

Anti-inflammatory effect of thymoquinone in a mouse model of allergic lung inflammation

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Received 6 December 2005; received in revised form 20 December 2005; accepted 2 February 2006

Abstract

Thymoquinone (TQ), the main active constituent of the volatile oil extracted from *Nigella sativa*'s seeds, has been reported to have an anti-inflammatory and immune stimulatory effect on bronchial asthma and inflammation. However, little is known about the factors and mechanisms underlying these effects. In the present study, we examined the effect of TQ on airway inflammation in a mouse model of allergic asthma. Intraperitoneal injection of TQ before airway challenge of ovalbumin (OVA)-sensitized mice resulted in a marked decrease in lung eosinophilia and the elevated Th2 cytokines observed after airway challenge with OVA antigen; both in vivo, in the bronchoalveolar lavage (BAL) fluid and in vitro, following stimulation of lung cells with OVA. TQ also decreased the elevated serum levels of OVA-specific IgE and IgG1. Histological examination of lung tissue demonstrated that TQ significantly inhibited allergen-induced lung eosinophilic inflammation and mucus-producing goblet cells. While TQ showed a significant effect in inhibiting IL-4, IL-5 and IL-13 and some effect in inducing IFN- γ production in the BAL fluid, it did show a slight effect on in vitro production of IL-4 by cultured lung cells stimulated with OVA antigen. These data suggest that TQ attenuates allergic airway inflammation by inhibiting Th2 cytokines and eosinophil infiltration into the airways; thus demonstrating its potential anti-inflammatory role during the allergic response in the lung.

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Keywords: Allergy; Lung inflammation; Th2 cytokines; Eosinophils; Thymoquinone

1. Introduction

Allergic inflammation associated with airway hyper-reactivity is the main feature of allergic asthma. The inflammatory response is characterized by an increase in the numbers of eosinophils and mast cells [1], mucus hypersecretion and activation of T cells [2]. Several

studies have shown that T-helper type (Th2) cells play a major role in the initiation and maintenance of allergic airway inflammation and asthma through their increased production of Th2-type cytokines (IL-4, IL-5, and IL-13) [3]. These inflammatory cytokines, also produced in the bronchial tissue by mast cells, alveolar macrophages and epithelial cells, play a significant role in the pathogenesis of airway inflammation [4]. This role has been highlighted in several studies using gene knockout and cytokine ablation approaches [5–7]. Th2 cytokines mediate a series of events in the inflammatory cascade leading to the development of allergic asthma. Such

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events include B cell maturation and IgE isotype switching, activation and regulation of mast cell, eosinophil and neutrophil function, and regulation of chemokine and adhesion molecules and mucus production [8–12].

Previous studies have reported that the seed extract of *Nigella sativa*, which is traditionally used to treat some conditions such as asthma, inflammation, bronchitis and eczema [13], possesses some important biological effects. These include immune stimulation [14], anti-inflammatory [15,16], anti-tumor [17], anti-oxidant [18], and respiratory stimulatory effects [19]. Most of these biological activities have been attributed to thymoquinone, the main active constituent of the volatile oil extracted from the seeds. TQ has been shown to inhibit generation of the inflammatory mediators, thromboxane B₂ and leukotriene B₄, by targeting the cyclooxygenase and 5-lipoxygenase pathways of arachidonic acid metabolism [15]. Also, TQ has been reported to induce relaxation in the guinea pig isolated trachea by inhibiting the effect of histamine and serotonin on smooth muscles [20]. Despite the information that implicates TQ in the treatment of inflammation and bronchial asthma, the factors and mechanisms involved remained largely unclear.

The present study aimed at investigating the effect of TQ on allergen-induced airway inflammation in a mouse model of allergic asthma.

2. Materials and methods

2.1. Mice

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.

2.2. Experimental protocol

Induction of allergic airway inflammation was performed by intraperitoneal (i.p.) sensitization and airway challenge through nasal inhalation. Mice (3 males and 3 females/group) were sensitized on day 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 eosinophilia, Th₂ cytokines, mucus production and airway hyperreactivity [21,22]. For TQ treatment, mice were injected (i.p.) once daily for 5 days preceding the first OVA challenge, with 3 mg/kg TQ (Sigma-Aldrich) in 10%

DMSO, or an equal volume of 10% DMSO alone (Naïve and S/Ch groups). TQ (2-isopropyl-5-methyl-1,4-benzoquinone; molecular formula C₁₀H₁₂O₂) was 99% pure, as indicated by the manufacturer (Sigma). All mice were analysed 24 h after the last OVA challenge. The mice groups are described as follows: 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 and received 5 injections of TQ in DMSO before the first OVA challenge. The sensitization and airway challenge experiment was repeated three times.

2.3. Bronchoalveolar lavage (BAL)

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 numbers were determined with crystal violet stained cells using a hemocytometer. For differential cell count, cytocentrifuged preparations were fixed and stained with Diff-Quick (Dade Behring, Dudingen, Switzerland) and differentiated morphologically by counting 300 cells/slide. Differential counts were reported as percentages of individual cell type.

2.4. Measurement of cytokines

Levels of IL-4, IL-5, IL-13 and IFN-γ in BAL fluid and culture supernatants 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 capture 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, biotin-conjugated detection antibodies and avidin–HRP were added and left at room temperature for 1 h. The plates were washed and the reactions were stopped 30–40 min after adding the substrate solution, and then read at 450 nm. The lowest detection limits were 7.8 pg/ml for IL-4 and IL-13, 15.5 pg/ml for IL-5 and 31.1 pg/ml for IFN-γ.

2.5. In vitro antigen-induced cytokine production by lung cells

Lung cells were prepared after cannulating the trachea and perfusing the airways with cold PBS to collect BAL fluid. Lungs were then sliced and incubated in 5 ml 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 20-gauge needle and filtered through a 45-µm nylon mesh. The resulting cell suspension was centrifuged at 1500 rpm for 5 min at 4 °C. Cells (total lung cells) were then resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 10 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, and 0.04 mM 2-mercaptoethanol (Invitrogen), in the presence or absence of 10 µg/ml OVA

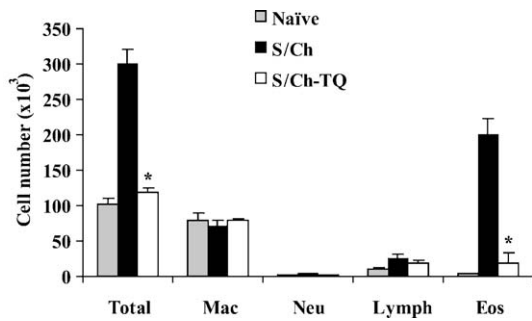


Fig. 1. TQ inhibits BAL inflammation in sensitized and challenged mice. Total and differential cell numbers were determined in BAL fluid obtained 24 h after the last OVA challenge in mice sensitized and challenged with saline and administered with DMSO alone (Naïve); sensitized and challenged with OVA and administered with DMSO alone (S/Ch); and sensitized and challenged with OVA and administered with TQ in DMSO (S/Ch-TQ). Data are expressed as mean $\pm$ S.E.M. of 6 mice per group, and represent data from three separate experiments. Mac, macrophage; Neu, neutrophil; Lymph, lymphocyte; Eos, eosinophil. *Significant difference compared with S/Ch group; $p < 0.05$.

and/or 10 μ M TQ, or DMSO alone, at 37 $^{\circ}$ C. Cells (3×10^5 /well) were cultured in 96-well round-bottom plates. Culture supernatants were collected after 72 h, and stored at -70° C until cytokines were analysed by ELISA as described above.

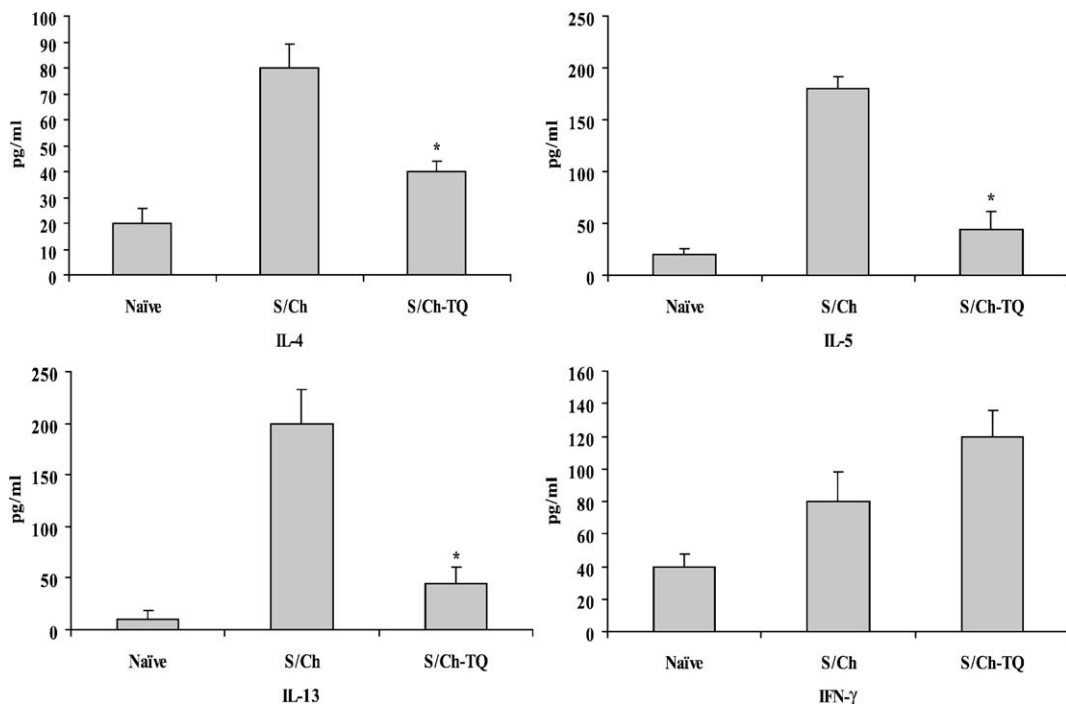


Fig. 2. Effect of TQ on cytokine profile in BAL fluid of sensitized and challenged mice. BAL fluid was collected 24 h after the last OVA challenge from the same groups described in the legend of Fig. 1. Levels of IL-4, IL-5, IL-13, and IFN- γ were measured by ELISA. Mean $\pm$ S.E.M. of 6 mice per group representing data from three experiments are shown. *Significant difference compared with S/Ch group; $p < 0.05$.

2.6. Determination of OVA-specific antibodies

Blood was collected by cardiac puncture, and serum levels of OVA-specific IgE, IgG1, and IgG2a were measured by ELISA. 96-well flat-bottom microtiter plates (Sigma-Aldrich) were coated overnight at 4 $^{\circ}$ C with 100 μ l of 100 μ g/ml OVA in NaHCO₃ buffer (pH 9.6). Plates were washed three times with PBS–0.05% Tween 20, and then blocked by incubation with 200 μ l 0.05% PBS–Tween and 1% BSA for 1 h at room temperature. After washing, different dilutions of serum samples and standard murine IgE, IgG1 and IgG2a (BD PharMingen, San Diego, CA) were added, and the plates were incubated for 2 h at room temperature. OVA-specific immunoglobulins were detected with biotin-conjugated rat anti-mouse (1 μ g/ml) IgE, IgG1, and IgG2a antibodies (BD PharMingen) by incubation for 2 h at room temperature. The plates were then washed and incubated with 1:1000 avidin–HRP-conjugated secondary antibody for 45 min at room temperature. The reaction was terminated with 2 N H₂SO₄, and then read at 450 nm. Total IgE was measured and compared with known mouse IgE standard (BD PharMingen). Detection limit for total IgE was 100 pg/ml.

2.7. Lung histopathology

Lungs were inflated and fixed in 10% neutral formalin. 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.

2.8. Statistical analysis

Data are expressed as means $\pm$ S.E.M. Levels of difference between all groups were determined by one way analysis of variance followed by two-tailed Student's *t* test, to determine the significant differences between pairs of groups. *P* values <0.05 were considered significant.

3. Results

3.1. Thymoquinone inhibits lung inflammation in sensitized and challenged mice

To evaluate the effect of TQ on lung inflammation in OVA allergic mice, total and differential cell numbers in the BAL fluid were compared. OVA sensitization and challenge resulted in a substantial increase in the number of total leukocytes (Fig. 1). This was largely due to an increase in the number of eosinophils. In addition, there was a slight increase in the numbers of lymphocytes and neutrophils. Administration of TQ (3 mg/kg), after OVA sensitization but before airway challenge, markedly decreased the numbers of total cells and eosinophils in the airways (Fig. 1). Treatment

with DMSO alone did not show any marked effect on eosinophil or total cell numbers (compare S/Ch with S/Ch-TQ). These data demonstrate that TQ plays a role during the inflammatory response in allergic lungs, by reducing the number of inflammatory cells in the airways of allergic mice.

3.2. TQ inhibits *in vivo* production of Th2 cytokines in the lung of immunized and challenged mice

BAL fluid was collected 24 h after the last OVA challenge. Th2 (IL-4, IL-5, and IL-13) and Th1 (IFN- γ) cytokine levels were assessed using ELISA. OVA sensitization and challenge significantly induced the production of IL-4, IL-5, and IL-13, whereas had a modest effect on IFN- γ production (Fig. 2). Treatment of mice with TQ, for five days before airway challenge with OVA antigen, not only normalized the increased levels of Th2 cytokines but also induced IFN- γ production (Fig 2). Treatment with DMSO alone did not alter the increased levels of Th2 cytokines or IFN- γ (compare S/Ch with S/Ch-TQ).

3.3. Effect of TQ on *in vitro* cytokine production by lung cells

The data presented in Fig. 2 showed that TQ inhibited *in vivo* Th2 cytokine production by lung cells. To investigate

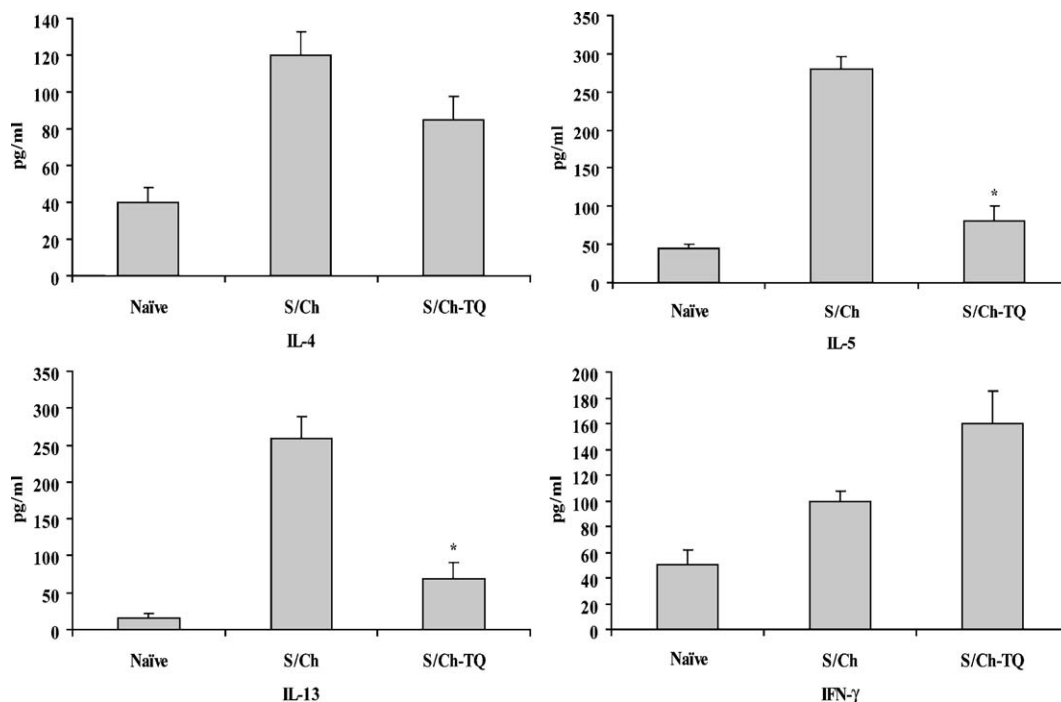


Fig. 3. TQ effect on cytokine production by cultured lung cells. Lung homogenates were prepared 24 h after the last OVA challenge, from naïve (Naïve group), sensitized and challenged (S/Ch group), and sensitized and challenged mice that were administered with TQ (S/Ch-TQ group) as described in Materials and methods. A total of 3×10^5 cells/well were cultured (in triplicates) and stimulated with 10 μ g/ml OVA for 72 h, in the presence or absence of 10 μ M of TQ in DMSO, or DMSO alone. Supernatants were removed and analysed by ELISA, for IL-4, IL-5, IL-13, and IFN- γ . Samples were assayed in triplicates. Data shown are mean $\pm$ S.E.M. of two separate cultures from two independent experiments, with 6 mice/group.

*Significant difference compared with cultures stimulated with OVA alone; *p* <0.05 .

Table 1

Treatment of mice with TQ decreases the elevated serum levels of OVA-specific Igs observed after sensitization and challenge

Group	Total IgE (ng/ml)	OVA-specific (EU/ml)		
		IgE	IgG1	IgG2a
Naïve	36±10	<10	<10	<10
S/Ch	112±43	580±91	912±120	1211±152
S/Ch-TQ	26±14 *	34±8 *	58±17 *	224±28 *

Mice were sensitized, injected with TQ and challenged with OVA allergen as described in Materials and methods. Levels of serum immunoglobulins (Igs) were measured 24 h after the last OVA challenge. Data represent mean±S.E.M. ($n=6$), and represent two separate experiments.

EU, ELISA unit; Naïve, sensitized and challenged with saline; S/Ch, sensitized and challenged with OVA; S/Ch-TQ, sensitized and challenged with OVA but received TQ for 5 days before the first OVA challenge.

* Significant difference ($P<0.05$) compared with S/Ch group.

whether TQ has a similar inhibitory effect on cytokine production in vitro, total lung cells were isolated from OVA-immunized and -challenged mice (S/Ch group) and cultured in the presence of 10 µg/ml OVA and 10 µM TQ in DMSO,

or DMSO alone, and cytokine levels in the culture supernatant were measured by ELISA. As shown in Fig. 3, OVA alone was able to significantly induce the production of IL-4, IL-5 and IL-13, and to a lesser extent IFN- γ . Addition of TQ, but not DMSO alone, to the cultured cells markedly decreased IL-5 and IL-13 and slightly increased IFN- γ production (compare S/Ch with S/Ch-TQ). However, TQ had a slight inducing effect on IL-4 production in contrast to what was observed in vivo (Figs. 2 and 3).

3.4. Sensitized mice administered with TQ do not develop a Th2-mediated humoral immune response after OVA challenge

Serum concentrations of total IgE and OVA-specific IgE, IgG1 and IgG2a were measured. OVA sensitization and challenge elevated the levels of total IgE as well as OVA-specific IgE, IgG1 and IgG2a (Table 1). Intraperitoneal injection of TQ (but not DMSO alone) after OVA sensitization significantly lowered the levels of these immunoglobulins, suggesting the involvement of TQ in the humoral immune response to OVA antigen.

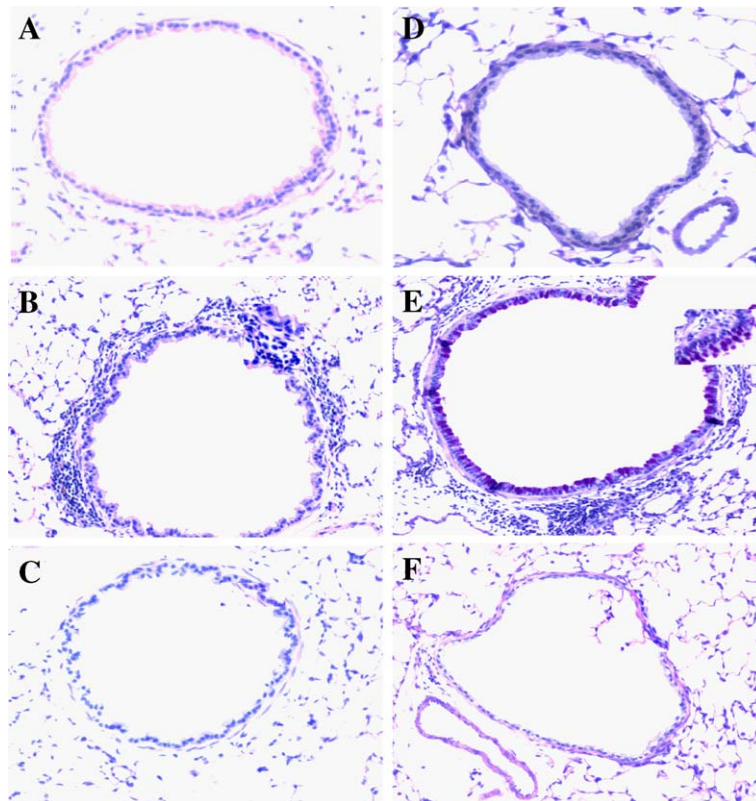


Fig. 4. Effect of TQ administration on lung tissue eosinophilia and goblet cell hyperplasia. Lung tissues from Naïve (A and D), S/Ch (B and E) and S/Ch-TQ (C and F) mice were processed 24 h after the last OVA challenge. OVA sensitization and airway challenge was repeated three times and representative sections from each group are shown. Tissue eosinophilia was detected with H&E staining (A, B, and C) and goblet cells were detected with PAS staining (D, E, and F). Note the peribronchial eosinophil infiltration (B) and the hyperplasia of goblet cells in the airways (E) of sensitized and challenged mice (S/Ch group). Administration of TQ five times after sensitization but before OVA challenge markedly attenuated airway inflammation (C and F).

3.5. Effect of TQ on OVA-induced lung tissue eosinophilia and mucus production

Lung tissues were collected 24 h after the last OVA challenge and processed as described in Materials and methods. H&E staining of lung sections obtained from sensitized and challenged mice (S/Ch group) showed a marked infiltration of the peribronchial tissue with inflammatory cells, including lymphocytes and eosinophils (Fig. 4B). In addition, lung tissues stained with PAS demonstrated a large number of mucus-producing goblet cells in the airway mucosa (Fig. 4E). In contrast, TQ markedly decreased the numbers of eosinophil infiltrates (Fig. 4C) and goblet cell hyperplasia (Fig. 4F) in the lung tissue of OVA-sensitized and -challenged mice (S/Ch-TQ group), whereas DMSO alone had no effect on lung tissue eosinophilia or goblet cell hyperplasia (compare Naïve and S/Ch with S/Ch-TQ).

4. Discussion

Seed extract of *Nigella sativa* and the main constituent of its volatile oil, thymoquinone, have traditionally been used to treat some illnesses such as bronchial asthma [13], and this may be due to its effect on some mediators of inflammation [15]. Our findings show that TQ attenuates allergen-induced inflammation by inhibiting Th2 cytokines, eosinophilic infiltration and goblet cell hyperplasia in the airways of mice sensitized and challenged with OVA antigen.

Th2 cytokines, including IL-4, IL-5, and IL-13, have been demonstrated to play an important role in the pathogenesis of airway inflammation and responsiveness in animal models and human [23,24,4]. These cytokines are produced by the many cell types that are found in, or infiltrating the lung microenvironment, including T cells, mast cells, macrophages, and epithelial cells. Increased production of IL-4, IL-5, and IL-13 has been reported in a variety of allergic disorders, including asthma [25–28]. IL-4 is necessary for differentiation of T cells to the Th2 type and is a key factor for isotype switching to IgE in B cells [29,6], regulation of chemokines required for eosinophil migration [30], and may increase mucus secretion in the allergic airways [31]. Blocking IL-4 with anti-IL-4 antibody has been shown to decrease serum IgE levels and eosinophilic infiltration in the airways of allergen-sensitized mice [32,33]. IL-5 is the key cytokine for activation and survival of eosinophils [34], and its level is elevated in allergic asthma [35]. IL-13 is a pleiotropic cytokine produced by Th2 cells and other cell types and is involved in the development of allergic inflammation and asthma [36,37]. Most of the functions ascribed to IL-13 overlap with

those of IL-4 [38]. For instance, IL-13 regulates IgE isotype switching in B cells and eosinophil function, and increases mucus production by goblet cells [9,11]. Our data demonstrated that TQ decreased the production of IL-4, IL-5, and IL-13 in the BAL fluid as well as in the supernatants of cultured lung cells from sensitized and challenged mice, thus indicating its effect in attenuating the inflammatory response. It is not clear, however, why TQ did not significantly affect IL-4 production in vitro by cultured lung cells, as seen in vivo. It is possible that other cellular or biochemical factors present in the lung microenvironment are required for TQ to exert its inhibitory effect on IL-4.

Airway eosinophilia is a main factor in the development of allergic airway inflammation [39,40]. Eosinophil recruitment into, and subsequent activation in, the airways is regulated mainly by IL-5 and IL-13 as well as chemokines and adhesion molecules such as eotaxin and VCAM-1 [4,41]. Previous studies have shown that eosinophilic inflammation does not develop in the absence of IL-5 in the airways of OVA-sensitized and -challenged mice [42,43]. In addition, IL-13 induces expression of VCAM-1 and eotaxin in the airway epithelium, thereby facilitating the recruitment of eosinophils into the airways [44]. Our results showed that TQ attenuated airway eosinophilia, and that such decrease in eosinophil numbers was associated with a decrease in the levels of IL-5 and IL-13 in the airways of sensitized and challenged mice.

Increased mucus production by goblet cells in the airway epithelium and elevated serum levels of IgE and IgG1 are associated with airway inflammation and asthma. The data presented here demonstrated that TQ reduced the level of OVA-specific IgE and IgG1 as well as goblet cell hyperplasia. Previous studies have implicated IL-4 and IL-13 in inducing IgE isotype switching in B cells [28,6,9] and increasing airway mucus secretion by goblet cells [45,9]. Also, IL-5 and airway eosinophilia contribute to the increased mucus production in the airways of allergic mice [46]. Thus, the decrease in the serum IgE level and goblet cells observed in mice treated with TQ may be due to the inhibition of Th2 cytokine.

Our data also showed that TQ not only inhibited Th2 response and cytokines but also shifted the response toward Th1 type, by inducing IFN- γ both in vivo and in vitro. Increased amounts of IFN- γ have been reported in asthmatic subjects [47]. In addition, administration of IFN- γ during allergic sensitization has been shown to inhibit airway inflammation [48,49]. It is possible that IFN- γ induced by TQ in this study will play a protective

role in ameliorating the inflammatory response seen in OVA-sensitized and -challenged mice.

In this study, TQ was administered before the induction of airway inflammation with allergen challenge and was effective in preventing the development of airway inflammation. As in our experiments, previous studies [16,50] also administered TQ before the induction of the inflammatory response. It is not clear from these studies whether TQ would elicit similar effects after induction an inflammatory response. However, a recent study [51] reported that *Nigella sativa* oil, with its active principle TQ, was effective in ameliorating the allergic symptoms in more than 50% of patients with developed allergic rhinitis, atopic eczema, and bronchial asthma. It is yet to be seen, in the context of the model used in our study, whether TQ has a comparable anti-inflammatory effect in the presence or after the development of airway inflammation.

In conclusion, these findings demonstrate that TQ inhibits allergen-induced lung inflammation by reducing airway eosinophilia, Th2 cytokines, mucus secretion, and allergen-specific antibodies, thus supporting its anti-inflammatory role during the allergic response in the lung.

Acknowledgements

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

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