howarth, and S. Holgate. 1994. Interleukin-4,-

- 5, and-6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *American Journal of Respiratory Cell and Molecular Biology* 10: 471–480.
8. Li, Q., and I.M. Verma. 2002. NF- κ B regulation in the immune system. *Nature Reviews Immunology* 2: 725–734.
 9. Poynter, M.E., R. Cloots, T. van Woerkom, K.J. Butnor, P. Vacek, D.J. Taatjes, C.G. Irvin, and Y.M. Janssen-Heininger. 2004. NF- κ B activation in airways modulates allergic inflammation but not hyperresponsiveness. *The Journal of Immunology* 173: 7003–7009.
 10. Desmet, C., P. Gosset, B. Pajak, D. Cataldo, M. Bentires-Alj, P. Lekeux, and F. Bureau. 2004. Selective blockade of NF- κ B activity in airway immune cells inhibits the effector phase of experimental asthma. *The Journal of Immunology* 173: 5766–5775.
 11. Oh, S.-W., J.-Y. Cha, J.-E. Jung, B.-C. Chang, H.-J. Kwon, B.-R. Lee, and D.-Y. Kim. 2011. Curcumin attenuates allergic airway inflammation and hyper-responsiveness in mice through NF- κ B inhibition. *Journal of Ethnopharmacology* 136: 414–421.
 12. Chen, Y.-C., L.-L. Yang, and T.J. Lee. 2000. Oroxylin A inhibition of lipopolysaccharide-induced iNOS and COX-2 gene expression via suppression of nuclear factor- κ B activation. *Biochemical Pharmacology* 59: 1445–1457.
 13. Song, X., Y. Chen, Y. Sun, B. Lin, Y. Qin, H. Hui, Z. Li, Q. You, N. Lu, and Q. Guo. 2012. Oroxylin A, a classical natural product, shows a novel inhibitory effect on angiogenesis induced by lipopolysaccharide. *Pharmacological Reports* 64: 1189–1199.
 14. Tseng, T.L., M.F. Chen, M.J. Tsai, Y.H. Hsu, C.P. Chen, and T.J. Lee. 2012. Oroxylin-A rescues LPS-induced acute lung injury via regulation of NF- κ B signaling pathway in rodents. *PLoS One* 7(10): e47403.
 15. Huang, H., X. Zhang, and J. Li. 2015. Protective effect of oroxylin A against lipopolysaccharide and/or D-galactosamine-induced acute liver injury in mice. *Journal of Surgical Research* 195: 522–528.
 16. Wong, C., C. Ho, F. Ko, C. Chan, A. Ho, D. Hui, and C. Lam. 2001. Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN- γ , IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clinical and Experimental Immunology* 125: 177–183.
 17. Barnes, P.J. 2001. Th2 cytokines and asthma: an introduction. *Respiratory Research* 2: 64–65.
 18. Ray, A., and L. Cohn. 1999. Th2 cells and GATA-3 in asthma: new insights into the regulation of airway inflammation. *Journal of Clinical Investigation* 104: 985.
 19. Moraes, T.J., J.H. Zurawski, and G.P. Downey. 2006. Neutrophil granulocyte contents in the pathogenesis of lung injury. *Current Opinion in Hematology* 13: 21–27.
 20. Leuppi, J.D., C.M. Salome, C.R. Jenkins, S.D. Anderson, W. Xuan, G.B. Marks, H. Koskela, J.D. Brannan, R. Freed, and M. Andersson. 2001. Predictive markers of asthma exacerbation during stepwise dose reduction of inhaled corticosteroids. *American Journal of Respiratory and Critical Care Medicine* 163: 406–412.
 21. Liao, S.-C., Y.-C. Cheng, Y.-C. Wang, C.-W. Wang, S.-M. Yang, C.-K. Yu, C.-C. Shieh, K.-C. Cheng, M.-F. Lee, and S.-R. Chiang. 2004. IL-19 induced Th2 cytokines and was up-regulated in asthma patients. *The Journal of Immunology* 173: 6712–6718.
 22. Duan, W., J.H. Chan, C.H. Wong, B.P. Leung, and W.F. Wong. 2004. Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model. *The Journal of Immunology* 172: 7053–7059.
 23. Karin, M., and Mireille D. The I κ B kinase (IKK) and NF- κ B: key elements of proinflammatory signalling. In *Seminars in immunology*, 85–98. Elsevier, 2000.
 24. Tak, P.P., and G.S. Firestein. 2001. NF- κ B: a key role in inflammatory diseases. *Journal of Clinical Investigation* 107: 7.
 25. Vallabhapurapu, S., and M. Karin. 2009. Regulation and function of NF- κ B transcription factors in the immune system. *Annual Review of Immunology* 27: 693–733.
 26. Edwards, M.R., N.W. Bartlett, D. Clarke, M. Birrell, M. Belvisi, and S.L. Johnston. 2009. Targeting the NF- κ B pathway in asthma and chronic obstructive pulmonary disease. *Pharmacology & Therapeutics* 121: 1–13.
 27. Henderson, W.R., E.Y. Chi, J.-L. Teo, C. Nguyen, and M. Kahn. 2002. A small molecule inhibitor of redox-regulated NF- κ B and activator protein-1 transcription blocks allergic airway inflammation in a mouse asthma model. *The Journal of Immunology* 169: 5294–5299.
 28. Das, J., C.-H. Chen, L. Yang, L. Cohn, P. Ray, and A. Ray. 2001. A critical role for NF- κ B in GATA3 expression and TH2 differentiation in allergic airway inflammation. *Nature Immunology* 2: 45–50.