Mastic Alleviates Allergic Inflammation in Asthmatic Model Mice by Inhibiting Recruitment of Eosinophils

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The pathogenesis of allergic asthma is characterized by airway inflammation, eosinophilia, and airway hyperresponsiveness. In the present study, we investigated the anti-inflammatory effects of mastic, obtained from the stem and the leaves of *Pistacia lentiscus* trees, on allergic asthma. In an ovalbumin-induced mouse asthma model, mastic significantly inhibited eosinophilia, while reducing airway hyperresponsiveness and suppressing the production of inflammatory cytokines (IL-5 and IL-13) as well as chemokines (eotaxin, eotaxin2, and regulated upon activation, normal T-cell expressed and secreted) in bronchoalveolar lavage fluid. Moreover, mastic potently inhibited eotaxin-induced eosinophil chemotaxis *in vitro* without influencing eotaxin receptor, chemokine receptor 3, expression. These results suggest that mastic may contribute to the treatment of inflammatory diseases.

Keywords: mastic; allergic asthma; eosinophil

The pathogenesis of allergic asthma is characterized by airway inflammation, eosinophilia, and airway hyperresponsiveness (AHR). Eosinophils are important effector cells in allergic asthma, particularly in late-phase reactions (1, 2). There is an increasing awareness of the essential role played by eosinophil-mediated inflammation of airways in the pathobiology of asthma. Recent evidence supports that patients with asthma whose treatments are based on sputum eosinophil counts have significantly fewer severe asthma exacerbations than those patients treated according to standard management therapy (3). In addition, selective ablating eosinophil lineage by antibody blockade (4) or cell genetic modification (5, 6) in animal studies have proven effective in protecting against the development of AHR and exacerbation of asthma.

Mastic is a natural resin that is obtained from the stem and leaves of *Pistacia lentiscus* trees. It has been extensively used for centuries in Mediterranean and Middle Eastern countries, both as a dietary supplement and traditional medicine. Medical trials demonstrate that mastic has cytoprotective or anti-acid effects on the gastrointestinal system, such as relief of ulcers (7) and reduction of intensity of gastric mucosal damage caused by antiulcer drugs, with little or no side effects. Moreover, it is already known for its antioxidant (8), antibacterial (9, 10), and antitumor (11, 12) properties. In particular, mastic has recently been reported to carry our anti-inflammatory activities in human aortic endothelial cells (13) and activated macrophages (14), and show immunomodulatory effects on neutrophils (15). Mastic has been observed to inhibit neutrophil- or LPS-activated RAW 264.7 macrophage function by nitric oxide (NO) production, and to regulate cytokine secretion (TNF-α and macrophage migration inhibitory factor [MIF]) of peripheral blood mononuclear cells from patients with Crone's disease (16). The extracts of mastic significantly inhibited the expression of vascular cell adhesion molecule–1 and intercellular adhesion molecule–1, and the binding ability to U937 cells in TNF-α–stimulated human aortic endothelial cells (13).

Considering the anti-inflammatory and immunomodulatory effects of mastic, we sought to investigate whether mastic would suppress eosinophilic inflammation. To test our hypothesis, in the present study, we used a mouse allergic asthma model of airway inflammation *in vivo*, as well as *in vitro* study of eosinophil migration. In an ovalbumin (OVA)-induced asthma model, we show that mastic ameliorated allergic asthma by reducing recruitment of eosinophils into the airways, suppressing AHR, and decreasing production of chemokines (eotaxin, eotaxin2, and RANTES [regulated upon activation, normal T-cell expressed and secreted]) and inflammatory cytokines (IL-5 and -13). Interestingly, *in vitro* studies demonstrated that mastic potently inhibited eotaxin-induced eosinophil migration without influencing the expression of eotaxin receptor, chemokine receptor (CCR) 3. These results suggest that mastic may contribute to the treatment of inflammatory diseases.

MATERIALS AND METHODS

Animals

BALB/c mice (6–8 wk old) were purchased from the Chinese Academy of Sciences (Shanghai, People's Republic of China). All studies were reviewed and approved by the animal ethics committee of the Shanghai Jiao Tong University–Affiliated Ninth People's Hospital.

Induction of Animal Experimental Asthma

BALB/c mice ($n = 6$) were sensitized with 20 μg of OVA (grade V; Sigma-Aldrich, St. Louis, MO) absorbed onto 2.25 mg of alum (Alum Inject; Pierce, Rockford, IL) by intraperitoneal injections on Days 0 and 7. On Days 28–30, mice were challenged by either PBS or an aerosol of 2% OVA (wt/vol) for 30 minutes. Mastic gum (50 or 100 mg/kg; no. G0878; Sigma) dissolved in 1% DMSO in saline was given intraperitoneally 4 hours before each OVA aerosol challenge. At 24 hours after last challenge, mice were killed.

Preparation of Bronchoalveolar Lavage Fluid

Mice were anesthetized 24 hours after the last challenge and bronchoalveolar lavage fluid (BALF) was collected. Shortly, the trachea was cannulated and lungs were washed with Hanks' balanced salt solution. The count of total leukocytes was immediately performed in a hemocytometer. Differential cell counts were obtained by using May-Grunwald-Giemsa staining, and enumerated with the standard hematological procedure by counting at least 500 cells under a light microscope.

Measurement of AHR

Mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (60 mg/kg) and then given a neuromuscular blockade (0.8 mg/kg

pancuronium bromide). AHR was measured by recording the enhanced pause (Penh) values (using a whole-body plethysmograph [Buxco Electronics, Inc., Wilmington, NC]) in response to inhaled methacholine (12.5–50 mg/ml).

Measurements of Cytokine and Chemokine Levels in BALF

Levels of IL-4, -5, -13, eotaxin, eotaxin2, and RANTES in BALF supernatant were determined by using ELISA kits from R&D Systems (Minneapolis, MN), according to the manufacturer's instructions and specifications.

Isolation of Human Eosinophils

Human eosinophils were isolated as previously described (17).

Chemotaxis Assay

The eosinophil migration assay was performed in a 48-well Transwell plate with a membrane pore size of 5 μm (NeuroProbe Inc., Gaithersburg, MD). Eosinophils (1 $\times$ 106) were loaded to the upper well. RPMI1640, with or without 30 nM mouse recombinant eotaxin (R&D Systems), was added into the lower chambers. Plates were incubated at 37°C with 5% CO_2 for 90 minutes, and then the membrane was removed and stained with Diff-Quick (Shanghai Biotech Inc., Shanghai, China). For inhibition assay, eosinophils were preincubated with mastic (5–20 μg/ml) for 20 minutes. Each incubation was performed in triplicate, and migration was determined by counting eosinophils migrating completely through the filter in five random high-power fields (1,000$\times$).

Flow Cytometry of Chemokine Receptor 3 Expression

BAL was performed with Ca^{2+}- and Mg^{2+}-free PBS with 0.5% BSA. After RBCs were lysed, BALF cells (1.5 $\times$ 10^5 cells/100 μl) were washed and stained for 30–45 minutes at 4°C with CCR3-FITC monoclonals (5 μg/ml; R&D Systems, Abingdon, UK). To prevent nonspecific binding to Fc receptors, 2.4G2 blocking reagent (10 μg/ml) was added to the monoclonal mix. Cells were analyzed on FACS (FCM-500; Beckman Coulter, Kraemer Blvd Brea, CA) using Cell Quest software.

Statistical Analysis

Data are presented as means ($\pm$SEM). Differences among groups were identified by ANOVA for multiple comparisons, followed by Bonferroni analysis. A P value less than 0.05 was considered significant.

RESULTS

Mastic Suppresses OVA-Induced Eosinophil Recruitment in Asthmatic Mice

To investigate if mastic could inhibit allergic airway inflammation, BALB/c mice were challenged three times with OVA to induce a clear and strong inflammation. Mice ($n = 6$) challenged by means of inhalation of OVA showed marked increase in the number of total cells (Figure 1), especially the number of eosinophils in BALF, when compared with PBS aerosol control (0.21 versus 4.56 $\times$ 10^4 cells/ml BALF; $P < 0.001$). As shown in Figure 1, the application of mastic drastically reduced the number of infiltrating eosinophils in comparison with OVA group; 50 and 100 mg/kg of mastic decreased the number of eosinophils in BALF by 30 and 55%, respectively. However, mastic did not reduce macrophage, lymphocyte, or neutrophil counts in the BALF as compared with OVA group ($P > 0.05$). Thus, mastic efficiently inhibits the airway recruitment of eosinophils in this murine model of allergic inflammation.

Mastic Inhibits OVA-Induced AHR in Mice

Next, to investigate the effect of mastic on AHR in response to increasing concentrations of methacholine (12.5–50.0 mg/ml), we measured Penh values 24 hours after the final OVA challenge. As shown in Figure 2, OVA-challenged mice ($n = 5$) developed
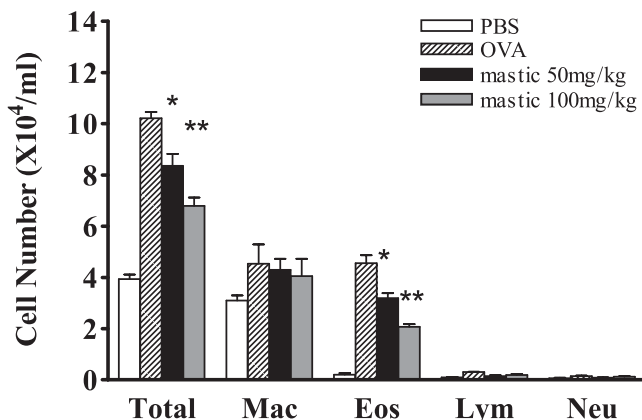

Figure 1. Inhibitory effect of mastic on eosinophil infiltration into the lung. Bronchoalveolar lavage fluid (BALF) was obtained from challenged mice ($n = 6$) 24 hours after last challenge. Differential cell counts were performed on a minimum of 500 cells to identify eosinophil (Eos), macrophage (Mac), neutrophil (Neu), and lymphocyte (Lym). Data are means ($\pm$SE) for six mice per group (*$P < 0.05$ versus OVA-challenged mice, **$P < 0.01$ versus OVA-challenged mice according to ANOVA with Bonferroni correction).

AHR, which is typically reflected by high Penh value. Mastic (100 mg/kg) dramatically reduced Penh values in OVA-challenged mice in response to methacholine ($P < 0.01$), suggesting that immune-mediated airway pathology *in vivo* was modified.

Mastic Reduces T Helper Type 2 Proinflammatory Cytokine and Chemokine Levels in BALF

Reduced cellular airway inflammation was associated with a significant reduction in T helper (Th) 2 proinflammatory cytokines (18). Thus, we detected Th2 proinflammatory cytokines (IL-4, -5, and -13) in BALF. OVA inhalation in challenged mice ($n = 4$) caused a notable concentration increase of
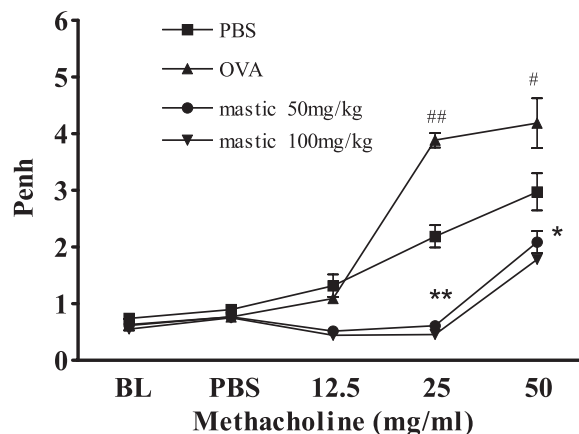
Figure 2. Mastic inhibits airway hyperresponsiveness (AHR) in ovalbumin (OVA)-challenged mice. The changes in enhanced pause (Penh) values, an index of airway obstruction, were measured. At 24 hours after the last PBS aerosol or OVA aerosol with pretreatment of 100 mg/kg mastic, mice ($n = 5$) were placed in a barometric plethysmographic chamber, and Penh was determined and plotted against the increasing concentration of methacholine. Each data point represents the mean ($\pm$SE). #$P < 0.01$ versus control (PBS); ##$P < 0.001$ versus control (PBS); *$P < 0.01$ versus OVA-challenged mice; **$P < 0.001$ versus OVA-challenged mice. BL, baseline.
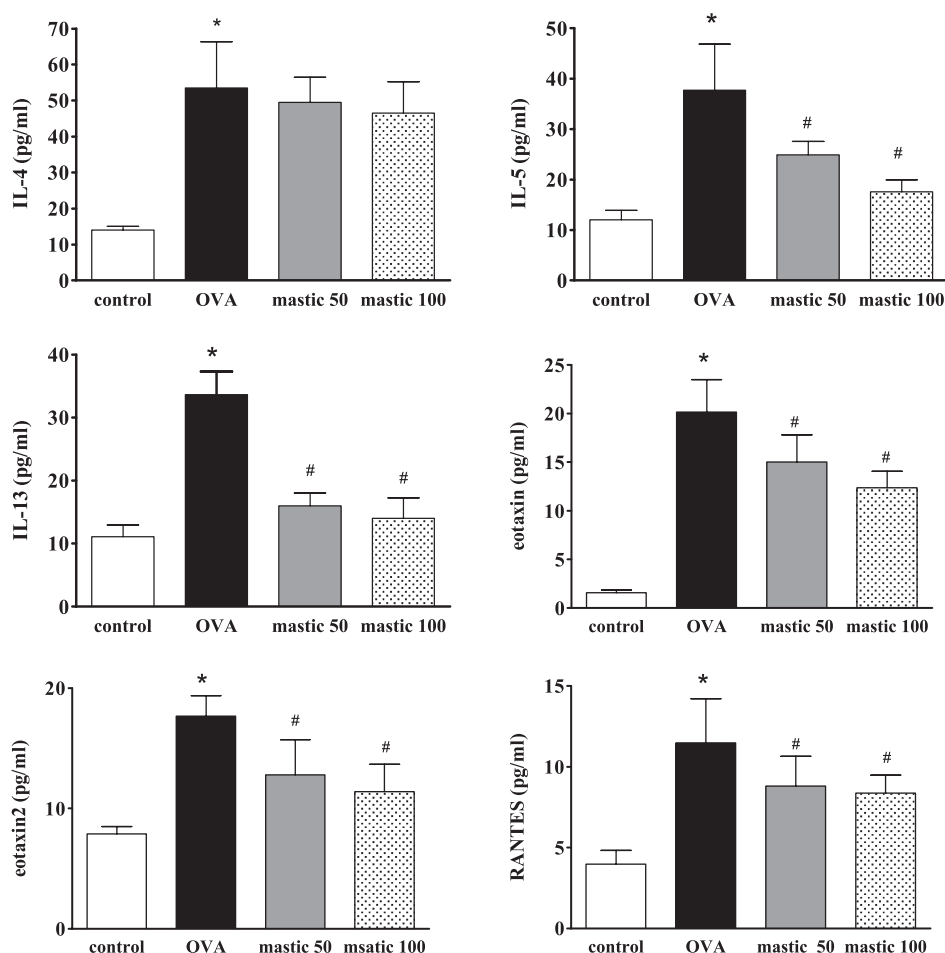
Figure 3. Mastic (either 50 or 100 mg/kg) blocks cytokine and chemokine production in BALF of OVA-challenged mice ($n = 4$). Cytokines (IL-4, -5, and -13) and chemokines (eotaxin, eotaxin2, and RANTES [regulated upon activation, normal T-cell expressed and secreted]) in the BALF were measured using R&D Systems and IL-4, IL-13, and eotaxin2 ELISA kits. Data are given as means ($\pm$SE) ($n = 4$); *$P < 0.01$ versus control (PBS) mice; #$P < 0.005$ versus OVA-challenged mice.

these cytokines as compared with PBS aerosol control (Figure 3). Mastic significantly ($P < 0.05$) reduced the levels of IL-5 and -13 in BALF. However, we did not find any difference in the level of IL-4. In addition, we measured the levels of BALF chemokines (eotaxin/CCL11, eotaxin2/CCL24, and RANTES/CCL5), which immunomodulate inflammation and play significant roles in allergic response, and found that the levels of these three chemokines dropped significantly after mastic injection (Figure 3).

Eosinophil Migration toward Eotaxin Is Suppressed by Mastic *In Vitro*

Reduction of eosinophil recruitment may be modulated by a decrease of chemokines, but we wanted to know the direct role of mastic in eosinophil migration. To address whether mastic directly modulated eosinophil chemotactic response to chemoattractants, we performed *in vitro* chemotaxis experiments using BALF cells from OVA-challenged mice ($n = 5$), which had increased eosinophils. Pretreatment of the mouse BALF cells with mastic (0–20 µg/ml) significantly inhibited eotaxin-induced eosinophil migration in a dose-dependent way (Figure 4A). This finding suggests that mastic functions directly as an antichemoattractant for eosinophils. Mastic possibly inhibited eotaxin-induced eosinophil migration by blocking eotaxin receptor, CCR3. However, when BALF cells were preincubated with various concentrations of mastic, no significant inhibition of CCR3 expression was seen compared with untreated cells ($P > 0.05$; Figure 4B). These results suggest that mastic directly inhibited eotaxin-induced eosinophil chemotaxis without affecting CCR3 expressions.

### Mastic Inhibits the Migration of Human Eosinophils

To confirm the effects of mastic on eotaxin-induced eosinophil chemotaxis, purified human eosinophils were mixed with vehicle or three dilutions of mastic (5, 10, and 20 µg/ml) for 20 minutes at 37°C, and were allowed to migrate for 90 minutes in a chemotaxis chamber toward 10 ng/ml of eotaxin placed in the bottom wells. Migrated cells in the bottom wells were then enumerated. Figure 5 demonstrates that mastic potently inhibited the migration of human eosinophils toward eotaxin. Similarly, when human eosinophils were incubated with mastic, no significant inhibition of CCR3 expression was observed compared with untreated cells (data not shown).

DISCUSSION

We demonstrate, for the first time, that mastic effectively inhibited eosinophilic inflammation in a murine model of allergic asthma via the inhibition of eosinophil migration into the airway.

The initial symptoms of asthma include airway inflammation in which eosinophils play a crucial role. Eosinophils are always present in excess in the airways of patients with asthma; however, their accumulation decreases as the symptoms of asthma decrease (1).

Natural products have long been used as traditional medicine to treat inflammatory diseases. Mastic has been reported to have antioxidant (8), antibacterial (9, 10), and anticancer (11, 12) properties, as well as beneficial pharmaceutical properties, such as peptic ulcers (7). Its anti-inflammatory and immunomodulatory effects have been described recently in several studies (13–16).

A

B
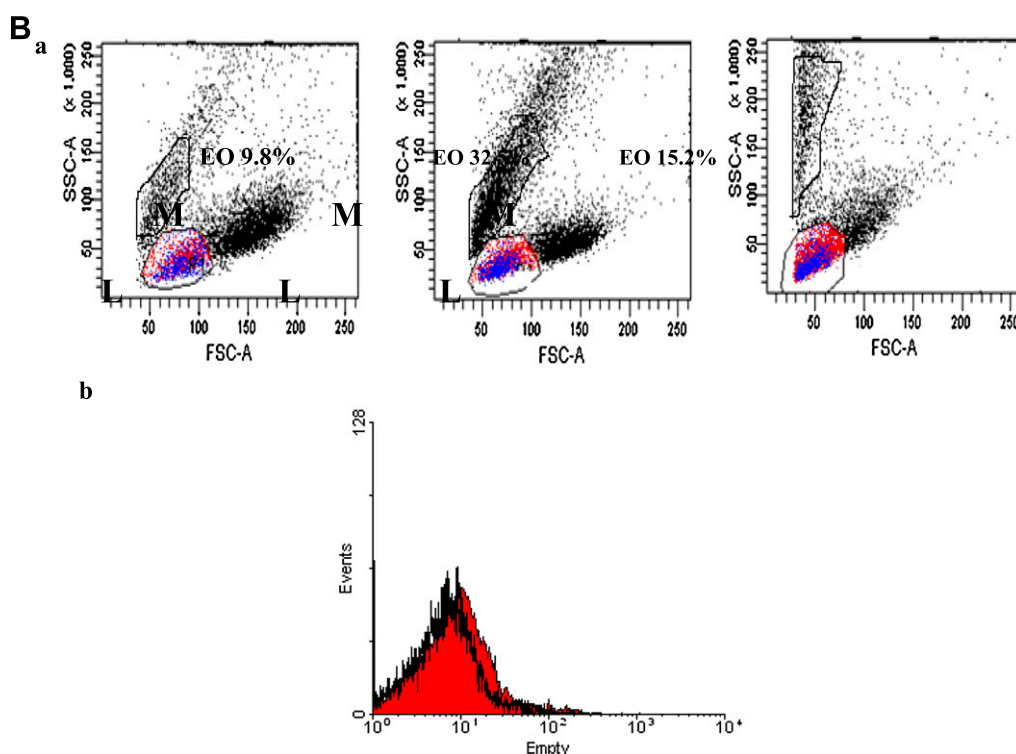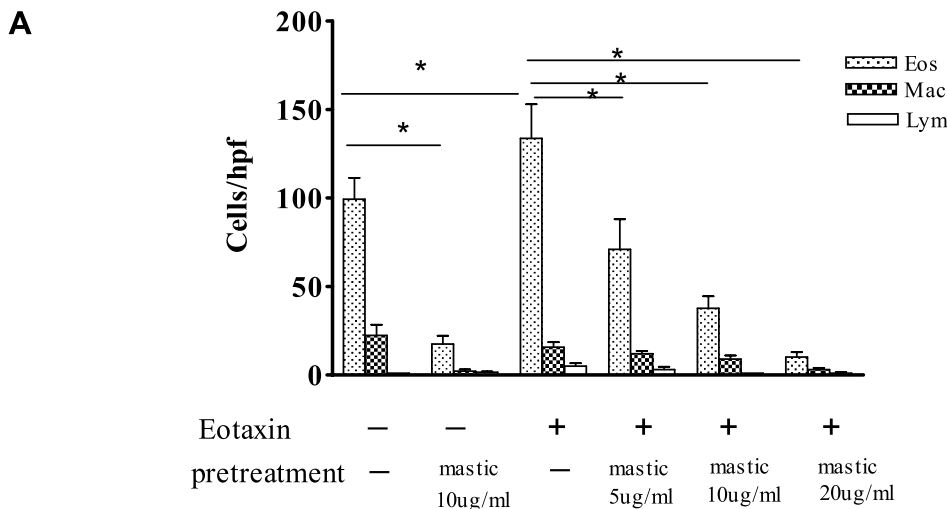
Figure 4. Effect of mastic on mouse BALF eosinophils *in vitro*. (*A*) Dose-dependent inhibition of eotaxin-induced chemotaxis of mouse BALF eosinophils by mastic *in vitro*. Cells collected from OVA-challenged mouse ($n = 5$) BALF were incubated with mastic (0–20 μg/ml) for 90 minutes. Eotaxin (10 ng/ml)-induced chemotaxis chamber assay was performed, and then the filters were removed and stained with Diff-Quick. The number of infiltrating cells was taken as the mean of counts in five immersion fields. Data are means ($\pm$SE) of three experiments. *$P < 0.05$. (*B*) Effect of mastic on chemokine receptor (CCR) 3 expression in mouse BALF eosinophils. (*a*) BALF cells collected from OVA-challenged mice were incubated with anti-mouse CCR3–fluorescein antibody and then sorted on a FACS. Lymphocytes (Lym), granulocytes (Eos; mainly eosinophils in this experiment), and macrophages (Mac) were sorted by forward scatter (FSC) and side scatter (SSC). Granulocytes were recognized as highly granular cells, and, in this model, almost all cells within this gate were eosinophils, which were defined as cells expressing the eotaxin receptor, CCR3. Lymphocytes (L) were identified as FSC^{low}/SSC^{low}, and macrophages (M) as FSC^{high}/SSC^{high} cells. The annotated numbers indicate the percentages of cells in each region. The data shown are representative of three independent experiments with similar results. (*b*) CCR3 expression in eosinophils. Histogram (log scale fluorescence) shows CCR3 expression in OVA-challenged/mastic-treated mice (*dotted line*) versus OVA-challenged mice (*solid line*) in gated eosinophils.

The objective of the present study was to observe if mastic could ameliorate allergic asthma in OVA-induced asthma, which is characterized by eosinophilia, increased AHR, and inflammatory cytokines and chemokines. Our findings showed that mastic prevented eosinophil infiltration into the airways, as shown by a significant drop in total cell counts and eosinophil counts in BALF. Because eosinophil transmigration into the airways is a multistep process that is orchestrated by cytokines and coordinated by specific chemokines, we examined various Th2 inflammatory cytokines (IL-4, -5, -13) and chemokines (eotaxin, eotaxin2, and RANTES) in BALF, and found that IL-5 and -13 concentrations were decreased after mastic administration. However, IL-4 level was unchanged. In BALB/c mice injected with mastic, the decrease in the levels of inflammatory cytokines and chemokines might be responsible for the decrease in eosinophil infiltration in BALF and lung tissue. Moreover, it is believed that inflammatory mediators released during the allergic inflammation play an important role in AHR development (19). Penh

values in response to inhaled methacholine were used to detect AHR. We demonstrate that mastic administration significantly inhibited Penh values compared with OVA-challenged mice. It has been well established that IL-5 plays a critical role in AHR by mobilizing and activating eosinophils, leading to the release of proinflammatory products that are closely associated with AHR (20, 21). IL-4 and -13 have also been shown to induce AHR in mouse asthma models (22). Therefore, the observed reduction of AHR by mastic might be associated with the reduction in cytokine production and eosinophilia in the airway. However, we cannot determine the origin of these cytokines, because IL-4, -5, and -13 can be produced by various resident cells, such as bronchial epithelial cells, tissue mast cells, and alveolar macrophages, as well as by infiltrated inflammatory cells, such as lymphocytes and eosinophils (23).

Persistent NF-κB activation has been observed in allergic airway inflammation, both in human and in animal models of asthma (24). Recently, we found that mastic reduced both
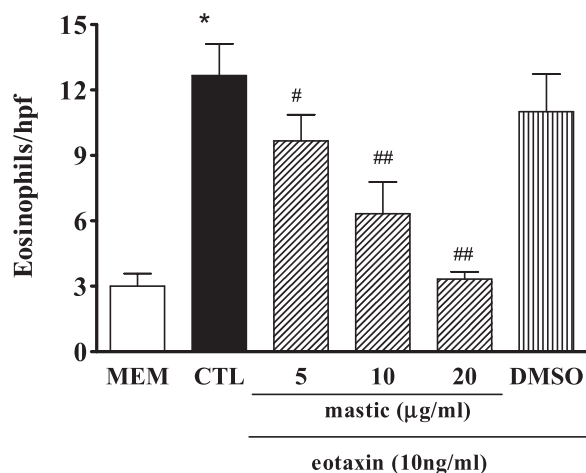
Figure 5. Inhibition of eotaxin (10 ng/ml)-induced human eosinophil migration by mastic. The human eosinophil suspension (5×10^5 cells/ml) was preincubated (37°C, 20 min) with mastic (5–20 μg/ml; *diagonally hatched columns*) or its vehicle (5 μl/ml of DMSO; *striped column*). Control migration (CTL) is represented by the *solid column*, whereas random chemotaxis (Minimum Essential Medium; in the absence of eotaxin) is represented by the *open column*. Each experiment was performed in triplicate. Eosinophil migration is expressed as the mean number of migrated cells per high-power field (hpf). Results are means (±SE). *$P < 0.001$ compared with minimum essential medium (MEM); #$P < 0.05$ and ##$P < 0.001$ compared with CTL.

nuclear p65 amount and DNA-binding activity in lung tissues obtained 24 hours after the last OVA or saline aerosol challenge, suggesting that mastic may exert its anti-inflammatory effects via inhibition of NF-κB activity (unpublished data). Furthermore, NF-κB is a critical transcription factor for Th2 cell differentiation (25). The observed reduction of IL-5, IL-13, and eotaxin levels in BALF from mastic-treated mice may be due to inhibition of NF-κB activation.

Besides cytokines, eosinophil transmigration from blood into lung tissue requires a series of chemokines. In particular, CC chemokine family members acting through CCR3 (26, 27) are crucial to the accumulation of eosinophils into airway walls (21). Therefore, we examined the production of chemokines (RANTES, eotaxin, and eotaxin2) in BALF, and found that mastic suppressed the production of these chemokines (Figure 3). Next, we investigated the direct effect of mastic on eosinophil. As shown in Figures 4 and 5, pretreatment of mastic *in vitro* dose-dependently suppressed eotaxin-induced chemotaxis of eosinophil (both murine and human eosinophils). In addition, administration of mastic *in vivo* inhibited eosinophil infiltration (Figure 1). These findings imply that administration of mastic to mice with allergic asthma inhibited the chemokine-induced migration of eosinophils from the bloodstream into the airway. The binding of CCR3 ligands to CCR3 on the surface of eosinophils and other CCR3-expressing cells is responsible for cell migration. The suppression of eosinophil chemotaxis may be induced by decreased expression of CCR3. To rule out this possibility, we performed FACS analysis, and found that mastic did not reduce the expression of the main eotaxin receptor, CCR3 (Figure 4). To validate the effects of the mastic on eosinophil, we used freshly isolated human blood eosinophils, a natural cell population expressing high surface levels of CCR3. CCR3 expression was not affected either (data not shown).

We report here for the first time that mastic effectively reduced several parameters of asthma-mediated lung inflammation. Moreover, mastic inhibited eotaxin-induced eosinophils

migration without influencing CCR3 expressions *in vitro*. Regardless of the exact mechanism, it is clear that mastic intervention may contribute in the treatment of inflammatory diseases. However, the results observed in animals are not always corroborated in humans. It is better to be more careful when translating animal data to potential human therapeutic approaches. As far as large animals or humans are concerned, we have, as of yet, no data at all. To confirm the antiasthmatic effect of mastic, to ascertain the precise mechanism of its action mode, and to prove the potential of mastic that is used in asthma treatment, further studies are definitely required.

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