



Zerumbone enhances the Th1 response and ameliorates ovalbumin-induced Th2 responses and airway inflammation in mice

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ABSTRACT

Zerumbone is a sesquiterpene compound isolated from the rhizome of wild ginger, *Zingiber zerumbet* Smith. The rhizomes of the plant are used as a spice and traditional medicine. Zerumbone was shown to possess anticarcinogenic, anti-inflammatory, and antioxidant properties. However, the antiallergic activity and the underlying mechanism of zerumbone have not been reported. Herein, we investigated the immunomodulatory effects of zerumbone on antigen-presenting dendritic cells (DCs) *in vitro* and its potential therapeutic effects against ovalbumin (OVA)-induced T helper 2 (Th2)-mediated asthma in mice. In the presence of zerumbone, lipopolysaccharide-activated bone marrow-derived DCs enhanced T cell proliferation and Th1 cell polarization in an allogeneic mixed lymphocyte reaction. In animal experiments, mice were sensitized and challenged with OVA, and were orally treated with different doses of zerumbone after sensitization. Circulating titers of OVA-specific antibodies, airway hyperresponsiveness to methacholine, histological changes in lung tissues, the cell composition and cytokine levels in bronchoalveolar lavage fluid, and cytokine profiles of spleen cells were assessed. Compared to OVA-induced hallmarks of asthma, oral administration of zerumbone induced lower OVA-specific immunoglobulin E (IgE) and higher IgG_{2a} antibody production, attenuated airway hyperresponsiveness, prevented eosinophilic pulmonary infiltration, and ameliorated mucus hypersecretion. Zerumbone treatment also reduced the production of eotaxin, keratinocyte-derived chemokine (KC), interleukin (IL)-4, IL-5, IL-10, and IL-13, and promoted Th1 cytokine interferon (IFN)- γ production in asthmatic mice. Taken together, these results suggest that zerumbone exhibits an antiallergic effect *via* modulation of Th1/Th2 cytokines in an asthmatic mouse model.

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1. Introduction

Asthma is a heterogeneous chronic inflammatory lung disease that results from a complex interplay of genetic and environmental factors. It is increasingly thought that asthma consists of multiple phenotypes and that these asthma phenotypes can be classified as T helper 2 (Th2)-associated and non-Th2 asthma. Th2-associated asthma includes early-onset (usually during preadolescence) allergic asthma, late-onset (often at age 20 years or later) eosinophilic asthma, and exercise-induced asthma [1]. It is recognized that Th2 cells and their cytokines

(IL-4, IL-5, and IL-13) are responsible for initiating and maintaining Th2-associated asthma [2]. These Th2 cytokines induce, prolong, and amplify inflammatory responses by increasing the production of allergen-specific IgE; enhancing recruitment, growth, and differentiation of eosinophils; and directly causing airway hyperresponsiveness (AHR) [3]. Therefore, inhibition of Th2 cell function and/or blockade of Th2 effector responses are therapeutic goals in Th2-associated asthma.

Although corticosteroid and β -agonists can improve asthma symptoms, they do not cure the disease. These agents also have side effects, particularly in children. Therefore, phytotherapy using alternative medicines has become increasingly popular for treating allergic asthma, and has been used to satisfy demands not provided by conventional medicine [4].

Zingiber zerumbet Smith, wild ginger that belongs to the Zingiberaceae family, is widely distributed in Southeast Asian countries. Its rhizomes have traditionally been used as a spice and a remedy to

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alleviate diarrhea, stomachaches, sores, loss of appetite, and inflammation [5]. Recently, several reports on the bioactivity of zerumbone, which was identified as a major compound of this plant, were published. Scientific researchers confirmed that zerumbone possesses many pharmacological activities, including suppression of cancer cell proliferation and downregulation of tumor invasion [6–9]. Zerumbone was also shown to contain anti-inflammatory [10–12] and antioxidant activities [13]. However, there is no report on the antiallergic activity of zerumbone in allergic diseases.

Dendritic cells (DCs), as professional antigen-presenting cells, play an important role in antigen presentation in the airways, and the cytokine profile secreted by DCs can influence differentiation of T cells [14, 15]. Inhaled “aero-antigens” are taken up by airway DCs that migrate to bronchial lymph nodes where they prime T-helper ($CD4^+$) cell precursors to differentiate into type 1 T-helper (Th1) cells, Th2 cells, or regulatory T cells (Tregs). To date, the protective effects of zerumbone against chronic inflammatory lung disease asthma and the underlying mechanisms have never been defined. Therefore, the aim of the current study was to clarify whether treatment with zerumbone can alter the regulatory ability of DCs on T cells and achieve antiallergic effects to alleviate the development of airway symptoms in a mouse model of allergic asthma. Changes in serum ovalbumin (OVA)-specific antibodies, AHR, airway inflammation, and cytokine production in zerumbone-treated mice were evaluated. Our data indicated that zerumbone modulates DC function to promote interferon (IFN)- γ production by activated T cells and convert T cells with Th1-regulatory activity in Th2-driven allergic diseases. These findings provide insights into how zerumbone affects the Th2-biased immune response and provide guidance on the use of zerumbone as an anti-inflammatory adjuvant in treating Th2-mediated allergic asthma.

2. Materials and methods

2.1. Preparation of zerumbone

Zerumbone ((2E,6E,10E)-2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-one; CID 5470187), with an MW of 218, was isolated and purified from rhizomes of *Z. zerumbet*, as previously reported [16]. The fresh rhizome (1.3 kg) of *Z. zerumbet* was refluxed with 95% ethanol ($40\text{ L} \times 2$) at 80°C for 6 h. The extract was filtered and concentrated with a rotary evaporator to remove the ethanol. The extract was diluted with water and apportioned with diethyl ether. The diethyl ether layer was coated onto a Celite 545 gel, and subjected to silica gel column chromatography ($7.0\text{ cm i.d.} \times 75\text{ cm}$) with an *n*-hexane-ethyl acetate (EtOAc) gradient ($10:0 \rightarrow 10:1 \rightarrow 5:1 \rightarrow 3:1 \rightarrow 2:1 \rightarrow 1:1 \rightarrow 0:10$). The *n*-hexane-EtOAc ($10:1$) elute was recrystallized with methanol, and 1.30 g of needle crystals of zerumbone was obtained. The purity of the zerumbone was determined by high-performance liquid chromatography (HPLC), and was shown to exceed 99.5%.

2.2. Mice

Female BALB/c and C57BL/6 mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and maintained in the Animal Center of Taipei Medical University. All mice were used at 5–8 weeks of age and were age-matched within each experiment. Animal care and handling protocols were approved by the Animal Committee of the College of Medicine, Taipei Medical University.

2.3. Preparation of bone marrow-derived DCs (BMDCs)

Tibial and femoral bone marrow from 5 to 6-week-old BALB/c mice was isolated. Bone marrow cells were cultured in RPMI-1640 and a 5% fetal bovine serum (FBS) complete medium with IL-4 (1000 U/ml) and GM-CSF (500 U/ml) on day 0. Every second day, fresh medium containing GM-CSF and IL-4 was used to replace the old medium, and non-

adherent cells were transferred to a new plate to decrease contamination by macrophages. On day 6 of culture, bone marrow-derived cells were analyzed by flow cytometry, gated on $CD11c^+$ (eBioscience, San Diego, CA) cells. The FACS analysis showed that there were 70%–80% of DCs in this cell population.

2.4. Isolation of allogeneic $CD4^+$ T cells

Spleens were harvested from female C57BL/6 mice and ground into single-cell suspensions. After depleting red blood cells using ACK lysis buffer, splenocytes were washed 3 times with Hank's balanced salt solution (HBSS) and resuspended with MACS buffer (0.5% bovine serum albumin (BSA) and 2 mM EDTA in $1 \times$ phosphate-buffered saline (PBS) solution) at 10^7 cells/ $90\text{ }\mu\text{L}$. Subsequent staining with anti- $CD4$ magnetic beads (Miltenyi Biotec, Auburn, CA) at 10^7 cells/ $10\text{ }\mu\text{L}$ was performed for 10 min at 4°C . According to the manufacturer's instructions, cells were then washed and resuspended in MACS buffer to purify naive $CD4^+$ T cells. Positively selected cells were collected for further analysis.

2.5. Mixed lymphocyte reaction (MLR)

The allogeneic MLR is an assay system for T-cell recognition of allogeneic cells in which the response is measured by proliferation in the presence of stimulating cells. This assay can be used to evaluate the stimulatory ability of DCs to alter T-cell proliferation and cytokine production. Approximately 10^6 day-6 BMDCs derived from BALB/c mice were seeded in a 24-well plate and treated with lipopolysaccharide (LPS; $1\text{ }\mu\text{g/ml}$), LPS plus zerumbone (0.2 and $1\text{ }\mu\text{g/ml}$), or medium only. After 24 h of culture, DCs were irradiated with 3000 rad (^{137}Cs source). After washing twice with HBSS, different proportions of irradiated BMDCs (0.5×10^4 , 10^4 , and 2×10^4 cells/well) and allogeneic naive $CD4^+$ T cells (2×10^5 cells/well) from C57BL/6 mice were incubated at 37°C in 96-well round-bottom plates. After 3 days of culture, tritiated thymidine (New England Nuclear, Boston, MA) was applied at $1\text{ }\mu\text{Ci/well}$ and incubated at 37°C for another 16–18 h. Cells were then harvested onto glass filters by an automated multi-sample harvester and counted with a dry scintillation counter (Packard Instrument, Meriden, CT). Additionally, the level of IFN- γ production in the culture supernatant was assayed by enzyme-linked immunosorbent assay (ELISA) kits (DuoSet, R&D Systems, Minneapolis, MN).

2.6. Experimental protocol for the asthma model

For systemic immunization, BALB/c mice were intraperitoneally (i.p.) immunized three times with $50\text{ }\mu\text{g}$ OVA (grade V; Sigma-Aldrich, St. Louis, MO) plus 2 mg alum (Thermo Scientific, Rockford, IL) on days 1, 14, and 28. Then, mice were intranasally (i.n.) challenged with $100\text{ }\mu\text{g}$ OVA on days 35 and 36. Subsequently, mice were exposed to an OVA aerosol challenge (5% OVA in a normal saline solution) for 3 consecutive days (days 37, 38, and 39) for 30 min daily, and the AHR was measured 1 d after the final challenge (on day 40). To examine the effects of various dosages (0.1 , 1 , and 10 mg/kg of body weight) of zerumbone, three groups of mice (ZER (0.1), ZER (1) and ZER (10)) were respectively orally fed three doses of zerumbone from days 23 to 39. OVA-sensitized and OVA-challenged mice were fed sterilized water instead of zerumbone as a positive control (PC). Negative control (NC) mice were neither sensitized with OVA nor administered zerumbone treatment although they received an OVA challenge. Serum samples were collected from the retro-orbital venous plexus on day 40.

2.7. Serum antibody assay

OVA-specific IgE, IgG $_1$, and IgG $_{2a}$ serum antibody titers were determined by an ELISA (Becton Dickinson Biosciences, San Jose, CA). Levels of antibodies were compared to IgE, IgG $_1$, and IgG $_{2a}$ standards of

predetermined concentrations (IgE at 1 µg/ml, IgG₁ at 25 µg/ml, and IgG_{2a} at 10 µg/ml). The concentration of standard serum was arbitrarily assigned as 1 ELISA unit (1 EU).

2.8. Determination of the airway responsiveness

At 24 h after the last aerosol exposure, airway function was measured by detecting changes in lung resistance in response to increasing doses of aerosolized methacholine (MCh, Sigma-Aldrich) in anesthetized mice. Mice were anesthetized, tracheostomized, and mechanically ventilated at a rate of 150 breaths/min, with a tidal volume of 0.3 ml and a positive end-expiratory pressure of 3–4 cmH₂O with a computer-controlled small-animal ventilator (model 683, Harvard Rodent Ventilator, Mount Holly, NJ). PE-50 tubing was inserted into the esophagus to the level of the thorax and coupled with a pressure transducer. Flow was measured by electronic differentiation of the volume signal, and changes in pressure, flow, and volume were recorded. Pulmonary resistance was calculated using software (model PNM-PCT100 W, LDS PONEMAH Physiology Platform, LDS Gould, Champaign, IL). MCh aerosol was generated with an inline nebulizer and directly administered through the ventilator. The resistance of the orotracheal tube was subtracted from all airway resistance measurements. Data are expressed as the pulmonary resistance (R_L).

2.9. Analysis of the cellular composition of bronchoalveolar lavage fluid (BALF)

Immediately after measuring the pulmonary function parameters, mice were sacrificed, and their tracheas were immediately lavaged three times via a tracheal cannula with 1 ml of HBSS that was free of ionized calcium and magnesium. The lavage fluid was cooled on ice and centrifuged (400 × g) at 4 °C for 10 min. After washing, the supernatants were collected for the chemokine and cytokine assays, and cell pellets were resuspended in 1 ml HBSS. The total number of cells in the BALF was counted with a standard hemocytometer. Differential cell counts were performed by counting at least 200 cells in cytocentrifuged preparations, staining with Liu's stain solution (Chi I Pao, Taipei, Taiwan), and differentiating according to standard morphological criteria.

2.10. Lung histology study

After the lavage, the lungs were immediately removed and fixed in 10% v/v buffered formalin (in 0.01 M PBS; pH 7.2). Pulmonary tissues were subsequently sliced, embedded in paraffin, and cut into 5-µm-thick sections. These frozen sections were stained with hematoxylin-eosin (H&E) to evaluate inflammatory cell infiltration, or with periodic acid-Schiff (PAS) to identify goblet cells. Light microscopy was used for the histopathological assessment.

2.11. Measurement of BALF and culture supernatant cytokines

Splenocytes (at 10⁷ cells/ml) from groups of mice were stimulated with 50 µg/ml OVA. After 72 h, supernatants were collected. Quantities of eotaxin, KC, IL-4, IL-5, IL-10, IL-13, IFN-γ, and tumor necrosis factor (TNF)-α in the BALF and culture supernatants of splenocytes were evaluated using commercially available ELISA kits (Duoset, R&D Systems).

2.12. Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. A *p* value of <0.05 was considered statistically significant.

3. Results

3.1. Zerumbone- and LPS-treated DCs enhance T-cell activation and Th1-cell polarization in vitro

Mature DCs have the capacity to induce proliferation and cytokine production in T cells. To ensure that zerumbone did not interfere with the survival of DCs, it was determined that the concentration used in the experiment was non-cytotoxic (data not shown). Then an allogeneic MLR assay was used to evaluate the effect of zerumbone on DC-mediated T-cell activation. BALB/c mouse-derived DCs were co-cultured with C57BL/6 mouse-derived naïve CD4⁺ T cells at different ratios (DC/T-cell ratios of 1:10, 1:20, and 1:40) *in vitro* to determine if zerumbone-treated DCs had an increased ability to activate T cells. Compared to control DCs (medium group) or only LPS-stimulated DCs (LPS group), LPS-activated DCs cultured in the presence of 1 µg/ml zerumbone exhibited significantly enhanced T-cell proliferation (Fig. 1A). Additionally, there was a significant increase in IFN-γ secretion by T cells at a DC/T-cell ratio of 1:10 (Fig. 1B). These results showed that zerumbone can enhance the Th1 response through DCs.

3.2. Zerumbone reduces serum anti-OVA IgE and enhances anti-OVA IgG_{2a} levels in an animal model of asthma

To investigate the *in vivo* impacts of zerumbone on regulating immune responses of allergic diseases, groups of mice were exposed to OVA sensitization and challenge (Fig. 2A). These OVA-sensitized mice were orally fed different doses (0.1, 1, and 10 mg/kg) of zerumbone

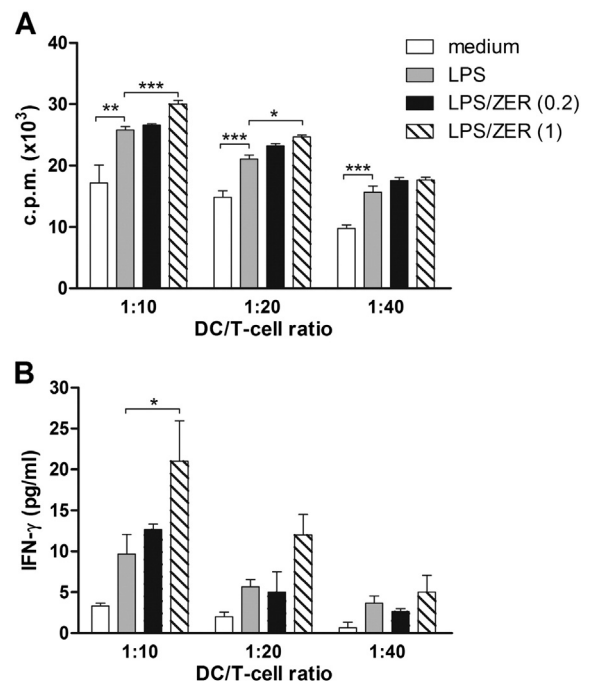


Fig. 1. Treatment of dendritic cells with zerumbone combined with lipopolysaccharide enhanced T cell proliferation and Th1 cell polarization in an allogeneic mixed lymphocyte reaction. (A) Modulatory effects of zerumbone on T cell proliferation. DCs were pretreated with medium, LPS (1 µg/ml), or LPS plus zerumbone (0.2 and 1 µg/ml) for 24 h. Allogeneic naïve CD4⁺ T cells (2 × 10⁵ cells/well) were co-cultured with different proportions of γ-irradiated immature DCs, LPS-pulsed DCs, or LPS plus zerumbone-treated DCs at DC/T-cell ratios of 1:10, 1:20, and 1:40 in 96-well round-bottomed plates. After 3 days of culture, cells were pulsed with 1 µCi/well of [³H]-thymidine for 16–18 h. Specific incorporation of [³H]-thymidine was determined with a β-counter, and results are expressed as counts per minute (cpm). (B) Modulatory effects of zerumbone on T-cell cytokine secretion. Concentrations of IFN-γ secreted by naïve CD4⁺ T cells, which were co-cultured with γ-irradiated DCs in 96-well plates for 3 days, were determined by an ELISA. Results in all panels are expressed as the mean ± SEM of three independent experiments. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001 vs. LPS-stimulated DCs.

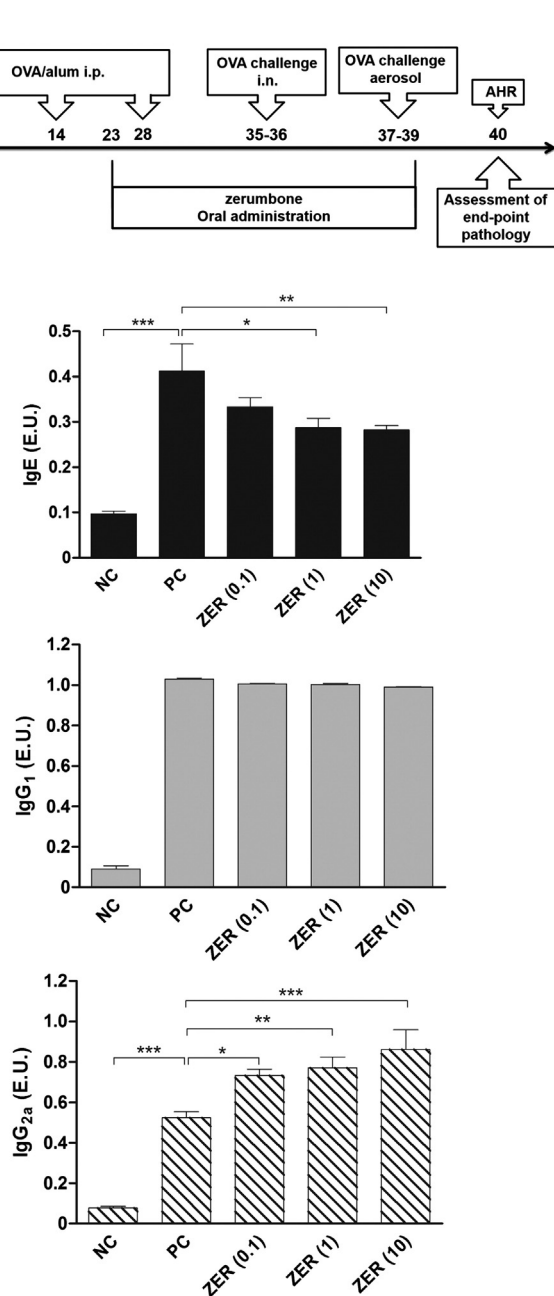


Fig. 2. Anti-OVA antibody expression levels of zerumbone-treated OVA-induced allergic asthmatic mice. (A) Brief description of the protocol of animal sensitization and challenge. On days 1, 14, and 28, all groups of mice were sensitized by an intraperitoneal injection of the OVA allergen. Three groups of mice ((ZER (0.1), ZER (1), and ZER (10)) were respectively orally fed 0.1, 1, and 10 mg/kg of zerumbone from days 23 to 39. Positive control (PC) mice were administered sterilized water instead of zerumbone. Then mice were intranasally challenged with OVA on days 35 and 36. Subsequently, mice were exposed to OVA aerosols for 3 consecutive days, and AHR was measured 1 day after the last challenge. BALF was collected after measuring the AHR. Negative control (NC) mice were neither sensitized with OVA nor administered zerumbone treatment but were challenged with OVA. (B) IgE, IgG₁, and IgG_{2a} anti-OVA antibody expressions of all groups of mice were measured by an ELISA. Results are expressed as the mean $\pm$ SEM ($n = 5$ mice in the NC group, $n = 7$ mice in all other groups). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the PC group.

(ZER (0.1), ZER (1) and ZER (10), respectively). PC mice received distilled water instead of zerumbone. NC mice were neither sensitized with OVA nor administered zerumbone treatment, although they received an OVA challenge. Serum samples were collected on day 40, and OVA-specific IgE, IgG₁, and IgG_{2a} serum antibody titers were determined by an ELISA. In mice, the Th2 cytokine, IL-4, promotes IgG₁ and

IgE production. However, the Th1 cytokine, IFN- γ , induces the production of IgG_{2a}. Therefore, expression levels of subclasses (IgG₁ and IgG_{2a}) or classes (IgE) of antibodies can be used as a marker to identify what type of Th response is induced in these mice. In the PC group, strong IgE and IgG₁ production and low IgG_{2a} expression were induced (Fig. 2B). In contrast, zerumbone treatment efficiently inhibited OVA-specific IgE production. Additionally, although the administration of zerumbone did not downregulate IgG₁ secretion, IgG_{2a} synthesis was markedly upregulated compared to the PC group.

3.3. Zerumbone decreases the severity of AHR and airway inflammation

To assess the therapeutic effects of zerumbone on allergic asthma, both AHR and the accumulation of inflammatory cells in BALF were investigated. One day after the last OVA challenge, the airway responsiveness to aerosolized MCh of each group of mice was measured. PC mice, which were administered distilled water instead of zerumbone and sensitized and challenged with OVA, developed markedly increased airway responsiveness to MCh inhalation compared to that of non-allergic NC mice (Fig. 3A). However, oral treatment with zerumbone dose-dependently alleviated the development of AHR compared to the PC group. Furthermore, in the NC group, cells in the BALF were mostly composed of macrophages (Fig. 3B). On the other hand, in the PC group, exposure to aerosolized OVA induced marked increases in cell numbers of eosinophils and neutrophils in the BALF. In contrast, mice administered zerumbone showed significantly reduced increases in these infiltrating cells.

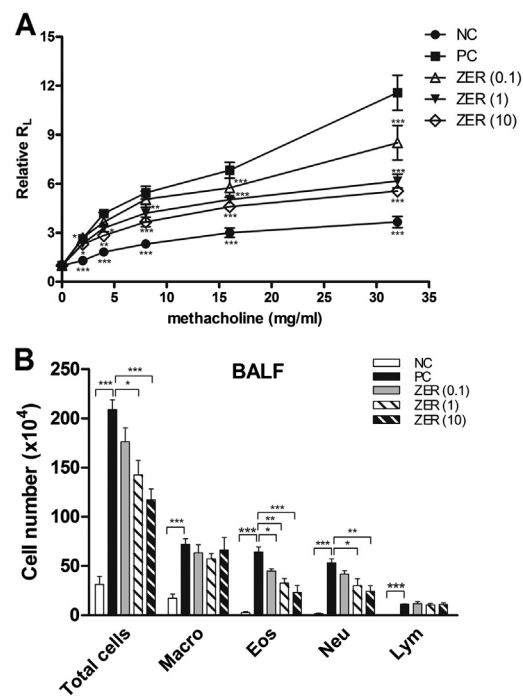


Fig. 3. Effects of zerumbone on the airway hyperresponsiveness and various cell types in bronchial alveolar lavage fluid. (A) Oral administration of zerumbone suppressed the development of AHR in OVA-sensitized mice. One day after the last OVA challenge, airway resistance was measured in response to increasing concentrations of methacholine (0–32 mg/ml) by invasive body plethysmography. Results are expressed as the mean $\pm$ SEM ($n = 5$ mice in the NC group, $n = 7$ mice in all other groups) of the pulmonary resistance (R_L) after PBS nebulization. (B) Changes in the cellular composition of the BALF of mice exposed to an aerosolized allergen. After measuring the pulmonary function parameters, each group of mice was sacrificed, and the BALF was collected. Cells were counted and classified as macrophages (Macro), eosinophils (Eos), neutrophils (Neu), and lymphocytes (Lym). Results are expressed as the mean $\pm$ SEM ($n = 5$ mice in the NC group, $n = 7$ mice in all other groups). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the PC group.

3.4. Zerumbone attenuates OVA-induced lung pathology

Histological analysis of lung tissue sections from the PC group exhibited increased numbers of inflammatory cells around the peribronchiolar and perivascular regions, and excessive mucus secretion from hyperplastic goblet cells (Fig. 4), relative to the NC group. Lungs of mice administered zerumbone showed significantly less inflammatory cell infiltration, a more-prominent decrease in goblet cell hyperplasia, and fewer PAS-positive cells than mice in the PC group.

3.5. The influence of zerumbone on OVA-induced chemokine and cytokine secretions in BALF

Eotaxin and KC are the most potent chemokines for respectively recruiting eosinophils and neutrophils. Thus, evaluating these chemokine levels in BALF showed that administration of zerumbone significantly decreased both eotaxin and KC production (Fig. 5). This result indicated that zerumbone reduced the secretions of eotaxin and KC, and subsequently decreased the numbers of recruited eosinophils and neutrophils. Furthermore, to determine the possible effect of zerumbone on T-cell responses, we assessed levels of the important Th2 cytokines, IL-4, IL-5, IL-10, and IL-13, in BALF. Data showed that zerumbone treatment had dose-dependent suppressive effects on the

production of Th2 cytokines compared to the PC group. In addition, we observed mild enhancement of BALF IFN- γ expression in mice treated with zerumbone compared to the PC group.

3.6. The effect of zerumbone on OVA-induced cytokine secretions by splenocytes

By studying the production of Th1 and Th2 cytokines in cell culture supernatants from the spleen, we determined whether it was possible to affect Th2 immune responses through inducing IFN- γ -producing T cells. Freshly isolated spleen cells from different groups of mice were cultured with OVA. After 3 days of culture, cell supernatants were collected and analyzed by an ELISA. Results showed that zerumbone administration markedly inhibited IL-4, IL-5, IL-10, and IL-13 production by splenocytes (Fig. 6). Furthermore, the data demonstrated that OVA-sensitized mice treated with zerumbone exhibited a slightly enhanced level of IFN- γ production. We also checked expression of the proinflammatory cytokine, TNF- α , by splenocytes and found that medium and high dosages of zerumbone significantly reduced TNF- α production relative to mice in the PC group. Taken together, these findings suggest that zerumbone treatment might induce protective immunity against asthma through induction of IFN- γ production by OVA-specific T cells.

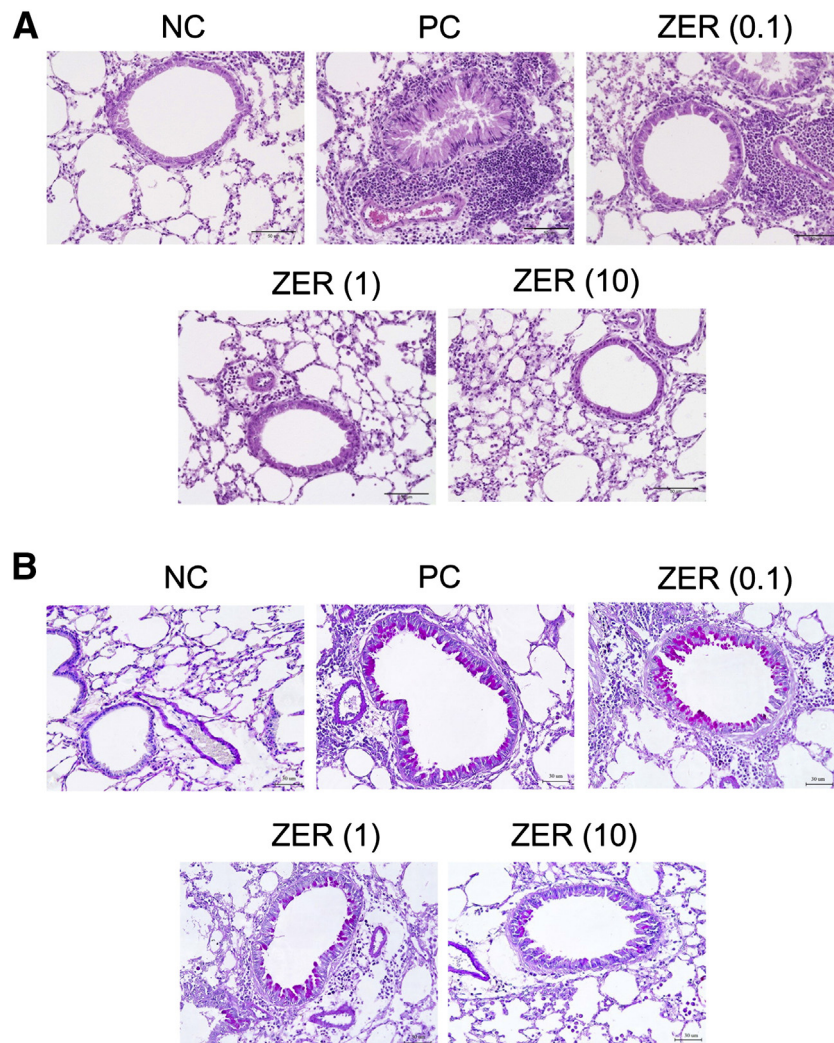


Fig. 4. Histological analysis of mouse lung sections. (A) Effect of zerumbone on lung inflammatory cell infiltration in OVA-induced allergic asthmatic mice (H&E staining). (B) Effect of zerumbone on lung mucus production in mice with OVA-induced allergic asthma (PAS staining). Lung sections were obtained from NC mice, sterilized water-treated PC mice, and those treated with different doses of zerumbone. Sections were stained with H&E and PAS stain for the morphological analysis. Tissues were examined by light microscopy (original magnification: $\times 200$).

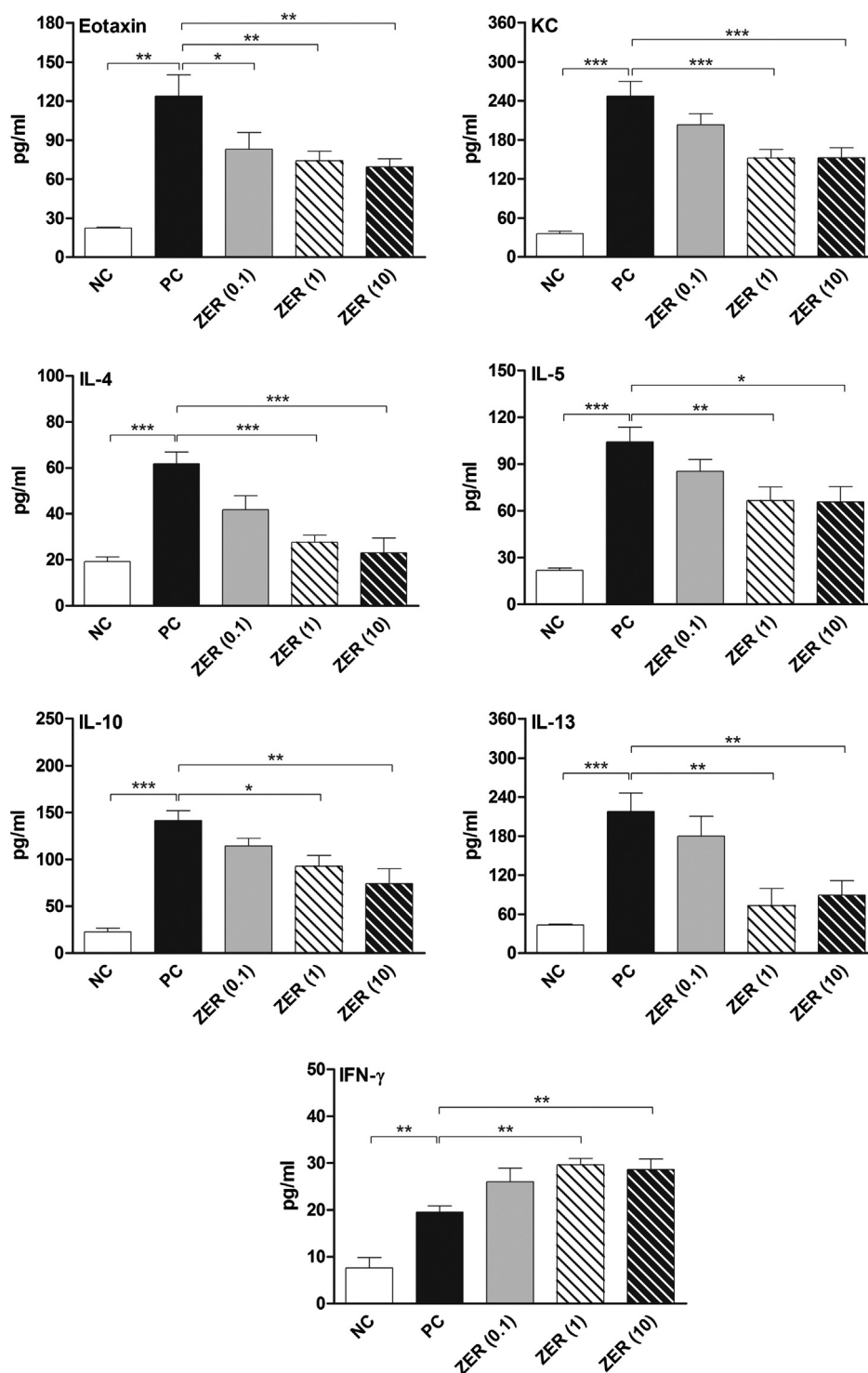


Fig. 5. Effect of zerumbone on levels of inflammatory mediators and cytokines in BALF. Immediately after measuring the pulmonary function parameters, each group of mice was sacrificed, and BALF was collected and analyzed for eotaxin, KC, IL-4, IL-5, IL-10, IL-13, and IFN- γ contents by an ELISA. Results are expressed as the mean $\pm$ SEM ($n = 5$ mice in NC group, $n = 7$ mice in all other groups). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the PC group.

3.7. The preventive effects of zerumbone against OVA-induced Th2-mediated asthma in mice

We next investigated the preventive effects of zerumbone in OVA-induced asthmatic mice. The protocol of animal sensitization and challenge is shown in Fig. S1A. To examine the protective effects of zerumbone, three groups of mice were additionally orally fed different doses (0.25, 1.25, and 3.75 mg/kg of body weight) of zerumbone for

30 consecutive days. Notably, administration of zerumbone efficiently inhibited OVA-specific IgE and IgG₁ production, and enhanced OVA-specific IgG_{2a} secretion (Fig. S1B). Our data also showed that zerumbone treatment dose-dependently alleviated the development of AHR compared to the PC group (Fig. S1C). Furthermore, mice treated with zerumbone demonstrated significantly reduced increases in eosinophils and neutrophils in the lungs (Fig. S1D). As shown in Fig. S1E, levels of chemokines and Th2 cytokines in the BALF significantly declined in

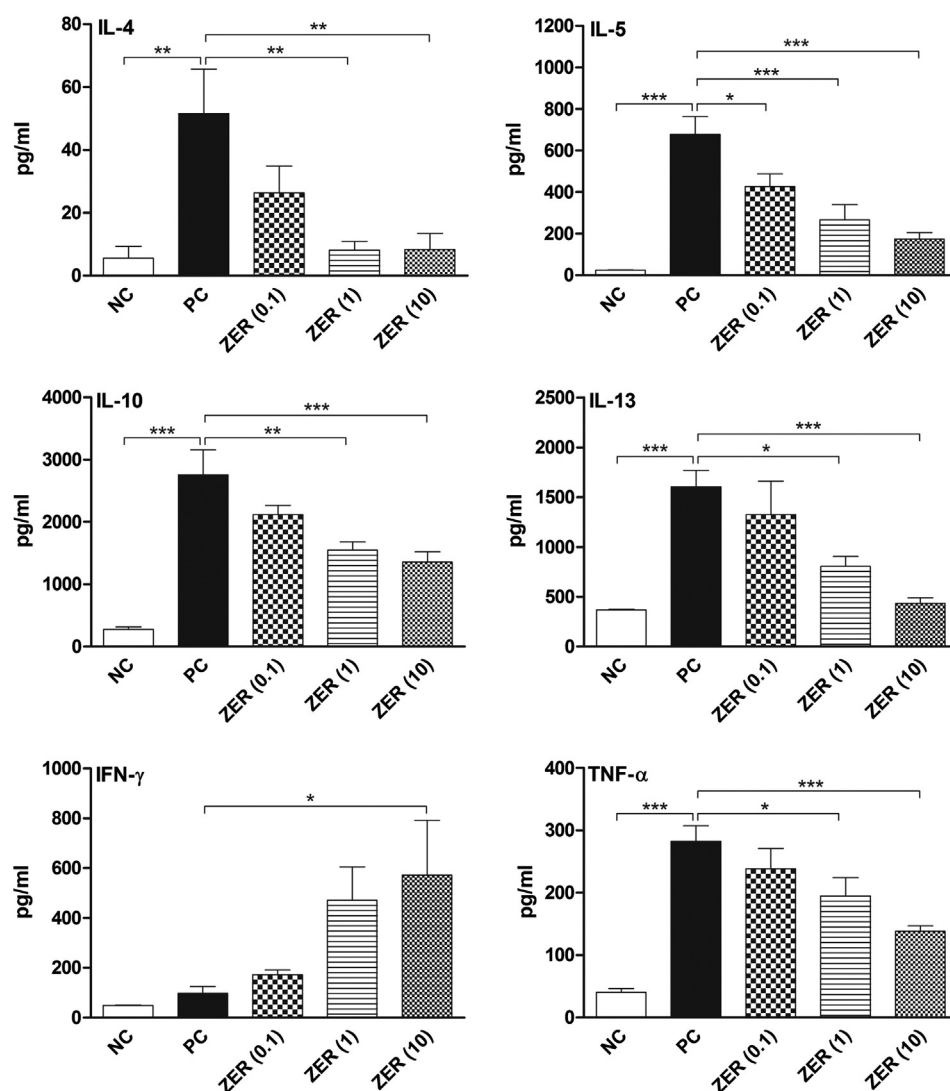


Fig. 6. Effect of zerumbone on the cytokine profile of antigen-restimulated splenocytes in OVA-induced allergic asthmatic mice. Splenocytes (10^7 cells/ml) from zerumbone-treated and control groups were stimulated with 50 μ g/ml OVA in 24-well plates, and culture supernatants were collected after 72 h. Levels of cytokine production of IL-4, IL-5, IL-10, IL-13, IFN- γ , and TNF- α were analyzed by an ELISA. Results are expressed as the mean $\pm$ SEM ($n = 5$ mice in the NC group, $n = 7$ mice in all other groups). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. the PC group.

zerumbone-treated mice, compared to PC mice. Similarly, levels of Th2 cytokines in the supernatant of splenocyte cultures also strongly decreased in zerumbone-treated mice (Fig. S1F). In addition, administration with any dose of zerumbone enhanced a moderate level of IFN- γ production by splenocytes.

4. Discussion

Asthma is a chronic inflammatory airway disease that involves multiple complex pathways. Because of its chronic nature, long-term medications are required for therapy. Currently available therapeutic agents such as inhaled β -adrenergic agonists and steroid treatments are effective, but have major adverse effects, require daily dosing, and depend on patient compliance and coordination. Therefore, it is imperative to find substances, preferably non-steroidal in nature, which have therapeutic effects on allergic asthma and can be taken for a long time with no adverse effects. Recently, phytochemical and pharmacological studies have identified many potential anti-inflammatory substances, especially those derived from plants used as folk medicines or foods, so natural products are becoming increasingly important as sources of pharmacotherapeutics.

The rhizome of *Z. zerumbet* is regularly used as a food flavoring and appetizer, and also has been used in folkloric medicine as a cure for various ailments. This natural agent has few side-effects, is readily available, and hence is cost effective. The main bioactive component in this ginger, known as zerumbone, is a monocyclic sesquiterpene, which was shown to possess antitumor activity. Several researchers reported that zerumbone possesses antiproliferative properties towards several cancer cell lines with minimal effects on normal cells [8,9]. Huang et al. demonstrated that zerumbone can induce DNA fragmentation in cultured P-388D₁ cells *in vitro*, and prolong the life of P-388D₁-bearing CDF1 mice [16]. In *in vivo* studies, zerumbone was shown to suppress azoxymethane-induced rat aberrant crypt foci [17] and phorbol ester-induced papilloma formation in mouse skin [18]. On the other hand, zerumbone also showed anti-inflammatory properties. It was demonstrated that zerumbone attenuated cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) expressions *via* modulating nuclear factor (NF)- κ B activation in cell culture systems [12,19]. In addition, oral feeding of zerumbone significantly lowered levels of IL-1 β , TNF- α , and prostaglandin E₂ (PGE₂) and suppressed dextran sodium sulfate (DSS)-induced colitis in mice [20]. Moreover, zerumbone showed inhibitory effects in acute and chronic models of inflammation in mice,

namely carrageenan-induced inflammatory and cotton pellet-induced granuloma tests, respectively [11]. In a recent study, it was reported that dietary zerumbone prevented mouse corneas from UVB-induced photokeratitis [21].

In the present study, for the first time, we explored the mechanism and immunomodulatory effects of zerumbone in a murine asthma model. Our study demonstrated an impressive therapeutic role of zerumbone in OVA-induced IgE- and Th2 cell-mediated allergic asthma in mice. Th2-associated asthma is generally regarded as a Th2 cell-mediated allergic disease. Allergens cause the differentiation of Th2 cells, which then secrete Th2 cytokines, such as IL-4, IL-5, and IL-13. These cytokines are responsible for IgE production, the development of AHR, eosinophil accumulation, and mucus hypersecretion [22]. In this study, oral administration of zerumbone significantly reduced the production of serum OVA-specific IgE in asthmatic mice. Isotype switching of B cells to IgE synthesis is a prerequisite of atopy and provides the critical trigger mechanism for the immediate allergic response upon allergen exposure. This requires the obligatory presence of the Th2 cytokine, IL-4. IL-4, which is pivotal in the pathogenesis of allergic disorders, acts on B cells to facilitate IgE production [23]. Our results showed that zerumbone decreased IL-4 production in the BALF and spleen. Thus, we speculated that zerumbone may have an antiallergic effect on allergic asthma by suppressing IL-4 secretion and consequently reducing IgE production by B cells. In particular, IL-13 is a Th2-type cytokine that appears to cause AHR through direct effects on resident airway cells [24]. We found herein that BALF and spleen IL-13 levels decreased after oral administration of zerumbone; therefore, it caused attenuation of AHR sensitized by an allergen in mice.

IL-10 was originally described as a Th2-associated cytokine that antagonizes Th1 responses. However, subsequent studies showed that IL-10 can downregulate Th2 clones and their production of IL-4 and IL-5 [25]. IL-10 was also shown to be produced by T-regulatory 1 (Tr1) cells, and the suppressive activity of Tr1 cells was found to be closely related to its IL-10-producing ability [26,27]. In our study, both local (BALF) and systemic (spleen) levels of IL-10 in mice treated with zerumbone were significantly lowered compared to those of PC mice. Therefore, it could be speculated that inducible Tr1 cells did not play a regulatory role of suppressing the immune response of allergen-specific Th2-cell populations in these zerumbone-treated mice.

Eosinophils are recruited from the circulation to inflammatory tissues in response to allergic stimuli, and they are the predominant inflammatory cells in asthmatic lung tissues. It was suggested that eosinophils contribute to the pathogenesis of allergic asthma including tissue damage and AHR [28]. In the lungs, endothelial cells, smooth muscle cells, epithelial cells, alveolar macrophages, and eosinophils can produce eotaxin. Notably, eotaxin can bind to CCR3 which is highly expressed by eosinophils, and it is more potent than other chemokines in inducing eosinophilic degranulation and leukotriene C4 release [29]. We found that zerumbone treatment inhibited airway inflammation through reducing eotaxin expression in our murine model of asthma. The reduced eotaxin levels may have been due to a direct or indirect influence exerted on the recruitment of eosinophils, which are the source of eotaxin. IL-5 is another key Th2 cytokine in the growth and differentiation of eosinophils in bone marrow and enhances eosinophil recruitment, activation, and survival at sites of inflammation [30]. In our study, IL-5 levels in the BALF and spleen of mice that received zerumbone were also significantly reduced. Thus, we speculated that the reduction in eosinophils may have been due to inhibition of IL-5 secretion from Th2 cells. Additionally, eosinophils are also a source of IL-5, and less eosinophil infiltration and activation in lungs of zerumbone-treated mice may have resulted in reduced IL-5 levels in the BALF. As expected, consistent with the results of the histological analysis of lung tissues, zerumbone successfully alleviated lung inflammation and reduced the infiltration of total inflammatory cells in peribronchiolar regions. On the other hand, mucus is produced by goblet cells in the airways. Mucus hypersecretion is a major cause of airway obstruction in asthma leading to

coughing and wheezing. Oral administration of zerumbone to OVA-induced asthmatic mice markedly reduced mucus hypersecretion and goblet cell hyperplasia. Taken together, these results demonstrate that zerumbone had profound regulatory effects on the development of airway inflammation in this OVA-induced asthma model.

By administering zerumbone, we demonstrated that zerumbone-treated DCs stimulated T-cell proliferation and enhanced IFN- γ production in T cells in an allogeneic MLR. Therefore, we speculated that zerumbone may influence allergen pathways by modulating the function of DCs which support the generation of Th1 cells. In addition, we observed that zerumbone had inductive effects on Th1 responses *in vivo*, including anti-OVA IgG_{2a} and IFN- γ production in zerumbone-treated asthmatic mice. IFN- γ , which is secreted by Th1 cells, promotes the proliferation of Th1 cells and induces IgG_{2a} production by B cells. It is well documented that the balance between IL-4 and IFN- γ plays a major role in regulating IgE synthesis in asthmatic patients [31]. IFN- γ can inhibit Th2 cell proliferation [32], induce eosinophil apoptosis [33], and also promote mucous cell apoptosis [34]. In models of asthma, IFN- γ downregulated OVA-specific IgE in serum, reduced the recruitment of lymphocytes and eosinophils, inhibited the development of AHR, and suppressed mucus production [35–37]. However, it was also reported that high levels of IFN- γ can induce AHR and promote established Th2-induced airway inflammation [38]. In our model, although there was a mild increase in IFN- γ , the level did not appear to be high enough to exacerbate AHR or increase airway inflammation. Thus, the key therapeutic approach is perhaps to achieve a balance between Th2 and Th1 cytokines, where there is a decrease in Th2 cytokines without a large increase in the Th1 response. In addition, it appears relevant to point out that the toxicity test showed no occurrence of death over a period of 17 days with 10 mg/kg zerumbone treatment. Macroscopic and microscopic observations of the organs appeared normal and showed no treatment-related organ abnormalities. These results indicate that zerumbone might have a reasonable safety margin in terms of toxicity. Indeed, published studies so far indicate that zerumbone exerts little cytotoxic or genotoxic effects at doses used in most experimental studies [39]. For instance, single injected doses of zerumbone at 100–200 mg/kg of body weight produced no toxic effects in liver and renal tissues of female Sprague-Dawley rats [8].

In conclusion, this is the first known report demonstrating that zerumbone has a markedly protective effect on OVA-induced allergic asthma in mice. DCs were stimulated with zerumbone plus allergens, and these DCs activated T cells and resulted in enhanced IFN- γ production. The produced IFN- γ affected the Th1/Th2 balance which is important for allergies, and promoted the negative regulation of Th2 cytokine production. The data reported in this article thus provide a rationale for extensive preclinical studies on zerumbone in IgE- and Th2 cell-mediated allergic asthma, and suggest that zerumbone can be used as a therapy for patients in the future.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.intimp.2014.12.027>.

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