

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

Biochimica et Biophysica Acta xx (2006) xxx–xxx

BIOCHIMICA ET BIOPHYSICA ACTA
BBA<http://www.elsevier.com/locate/bba>

Downregulation of leukotriene biosynthesis by thymoquinone attenuates airway inflammation in a mouse model of allergic asthma

Mohamed El Gazzar*, Rabab El Mezayen, Mark R. Nicolls, John C. Marecki, Stephen C. Dreskin

Department of Medicine, University of Colorado Health Sciences Center, 4200 E. Ninth Avenue, Denver, CO 80262, USA

Received 13 January 2006; received in revised form 1 March 2006; accepted 3 March 2006

Abstract

Chronic airway inflammation is a key feature of bronchial asthma. Leukotrienes are potent inflammatory mediators that play a role in the pathophysiology of asthma, and their levels are elevated in the airways in response to allergen challenge. We examined the anti-inflammatory effect of thymoquinone (TQ), the active principle in the volatile oil of *Nigella sativa* seeds, on leukotriene (LT) biosynthesis in a mouse model of allergic asthma. Mice sensitized and challenged with ovalbumin (OVA) antigen had an increased amounts of leukotriene B4 and C4, Th2 cytokines, and eosinophils in bronchoalveolar lavage (BAL) fluid. In addition, there was also a marked increase in lung tissue eosinophilia and goblet cell numbers. Administration of TQ before OVA challenge inhibited 5-lipoxygenase, the main enzyme in leukotriene biosynthesis, expression by lung cells and significantly reduced the levels of LTB4 and LTC4. This was accompanied by a marked decrease in Th2 cytokines and BAL fluid and lung tissue eosinophilia, all of which are characteristics of airway inflammation. These results demonstrate the anti-inflammatory effect of TQ in experimental asthma.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Inflammation; Th2 cytokines; Leukotrienes; 5-lipoxygenase; Thymoquinone

1. Introduction

Studies on the mediators of allergic lung inflammation have identified a number of effector cells, such as T cells, mast cells and eosinophils, and inflammatory mediators, such as cytokines, chemokines and leukotrienes that contribute to the development and maintenance of airway inflammation. Immune deviation from the T helper (Th)1 to the Th2 response underlies the allergen-mediated Th2-driven inflammation in the airway mucosa with a predominant Th2 cytokine pattern in lung T cells. When activated by allergen, inflammatory cells, including Th2 cells, in the airways produce Th2 type cytokines such as IL-4, IL-5, IL-13, and IL-10 [1–4]. Several previous studies have emphasized the role of these cytokines as well as leukotrienes in the pathogenesis of allergic inflammation and asthma [2,5–8].

Leukotrienes (LT)s are lipid mediators involved in inflammation and asthma (reviewed in [9], [10]). These eicosanoid products are synthesized via 5-lipoxygenase (5-LO) metabolism of arachidonic acid, generated from membrane phospholipids by cytosolic phospholipases (reviewed in [9]). There are two known classes of LTs: leukotriene B4 (LTB4) and cysteinyle LT (CysLTs), which includes LTC4, LTD4 and LTE4 (reviewed in [9], [10]). LTA4 is the first metabolite in the arachidonic acid peroxidation pathway. It is converted either into LTB4 or LTC4. The latter is sequentially converted into LTD4 and LTE4. LTC4 and LTD4 produced in the lung are metabolized extracellularly to LTE4, the most stable form of CysLTs [11]. LTB4 and CysLTs are synthesized by mucosal mast cells and basophils during the early phase response to antigen and by eosinophils and alveolar macrophages during the late phase. LTB4 is also produced by polymorphonuclear leukocytes and airway epithelial cells [11,12].

Elevated levels of LTs have been observed in BAL fluid during early (acute) and late (chronic) asthmatic response to allergen [13,14], as well as in the sputum of patients with severe

* Corresponding author. ECHSC Research Bldg. 21, 1055 Clermont St. (151), Denver, CO 80220, USA. Tel.: +1 303 399 6985; fax: 303 394 5840.

E-mail address: mohamed.elgazzar@uchsc.edu (M. El Gazzar).

asthma [15]. In humans, increased levels of CysLTs have been reported in BAL fluid and in sputum of patients with asthma [13,16], and this correlated with an increase in eosinophil numbers. LTB₄ is a potent chemoattractant of eosinophils, macrophages, neutrophils, and T cells, through binding to its receptor (BLT1) on these cells [17–20] and is involved in inflammation and immune response to infection [10,20,21]. CysLTs induce bronchoconstriction [22], mucus secretion [23], and chemotaxis [24], which contribute to the recruitment of inflammatory cells in bronchial asthma by significantly increasing the number of eosinophils in the airway mucosa [25,26].

The seed extract of *Nigella sativa* has been used for many years as a natural remedy for the treatment of a number of illnesses and conditions due to its pharmacological properties as an immunostimulant, anti-tumor, anti-inflammatory, and respiratory stimulant agent [27–32]. The volatile oil extracted from the seeds and its main active principle, thymoquinone (TQ) has been shown to exert anti-inflammatory effects in a number of diseases including bronchial asthma [27,29,33–35]. In vitro studies using cultured leukocytes have demonstrated that TQ inhibits peroxidation of arachidonic acid and leukotriene synthesis and that this may be due to targeting the 5-lipoxygenase activity, the key enzyme in leukotriene biosynthesis. The in vivo effects of TQ on leukotriene production and its relation to the inflammatory response remains to be elucidated.

Using a mouse model of allergen-induced airway inflammation, we examined the effect of TQ on leukotriene biosynthesis and lung inflammation. We report that TQ attenuates lung inflammation by downregulating 5-LO expression and LTB₄ and LTC₄ production by lung cells. The inhibitory effect of TQ on leukotriene generation was accompanied by a significant decrease in airway inflammation, as shown by the decrease in Th2 cytokines and lung eosinophilia, demonstrating the anti-inflammatory effect of TQ in the lung.

2. Materials and methods

2.1. Experimental protocol

Males and females BALB/c mice 8 to 10 weeks of age were kept on 12 h light/dark cycle with free access to an OVA-free diet. Lung inflammation was induced by intraperitoneal (i.p.) sensitization and airway challenge through nasal inhalation with OVA allergen. Mice (3 males and 3 females/group) were sensitized on days 0 and 14 by i.p. injection of 20 µg of OVA (Grade V; Sigma-Aldrich, St. Louis, MO) in 2 mg Al(OH)₃ adjuvant (Pierce Biotechnology, Rockford, IL) in a volume of 0.1 ml saline. Two weeks after the second sensitization, mice were airway challenged three times (days 28–30) via nasal inhalation with 1% OVA/saline aerosol for 20 min, using De Vilbiss nebulizer (Sunrise Medical, Carlsbad, CA). This protocol has previously been shown to induce airway eosinophilic inflammation, Th2 cytokines, mucus production and airway hyperreactivity [36,37]. For TQ treatment, mice were (i.p.) injected once daily for the 5 days preceding the first OVA challenge, with 3 mg/kg TQ (Sigma-Aldrich) in 10% DMSO or an equal volume of DMSO alone. All mice were analysed 24 h after the last OVA challenge. The animal groups are described as follow: Naïve, mice sensitized and challenged with saline and injected with DMSO alone; S/Ch, mice sensitized and challenged with OVA and injected with DMSO alone; and S/Ch-TQ, mice sensitized and challenged with OVA but received 5 injection of TQ in DMSO before the first OVA challenge. TQ (2-Isopropyl-5-methyl-1,4-benzoquinone; molecular formula C₁₀H₁₂O₂) was 99% pure, as indicated by the manufacturer. Each experiment was repeated at least three times, and representative data are shown.

2.2. Bronchoalveolar lavage (BAL) fluid

Mice were anesthetized and lungs were lavaged by instillation and withdrawal of 1 ml PBS through a tracheal cannula and BAL fluid (BALF) was collected. Total BAL cell count was determined with crystal violet stained cells using a hemocytometer. For differential cell count, cytocentrifuged preparations were fixed and stained with Diff-Quick (Dade Behring, Duding, Switzerland) and differentiated morphologically by counting 300 cells/slide.

2.3. Western blot

To isolate lung cells, lungs were sliced and incubated in 5 ml of PBS containing 1.6 mg/ml collagenase (CLS4; Invitrogen, Carlsbad, CA), antibiotics, and 10% FBS at 37 °C for 1 h. The digested tissue was disrupted into a single cell suspension by passing through a 20-gauge needle and filtered through a 45-µm nylon mesh. The resulting cell suspension (whole lung cells) was pelleted by centrifugation at 1500 rpm for 5 min at 4 °C.

Whole lung cells (2×10⁶) were washed with PBS and lysed in a cold buffer containing 50 mM HEPES (pH 7.9), 150 mM NaCl, 1% Triton X-100, 1 mM MgCl₂, 1 mM EDTA, 1 mM PMSF, 10% glycerol, 10 µg/ml aprotinin, and 1 mM leupeptin. Lysates were centrifuged for 5 min at 10,000 rpm and 4 °C to remove cell debris, and the total protein concentration in the supernatants was determined by Bradford assay (Bio-Rad). Samples containing 50 µg protein/lane were resolved on 12% Tris–glycine denaturing SDS-PAGE, and transferred onto a nitrocellulose membranes (Amersham, Piscataway, NJ) at 100 V for 1 h in a buffer containing 25 mM Tris–HCl (pH 8.0), 192 mM glycine, and 20% methanol. Membranes were blocked in 20 ml of TBS (Tris-Buffered Saline) plus 0.1% Tween 20 (TBST) and 5% nonfat dry milk for 1 h at room temperature. After three washes in TBST (5 min each), membranes were incubated overnight at 4 °C with purified mouse anti-5-lipoxygenase Ab (BD PharMingen, San Diego, CA). Blots were washed three times in TBST and incubated for 1 h at room temperature with the horseradish peroxidase-conjugated secondary antibody (Amersham). The blots were then washed three times in TBST and incubated for 1–2 min with 5 ml of ECL reagent (Amersham) at room temperature. The bound proteins were visualized by exposure to Kodak X-AR film (Eastman Kodak, NY). Membranes were subsequently stripped in a buffer containing 62 mM Tris–HCl (pH 6.8), 20% SDS, and 100 mM β-mercaptoethanol and re-probed for GAPDH.

2.4. Measurement of 5-LO enzyme activity

Measurement of 5-LO activity was performed as described previously [38], with slight modification. Lung cells isolated from Naïve, S/Ch, and S/Ch-TQ groups were immediately washed in PBS. Cells were resuspended in 5 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and sonicated for 30 s. The resulting homogenate was cleared by centrifugation at 15,000 rpm for 20 min at 4 °C. The supernatant was removed and filtered through 0.45 µ filter, and the protein concentration in the supernatant was determined by Bradford assay.

For assaying 5-LO activity, the enzymatic reaction mixture contained 300 µM CaCl₂, 100 mM phosphate buffer (pH 7.4), 50 µM DTT, 200 µM ATP, 100 µM arachidonic acid (as a substrate), and 1 µg protein (supernatant). The reaction was carried out at 37 °C for 2 min. The formed arachidonic acid metabolite, 5-HETE, was measured spectrophotometrically at 236 nm, and the extinction co-efficient of 25 mM⁻¹ cm⁻¹ was used to calculate the specific activity of the enzyme (total conversion of arachidonic acid into 5-HETE). The activity was expressed as µmoles of 5-HETE formed/min/mg protein. The 5-LO activity was expressed as fold increase relative to the enzyme activity in the supernatant derived from Naïve mice, which was given the value 1.

2.5. Cytokine and leukotriene measurement

Levels of IL-4, IL-5, IL-13, and IL-10 in BAL fluid were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol, and compared with known standards. Briefly, microtiter plates were coated overnight at 4 °C with the first antibody, washed and then blocked at room temperature for 1 h. Samples and a series of diluted standards were incubated overnight at 4 °C. After washing, the

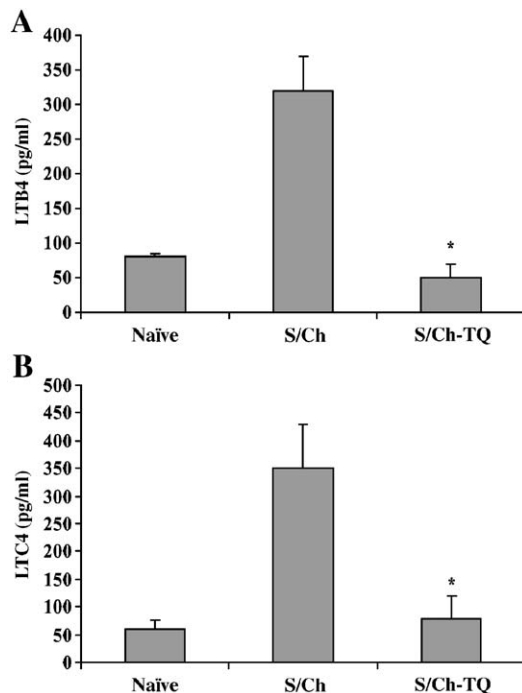


Fig. 1. Leukotriene levels in BAL fluid. BAL fluid was collected 24 h after the last OVA challenge, and levels of LTB4 (A) and LTC4 (B) were measured by EIA. Data (mean±S.E.M.) shown represent three independent experiments. Naïve, sensitized and challenged with saline, and injected with DMSO alone; S/Ch, sensitized and challenged with OVA and injected with DMSO alone; and S/Ch-TQ sensitized and challenged with OVA but administered with TQ in DMSO before the first OVA challenge. *Statistical significance compared to S/Ch group; $n=6$; $P<0.05$.

biotin-conjugated detection antibodies and avidin-HRP were added and left at room temperature for 1 h. After washing the plates, the substrate was added for the appropriate periods of time. The reactions were then stopped and read at 450 nm. The detection limits were 7.8 pg/ml for IL-4 and IL-13, 15.5 pg/ml for IL-5, and 4 pg/ml for IL-10. LTB4 and LTC4 levels in BAL fluid were measured using EIA kits according to the manufacturer's protocol (Cayman, Ann Arbor, MI). Limits of detection of LTB4 and LTC4 were 6 pg/ml and 10 pg/ml, respectively.

2.6. Histopathology

Lung tissue obtained 24 h after the last OVA challenge was fixed in 10% neutral formalin, and paraffin sections (0.6 μ m) were stained with Hematoxylin and Eosin (H&E), to detect eosinophilic infiltration and Periodic-Acid Schiff (PAS), to detect mucus-secreting goblet cells.

2.7. Statistical analysis

Data (means±S.E.M.) were analysed by one way analysis of variance followed by two-tailed Student's t test, to determine the statistical difference between pairs of groups. P values < 0.05 were considered significant.

3. Results

3.1. Effect of TQ on leukotriene production

Leukotrienes play an important role in the development and maintenance of allergic inflammation by upregulating adhesion molecules and stimulating chemotaxis, thereby promoting the recruitment and activation of inflammatory cells, predominantly eosinophils, in the airways. We measured the levels of two proinflammatory leukotrienes, LTB4 and LTC4, in the BAL fluid using EIA. OVA sensitization and challenge significantly increased the production of LTB4 and LTC4 in the lung by about 4- and 7-fold, respectively (Fig. 1). TQ administration decreased the production of the two leukotrienes in the lung (S/Ch-TQ group), with more potent effect on LTB4. DMSO alone (Naïve and S/Ch mice) did not affect LTs production.

3.2. TQ downregulates 5-lipoxygenase expression in the lung of OVA-sensitized and challenged mice

Arachidonic acid metabolism and leukotriene biosynthesis is catalyzed by 5-LO activity, the key enzyme in LTs biosynthesis. To determine whether the inhibitory effect of TQ on LTB4 and LTC4 production in the airways of sensitized and challenged mice was due to an effect on 5-LO expression. Protein expression of 5-LO by whole lung cells was analysed by western blotting. The protein expression was induced after OVA sensitization and challenge and TQ (in DMSO, but not DMSO alone) administration normalized the increased level of 5-LO expression to a level close to that seen in naïve mice (Fig. 2) (compare S/Ch with S/Ch-TQ). These results indicate that TQ target 5-lipoxygenase protein expression and eicosanoid generation.

3.3. Effect of TQ on 5-LO activity

5-LO catalyzes the conversion of arachidonic acid into 5-hydroeicosatetraenoic acid (5-HETE), which is further metabolized into leukotrienes. The data presented in Figs. 1 and 2 showed that TQ inhibited 5-LO protein expression and LT production by lung cells from OVA-sensitized and challenged mice (S/Ch-TQ). To investigate whether this inhibitory effect of TQ was accompanied by a parallel decrease in the 5-LO enzymatic activity, we measured the level of 5-HETE formation from arachidonic acid as an exogenous substrate. Fig. 3 shows that lung cells from OVA-sensitized and challenged mice have about 3-fold increase in 5-LO activity, whereas cells from mice treated with TQ before OVA sen-

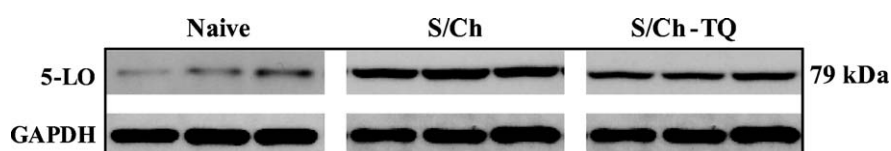


Fig. 2. TQ inhibits 5-LO expression by lung cells. Whole lung cells were isolated after the last allergen challenge and used in western blot. Cell lysate was run onto 12% SDS-PAGE. The 5-LO protein was probed with anti-5-LO antibody and visualized with ECL. Data from three mice per group are shown, and the experiment was repeated two times. Naïve, sensitized and challenged with saline and injected with DMSO; S/Ch, sensitized and challenged with OVA and injected with DMSO; S/Ch-TQ sensitized and challenged with OVA but administered with TQ in DMSO before the first OVA challenge.

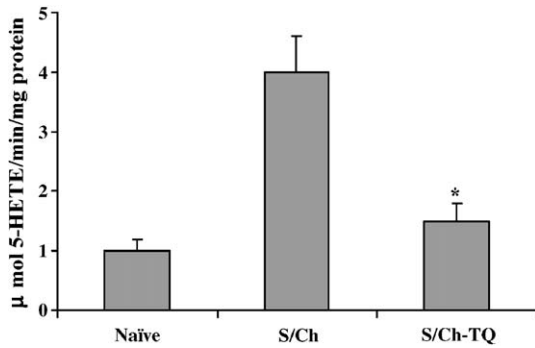


Fig. 3. 5-LO enzyme activity. Cell extract containing 5-LO was incubated with arachidonic acid as an exogenous substrate, and the formation of the 5-HETE metabolite was measured at 236 nm. The amount of 5-HETE formed by extract derived from Naïve mice was assigned 1, and the amounts from the treated groups were compared to this baseline. Data are mean $\pm$ S.E.M. of values from three independent experiments.

sitization and challenge have a significantly decreased enzyme activity. These data indicate that the downregulation of 5-LO protein expression by TQ is accompanied with a parallel decrease in the enzyme activity.

3.4. Effect of TQ on BAL fluid cytokines

LTs promote Th2 cytokine production, and because TQ showed an inhibitory effect on the LTB₄ and LTC₄ production,

we determined the effect of TQ on IL-4, IL-5, IL-13, and IL-10 protein levels in BAL fluid in response to OVA antigen. OVA-sensitized and challenged mice (S/Ch group) produced significant amounts of these cytokines compared to naïve mice (Fig. 4). Intraperitoneal administration of TQ before OVA challenge normalized the elevated cytokine levels (S/Ch-TQ), with greater effect on IL-5 and IL-13 than on IL-4, while administration of DMSO alone had no effect on the cytokine response (S/Ch group). In addition, IL-10 was significantly induced by OVA sensitization and challenge (S/Ch) and TQ treatment resulted in a marked increase in its level (S/Ch-TQ group) (Fig. 4D).

3.5. TQ inhibits inflammatory cell recruitment and mucus production in allergic lung

Data presented in Figs. 1 and 2 showed that TQ downregulated leukotriene biosynthesis by inhibiting 5-LO expression in the lung. LTs promote inflammatory cell recruitment to, as well as mucus production in, the airways in response to allergen. To assess BAL cell inflammation, BAL fluid was collected 24 h after the last allergen challenge and total and differential cell numbers were determined. Mice sensitized and challenged had a significant increase in the total and eosinophil cell numbers (Fig. 5). Treatment with TQ showed a potent effect in decreasing the number of eosinophils in the airways.

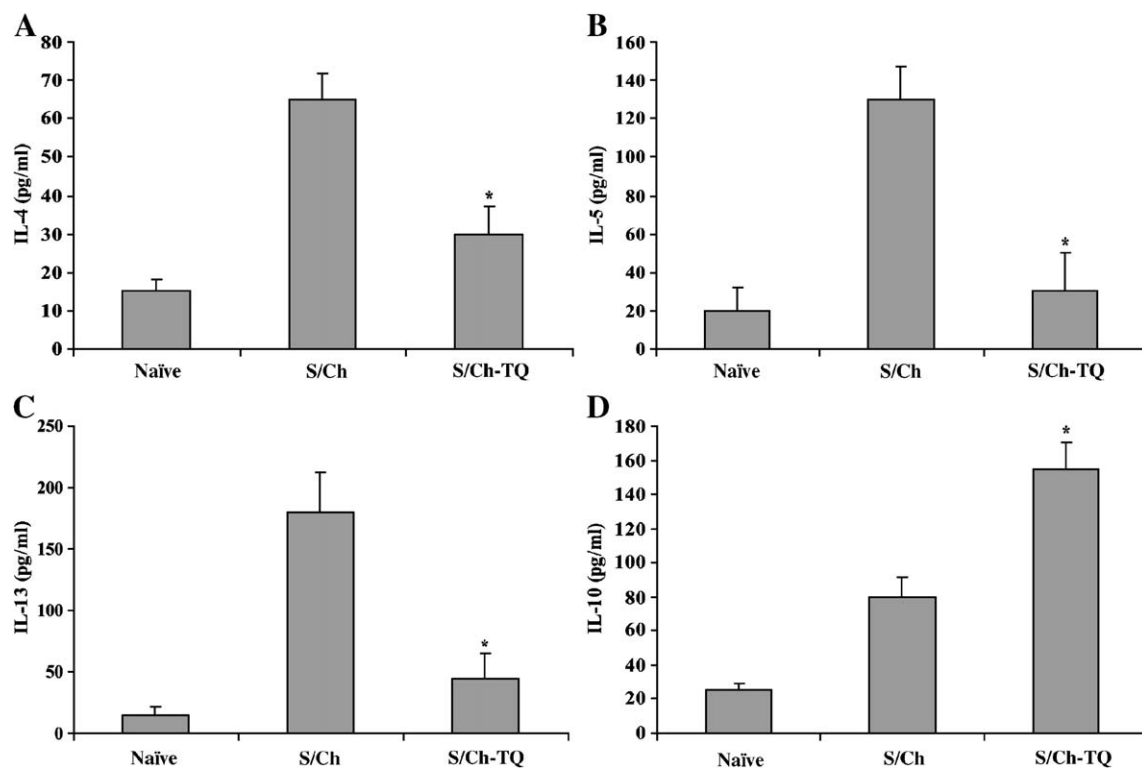


Fig. 4. Th2 cytokine levels in BAL fluid. Levels of IL-4 (A), IL-5 (B), IL-13 (C), and IL-10 (D) were measured by ELISA. Data are expressed as mean $\pm$ S.E.M. of 6 mice per group, and represent three different experiments. Naïve, sensitized and challenged with saline and injected with DMSO; S/Ch, sensitized and challenged with OVA and injected with DMSO; S/Ch-TQ, sensitized and challenged with OVA but administered with TQ in DMSO before the first OVA challenge. *Statistical significance compared to S/Ch group; $P < 0.05$.

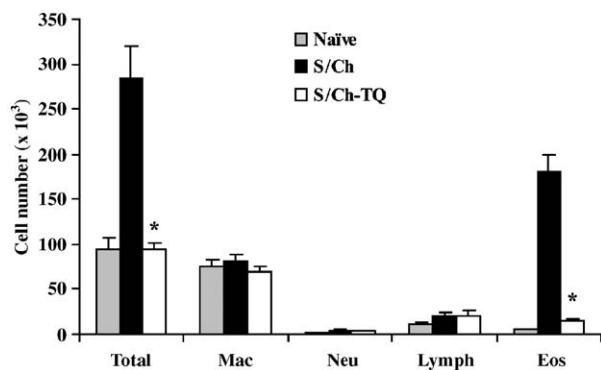


Fig. 5. Inflammatory cells in BAL fluid. Total and differential cell count in BAL fluid were determined 24 h after the last airway challenge. Data shown are the mean $\pm$ S.E.M. of 6 mice per group, and represent three experiments. Mac, macrophage; Neu, neutrophil; Lymph, lymphocyte; Eos, eosinophil. *Statistical significance compared to S/Ch group; $P < 0.05$.

Histological examination of lung tissues collected 24 h after OVA challenge showed a massive infiltration of the peri-bronchial tissues with inflammatory cells, mainly eosinophils (Fig. 6B). In addition, there was a marked increase in the number of mucus-secreting goblet cells in the airway mucosa. (E) TQ was effective in blocking lung tissue eosinophilia and goblet cell hyperplasia (C and F). In addition, treatment with DMSO alone did not affect lung inflammation (compare Naïve and S/Ch with S/Ch-TQ). These results demonstrate the anti-inflammatory effect of TQ on allergen-driven lung inflammation.

4. Discussion

Chronic airway inflammation is characterized by increased mucus secretion and infiltration and subsequent activation of inflammatory cells, typically eosinophils, in the allergic airways [39,40]. In vitro and in vivo studies have suggested that LTs, produced by activated eosinophils, macrophages and mast cells [40,41], play an important role in the pathogenesis of allergic airway inflammation and asthma through inducing Th2 cytokine production by activated T cells, mucus secretion by goblet cells, bronchoconstriction, and airway remodeling [41–43].

In this study, we found that TQ inhibited airway inflammation induced by OVA sensitization and challenge in BALB/c mice. This was shown by downregulation of 5-LO expression by lung cells and inhibition of LTB₄ and LTC₄ and Th2 cytokine production in the airways, as well as a significant decrease in eosinophil and goblet cell numbers in the lung tissue of sensitized and challenged mice.

Levels of LTB₄ and LTC₄ are increased in BAL fluid of allergic mice and human asthmatic subjects after allergen challenge [7,13]. Our data showed that levels of LTB₄ and LTC₄ in BAL fluid and 5-LO protein expression and enzyme activity by lung cells were significantly increased by OVA sensitization and challenge. TQ was effective in down-regulating 5-LO expression and LTB₄ and LTC₄ production. LTB₄ is a potent inducer of effector Th2 cell recruitment into

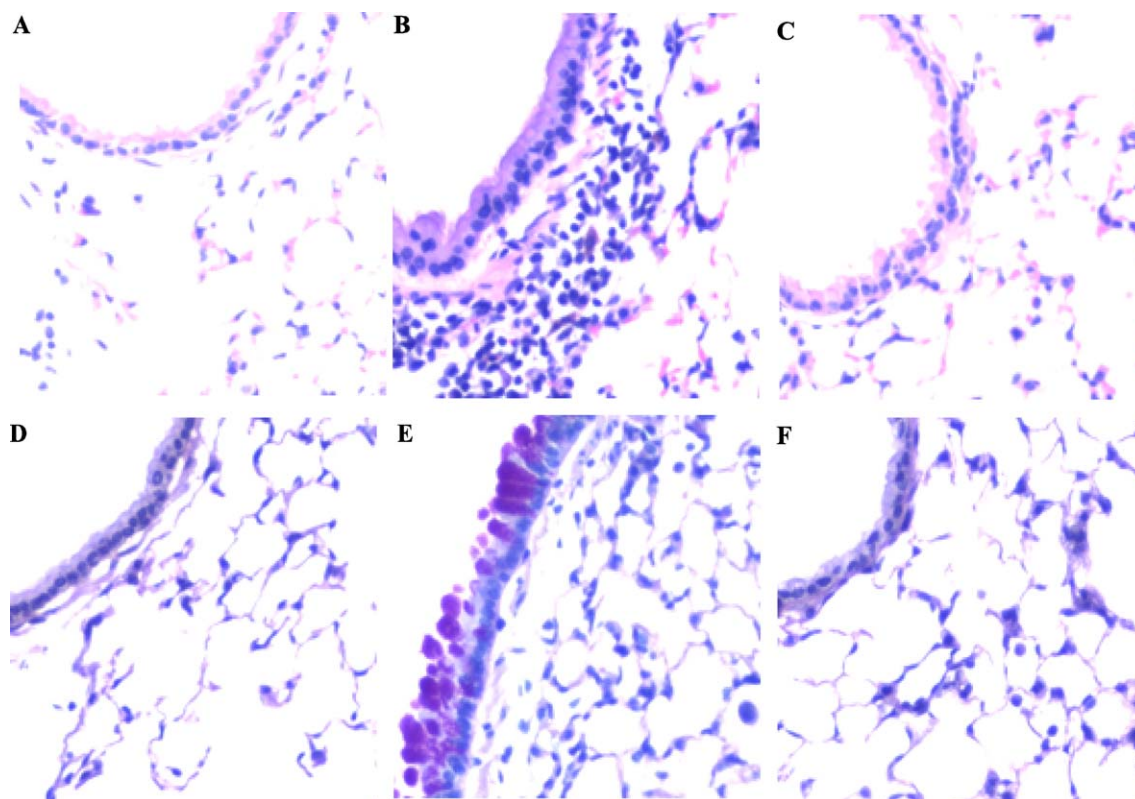


Fig. 6. TQ abrogates lung inflammation. Lung tissue were collected from Naïve group (A, D), S/Ch group (B, E), and S/Ch-TQ group (C, F) 24 h after the last allergen challenge. Tissue sections were stained with H&E, to detect lung tissue eosinophilia (A–C), and with PAS, to examine goblet cell hyperplasia (D–F). Administration of TQ before OVA challenge (S/Ch-TQ group) inhibited lung tissue eosinophilia (C) and goblet cell hyperplasia (F).

allergic lung [10,44]. It is also a potent agonist for eosinophils, which express high levels of LTB₄ receptor [18]. Th2 cells along with eosinophils produce the Th2 cytokines, IL-4, IL-5, and IL-13. These cytokines play an important role in the development and maintenance of the allergic inflammatory response [45]. IL-5 plays a central role in the recruitment, activation and survival of eosinophils in the allergic lung [46], while IL-4 and IL-13 induce IgE isotype switching in B lymphocytes [47,48] and mucus production by goblet cells [49,50], as well as upregulate the expression of adhesion molecules required for inflammatory cell recruitment [51,52]. Our results showed that IL-4, IL-5, and IL-13 protein levels in BAL fluid were significantly reduced in allergic mice after TQ administration. In addition, previous studies have shown that LTB₄ and LTC₄ augment Th2 cytokine secretion by activated T cells [53–55], and that leukotriene receptor antagonists significantly reduce IL-4, IL-5, and IL-13 expression in allergic lung [56,57]. In the current study, the inhibitory effect of TQ on LTs biosynthesis was accompanied by a significant decrease in Th2 cytokines, suggesting that the inhibition of Th2 cytokine production may be due to inhibition of leukotriene biosynthesis by TQ.

Previous studies identified eosinophils as the major effector cells underlying allergic airway inflammation [58,59]. LTB₄ and LTC₄ promote chemotaxis and chemokinesis of eosinophils in mice and human [60,61], thus contributing to airway inflammation. In addition, agents that block LT production or their receptors also inhibit eosinophil recruitment into the allergic lung. We found that the numbers of eosinophils were increased in BAL fluid and lung tissue of sensitized and challenged mice. TQ blocked eosinophil infiltration into the lung, as well as mucus-secreting goblet cells. Thus, it seems that the attenuation in airway eosinophilia and Th2 cytokine production by TQ was due to its inhibitory effect on 5-LO protein expression and LT production. TQ has also been shown to inhibit *in vitro* production of LTs by human leukocytes [62]. There is no information about the exact mechanism(s) by which TQ inhibits LTs and Th2 cytokines. From our data, TQ seems to mediate these effects, at least in part, by downregulating 5-LO expression. In addition, cytosolic phospholipase (cPLA₂) catalyses the conversion of membrane phospholipids to arachidonic acid, which is subsequently metabolized into LTs by 5-LO [11]. Western blot analysis of cPLA₂ expression in lung cells (data not shown) showed that cPLA₂ protein levels remained constant with or without TQ administration to OVA-sensitized and challenged mice, suggesting that TQ has no effects on the LT biosynthesis cascade upstream of 5-LO. In addition, in our ongoing experiment using cultured mast cell line, TQ inhibited the activation and nuclear translocation of NF- κ B that was induced by LPS stimulation (data not shown).

While Th2 cells, with the characteristic IL-4, IL-5, and IL-13 cytokine profile, are directly involved in the initiation and maintenance of allergic diseases, recent studies of allergic airway inflammation in mouse models have identified different types of T cells with different cytokine profiles and immuno-

regulatory function (reviewed in [63,64]). One of the T regulatory (T reg) cell types is the antigen-specific T reg cells, also known as IL-10-producing T cells [63]. As a pleiotropic cytokine produced by Th1 and Th2 cells, eosinophils, and macrophages [65,66], IL-10 has been described as a potent anti-inflammatory Th2 cytokine and more recently as an immunomodulatory cytokine of allergic asthma [64]. Its level is increased in asthmatics [67]. IL-10 inhibits allergen-induced airway inflammation by blocking Th2 cytokines and airway eosinophilia and inducing the switch from IgE to IgG4 antibody response [68–71]. Our results showed that IL-10 level was induced by OVA sensitization and challenge, and that TQ significantly elevated its level in BAL fluid, which was associated with attenuated airway inflammation. This indicates that TQ may stimulate the expansion of the Th cell clones toward the protective T reg cell type during the allergic response. Therefore, the increase in IL-10 production by TQ can be another possible mechanism that mediates the anti-inflammatory effect of TQ in the airways.

In conclusion, our study demonstrates that the anti-inflammatory effect of TQ allergic lung is mediated, at least in part, by inhibition of 5-LO expression and LT synthesis, which was accompanied by a decrease in Th2 cytokines and inflammatory cells in the airways, as well as an induction of IL-10. Inhibition of leukotriene biosynthesis by blocking 5-LO expression can be an important mechanism in ameliorating inflammatory responses. Several other plant extracts have been shown to have an inhibitory effect on 5-LO activity (reviewed in [72]).

Acknowledgement

This work was supported in part by National Institutes of Health grant HL51241.

References

- [1] D.B. Corry, G. Grunig, H. Hadeiba, V.P. Kurup, M.L. Warnock, D. Sheppard, D.M. Rennick, R.M. Locksley, Requirements for allergen-induced airway hyperactivity in T and B cell-deficient mice, *Mol. Med.* 4 (1998) 344–355.
- [2] N.A. Lee, E.W. Gelfand, J.J. Lee, Pulmonary T cells and eosinophils: coconspirators or independent triggers of allergic respiratory pathology? *J. Allergy Clin. Immunol.* 107 (2001) 945–957.
- [3] D.S. Robinson, Q.A. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A.M. Bentley, C. Corrigan, S.R. Durham, A.B. Kay, Predominant Th2-like bronchoalveolar T-lymphocyte population in atopic asthma, *N. Engl. J. Med.* 326 (1992) 298–304.
- [4] J. Oh, C.M. Seroogy, E.H. Meyer, O. Akbari, G. Berry, G. Fathman, R.H. DeKruyff, D.T. Umetsu, CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperactivity and inflammation, *J. Allergy Clin. Immunol.* 110 (2002) 460–468.
- [5] P.S. Foster, S.P. Hogan, A.J. Ramsay, K.I. Matthaei, I.G. Young, Interleukin 5 deficiency abolishes eosinophilia, airway hyperactivity, and lung damage in a mouse asthma model, *J. Exp. Med.* 183 (1996) 195–201.
- [6] E. Hamelmann, A. Oshiba, J. Paluh, K. Bradley, J. Loader, T.A. Potter, G.L. Larsen, E.W. Gelfand, Requirement for CD8⁺ T cells in the development of airway hyperresponsiveness in a murine model of airway sensitization, *J. Exp. Med.* 183 (1996) 1719–1730.
- [7] W.R. Henderson, D.B. Lewis, R.K. Albert, Y. Zhang, W.J. Lamn, G.K. Chiang, F. Jones, P. Eriksen, Y. Tien, M. Jonas, E. Chi, The importance of

- leukotrienes in airway inflammation in a mouse model of asthma, *J. Exp. Med.* 184 (1996) 1483–1494.
- [8] W.R. Henderson, L. Tang, S.J. Chu, H. Tsao, M.Y. Chi, Role of cysteinyl-leukotrienes in airway remodeling in a mouse asthma model, *Am. J. Respir. Crit. Care Med.* 165 (2002) 108–116.
- [9] S.E. Wenzel, The role of leukotrienes in asthma, *Prostaglandins Leukot. Essent. Fat. Acids* 96 (2003) 145–155.
- [10] A.M. Tager, S.K. Bromley, B.D. Medoff, S.A. Islam, S.D. Bercury, E.B. Friedrich, A.D. Carafone, R.E. Gerszten, A.D. Luster, Leukotriene B4 receptor BLT1 mediates early effector T cell recruitment, *Nat. Immunol.* 4 (2003) 982–990.
- [11] A.R. Leff, Regulation of leukotrienes in the management of asthma: biology and clinical therapy, *Annu. Rev. Med.* 52 (2001) 1–14.
- [12] M. Peters-Golden, W.R. Henderson, The role of leukotrienes in allergic rhinitis, *Ann. Allergy, Asthma, & Immun.* 94 (2005) 609–618.
- [13] S.E. Wenzel, G.L. Larsen, K. Johnston, N.F. Voelkel, J.Y. Wescott, Elevated levels of leukotriene C4 in bronchoalveolar lavage fluid from atopic asthmatic after endo-bronchial allergen challenge, *Am. Rev. Respir. Dis.* 142 (1990) 112–119.
- [14] P. Diaz, M.C. Gonzalez, F.R. Galleguillos, P. Ancic, O. Cromwell, D. Shepherd, S.R. Durham, G.J. Gleich, A.B. Kay, Leukocytes and mediators in bronchoalveolar lavage during allergen-induced late-phase asthmatic reactions, *Am. Rev. Respir. Dis.* 139 (1989) 1383–1389.
- [15] I. Vachier, C. Bonnans, C. Chavis, M. Farce, P. Godard, J. Bousquet, P. Chané, Severe asthma is associated with a loss of LX4, an endogenous anti-inflammatory compound, *J. Allergy Clin. Immunol.* 115 (2005) 55–60.
- [16] A.J. Macfarlane, R. Dworski, J.R. Sheller, Sputum cysteinyl leukotrienes increase 24 hours after allergen inhalation in atopic asthmatics, *Am. J. Respir. Crit. Care Med.* 161 (2000) 1553–1558.
- [17] A.W. Ford-Hutchinson, M.A. Bay, M.V. Doig, M.E. Shipley, M.J. Smith, Leukotriene B₄, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes, *Nature* 286 (1980) 264–265.
- [18] W.W. Huang, E.A. Garcia-Zepeda, A. Sauty, H.C. Oettgen, M.E. Rothenberg, A.D. Luster, Molecular and biological characterization of the murine leukotriene B₄ receptor expressed on eosinophils, *J. Exp. Med.* 188 (1998) 1063–1074.
- [19] A.M. Tager, A.D. Luster, BLT1 and BLT2: the leukotriene B₄ receptors, *Prostaglandins Leukot. Essent. Fat. Acids* 69 (2003) 123–134.
- [20] N. Miyahara, K. Takeda, S. Miyahara, C. Taube, A. Joetham, T. Koya, S. Matsubara, A. Dakhama, A.M. Tager, A.D. Luster, E.W. Gelfand, Leukotriene B₄ receptor-1 is essential for allergen-mediated recruitment of CD8⁺ T cells and airway hyperresponsiveness, *J. Immunol.* 174 (2005) 4979–4984.
- [21] X.S. Chen, J.R. Scheller, E.N. Johnson, C.D. Funk, Role of leukotrienes revealed by targeted disruption of 5-lipoxygenase gene, *Nature* 372 (1994) 179–182.
- [22] S.E. Dahlen, G. Hansson, P. Hedqvist, J. Björck, E. Granström, B. Hahlen, Allergen challenge of lung tissue from asthmatic elicits bronchial contraction that correlates with release of leukotrienes C₄, D₄, and E₄, *Proc. Natl. Acad. Sci. U. S. A.* 80 (1983) 1712–1716.
- [23] W.R. Henderson, The role of leukotrienes in inflammation, *Ann. Intern. Med.* 121 (1994) 684–697.
- [24] L.A. Laitinen, A. Laitinen, T. Haahtela, V. Vikka, B.W. Spur, T.H. Lee, Leukotriene E₄ and granulocytic infiltration into asthmatic airways, *Lancet* 341 (1993) 989–990.
- [25] A. Laitinen, A. Lindqvist, M. Halme, A. Altraja, L.A. Laitinen, Leukotriene E₄-induced persistent eosinophilia and airway obstruction are reversed by zafirlukast in patients with asthma, *J. Allergy Clin. Immunol.* 115 (2005) 259–265.
- [26] K. Goodarzi, M. Goodarzi, A.M. Tager, A.D. Luster, U.H. Von, BLT1 control cytotoxic effector T cell recruitment to inflamed tissue, *Nat. Immunol.* 4 (2003) 965–973.
- [27] P.J. Houghton, R. Zerka, B. DL-Heras, J.R. Hoult, Fixed oil of *Nigella sativa* and derived thymoquinone inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation, *Planta Med.* 61 (1995) 33–36.
- [28] B.H. Ali, G. Blunden, Pharmacological and toxicological properties of *Nigella sativa*, *Phytother. Res.* 19 (2003) 299–305.
- [29] A. Mutabagani, S.A. El-Mahdy, Study of the anti-inflammatory activity of *Nigella sativa* L and thymoquinone in rats, *Saudi Pharm. J.* 5 (1997) 110–113.
- [30] D.R. Worthen, O.A. Ghosheh, P.A. Crooks, The in vitro anti-tumor activity of some crude and purified compounds of black seed, *Nigella sativa* L., *Anticancer Res.* 18 (1998) 1527–1532.
- [31] S.M. Swamy, B.K. Tan, Cytotoxic and immunopotentiating effects of ethanolic extract of *Nigella sativa* L. Seeds, *J. Ethnopharmacol.* 70 (2000) 1–7.
- [32] K.E. El-Tahir, M.M. Ashour, M.M. AL-Harbi, The respiratory effects of the volatile oil of the black seed (*Nigella sativa*) in Guinea pigs: elucidation of the mechanism(s) of action, *Gen. Pharmacol.* 24 (1993) 1115–1122.
- [33] V. Hajhashemi, A. Ghannadi, H. Jafarabadi, Black cumin seed essential oil, as a potent analgesic and antiinflammatory drug, *Phytother. Res.* 18 (2004) 195–199.
- [34] A. Mahgoub, Thymoquinone protects against experimental colitis in rats, *Toxicol. Lett.* 143 (2003) 133–143.
- [35] U. Kalus, A. Pruss, J. Bystron, M. Jurecka, A. Smekalova, J. Lichius, H. Kiesewetter, Effect of *Nigella sativa* (black seed) on subjective feeling in patients with allergic diseases, *Phytother. Res.* 17 (2003) 1209–1214.
- [36] E.W. Gelfand, Z.H. Cui, K. Takeda, A. Kanehiro, A. Joetham, Effects of fexofenadine on T-cell function in a murine model of allergen-induced airway inflammation and hyperresponsiveness, *J. Allergy Clin. Immunol.* 12 (2003) S89–S95.
- [37] K. Kanehiro, K. Takeda, A. Joetham, A. Tomkinson, T. Ikemura, C.G. Irvin, E.W. Gelfand, Timing of administration of anti-VLA-4 differentiates airway hyperresponsiveness in the central and peripheral airways in mice, *Am. J. Respir. Crit. Care Med.* 162 (2000) 1132–1139.
- [38] H. Raghaverna, B.T. Diwakar, B.R. Lokesh, K.A. Naidu, Eugenol-the active principle from cloves inhibits 5-lipoxygenase activity and leukotriene-C₄ in human PMNL cells, *Prostaglandins Leukot. Essent. Fat. Acids* 74 (2005) 23–27.
- [39] L.G. wood, P.G. Gibson, M.L. Garg, Biomarkers of lipid peroxidation, airway inflammation and asthma, *Eur. Respir. J.* 21 (2003) 177–186.
- [40] J. Bousquet, P. Chané, J.Y. Lacoste, G. Barneon, N. Ghavanian, I. Enander, P. Venge, S. Ahlstedt, J. Simony-Lafontaine, P. Godard, Eosinophilic inflammation in asthma, *N. Engl. J. Med.* 323 (1990) 1033–1039.
- [41] A.P. Sampson, The leukotrienes: mediators of chronic inflammation in asthma, *Clin. Exp. Allergy* 26 (1996) 995–1004.
- [42] Z. Marom, J.H. Shelhamer, M.K. Bach, D.R. Morton, M. Kaliner, Slow-reacting substances, leukotrienes C₄ and D₄, increase the release of mucus from human airways in vitro, *Am. Rev. Respir. Dis.* 126 (1982) 449–451.
- [43] S.T. Holgate, M. Peters-Golden, R.A. Panettieri, W.R. Henderson, Roles of cysteinyle leukotrienes in airway inflammation, smooth muscle function, and remodeling, *J. Allergy Clin. Immunol.* 111 (2003) S18–S36.
- [44] J.Z. Haegstrom, A. Wetterholm, Enzymes and receptors in the leukotriene cascade, *Cell. Mol. Life Sci.* 59 (2002) 742–753.
- [45] A.B. Kay, Th2-type cytokines in asthma, *Ann. N. Y. Acad. Sci.* 796 (1996) 1–6.
- [46] A. Mori, M. Sujo, O. Maminuma, Y. Nishizaki, T. Nagahori, T. Mikami, T. Ohmura, A. Hosino, Y. Asakura, H. Okudaira, enhanced production and gene expression of IL-5 in bronchial asthma, possible management of atopic diseases with IL-5 specific gene transcription inhibitor, *Adv. Exp. Med. Biol.* 409 (1996) 439.
- [47] J.E. De Vries, J.F. Gauchat, G.G. Aversa, J. Paunnonen, H. Gascan, H. Yssel, Regulation of IgE synthesis, *Curr. Opin. Immunol.* 3 (1991) 351–358.
- [48] T.C. Van der Pouw Krean, J.S. Vander Zee, L.C. Boeijs, E.R. De Groot, S. O. stapel, L.A. Aarden, The role of IL-13 in IgE synthesis by allergic asthma patients, *Clin. Exp. Immunol.* 111 (1998) 129–135.
- [49] U. Temann, B. Prasad, M. Gallup, C. Basbaum, S. Ho, R. Flavell, J.A. Rankin, A novel role for murine IL-4 in vivo: induction of MUC5AC gene expression and mucin hypersecretion, *Am. J. Respir. Cell Mol. Biol.* 16 (1997) 471–478.
- [50] Z. Zhu, R.J. Homer, Z. Wang, Q. Chen, G.P. Geba, J. Wang, Y. Zhang, J.A. Elias, Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production, *J. Clin. Invest.* 103 (1999) 779–788.

- [51] T. Fukuda, Y. Fukushima, T. Numao, N. Anado, M. Arima, H. Nakajima, H. Sagara, T. Adachi, S. Motojima, S. Makino, Role of interleukin-4 and vascular adhesion molecule-1 in selective eosinophil migration into the airway in allergic asthma, *Am. J. Respir. Cell Mol. Biol.* 14 (1996) 84–94.
- [52] L. Li, Y. Xia, A. Nguyen, Y.H. Lai, L. Feng, T.R. Mosmann, D. LO, Effect of Th2 cytokines on chemokine expression in the lung: IL-13 potentially induces eotaxin expression by airway epithelial cells, *J. Immunol.* 162 (1999) 2477–2487.
- [53] F. Arcoleo, S. Milano, P. D'Agostino, E. Cillari, effect of exogenous leukotriene B4 (LTB4) on BALB/c mice splenocyte production of Th1 and Th2 cytokines, *Int. J. Immunopharmacol.* 17 (1995) 457–463.
- [54] K.A. Yamaoka, J.P. Kolb, Leukotriene B4 induces interleukin 5 generation from human T lymphocytes, *Eur. J. Immunol.* 23 (1993) 2392–2398.
- [55] W. Busse, M. Kraft, Cysteine leukotrienes in allergic inflammation, *Chest* 127 (2005) 1312–1326.
- [56] Y. Kanaoka, J.A. Boyce, Cysteine leukotrienes and their receptors: cellular distribution and function in immune and inflammatory responses, *J. Immunol.* 173 (2004) 1503–1510.
- [57] A.Y. Wu, S.C. Chik, A.W. Chan, Z. Li, K.W. Tsang, W. Li, Anti-inflammatory effects of high doses of montelukast in an animal model of acute asthma, *Clin. Exp. Allergy* 33 (2003) 359–366.
- [58] A.J. Wardlaw, Molecular basis of selective eosinophil trafficking in asthma: a multistep paradigm, *J. Allergy Clin. Immunol.* 104 (1999) 917–926.
- [59] A.J. Costa, P.F. Weller, S.J. Galli, The cells of the allergic response: mast cells, basophils, and eosinophils, *JAMA* 278 (1997) 1815–1822.
- [60] B.J. McEwen, B.P. Wilcock, P. Eyre, The effect of leukotriene B4, leukotriene C4, zymosan activated serum, histamine, tabanid extract and N-formyl-methionyl-fenyl-phenylalanine on the in vitro migration of equine eosinophils, *J. Vet. Res.* 54 (1990) 400–404.
- [61] B.B. Vargaftig, M. Singer, Leukotrienes mediate murine bronchopulmonary hyperactivity, inflammation, and part of mucosal metaplasia and tissue injury induced by recombinant murine interleukine-13, *Am. J. Respir. Cell Mol. Biol.* 28 (2003) 410–419.
- [62] M. Mansuor, S. Tornhamre, Inhibition of 5-lipoxygenase in human blood cells by thymoquinone, *J. Enzyme Inhib. Med. Chem.* 19 (2004) 431–436.
- [63] E.L. Lynch, F.F. Little, K.C. Wilson, D.M. Center, W.W. Cruikshank, Immunomodulatory cytokines in asthmatic inflammation, *Cytokine Growth Factor Rev.* 14 (2003) 489–502.
- [64] M. Akdis, K. Blaser, C.A. Akdis, T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases, *J. Allergy Clin. Immunol.* 116 (2005) 961–968.
- [65] K.W. Moore, A. O'Garra, M.R. de Waal, P. Vieira, T.R. Mosmann, Interleukin-10, *Annu. Rev. Immunol.* 11 (1993) 873–978.
- [66] G.F. Del Prete, M. De Carli, M. Ricci, S. Romagnani, Helper activity of immunoglobulin synthesis of T helper type 1 (Th1) and Th2 human T cell clones: the help of Th1 clones is limited by their cytolytic capacity, *J. Exp. Med.* 174 (1991) 809–813.
- [67] C.K. Wong, C.Y. Ho, C.H. Chan, A.S. HO, D.S. Hui, Pro-inflammatory cytokines (IL-17, IL-6, IL-18, and IL-12) and Th cytokines (IFN-gamma, IL-4, IL-10, and IL-13) in patients with allergic asthma, *Clin. Exp. Immunol.* 125 (2001) 177–183.
- [68] P.J. Barnes, Cytokine modulators as novel therapies for asthma, *annu. Rev. Pharmacol. Toxicol.* 42 (2002) 81–98.
- [69] K.G. Tournoy, J.C. Kips, R.A. Pauwels, Counterbalancing of Th2-driven allergic airway inflammation by IL-12 does not require IL-10, *J. Allergy Clin. Immunol.* 107 (2001) 483–491.
- [70] M. Adachi, N. Oda, F. Kokubu, K. Minoguchi, IL-10 induces a Th2 cell tolerance in allergic asthma, *Int. Arch. Allergy Immunol.* 118 (1999) 391–394.
- [71] D.S. Robinson, M. Larche, S.R. Durham, Tregs and allergic disease, *J. Clin. Invest.* 114 (2004) 1389–1397.
- [72] I. Schneider, F. Bucar, Lipoxygenase inhibitors from natural plant sources: Part 1. Medical plants with inhibitory activity on arachidonate 5-lipoxygenase and 5-lipoxygenase/cyclooxygenase.