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Antiallergic Asthma Properties of Brazilin through Inhibition of T_H2 Responses in T Cells and in a Murine Model of Asthma

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ABSTRACT: This study aimed to determine whether brazilin exhibits anti-inflammatory effects that inhibit T helper cell type II (T_H2) responses and whether it suppresses allergic inflammation reactions in a murine model of asthma. We found that brazilin inhibited the mRNA and protein expression of interleukin (IL)-4 and IL-5 induced by phorbol myristate acetate (PMA) and cAMP in EL-4 T cells in a dose-dependent manner. Following the intratracheal instillation of brazilin in ovalbumin (OVA)-immunized mice, we found that brazilin-treated mice exhibited decreases in the release of IL-4, IL-5, IL-13, eotaxin-1, and tumor necrosis factor-α in bronchoalveolar lavage fluid (BALF); inhibited T_H2 functioning via a decrease in IL-4 production; and exhibited attenuation of OVA-induced lung eosinophilia, airway hyperresponsiveness, and airway remodeling. These results suggest that brazilin exhibits anti-T_H2 effects both in vitro and in vivo and may possess therapeutic potential for allergic diseases.

KEYWORDS: *brazilin, airway remodeling, allergic inflammation, GATA-3*

INTRODUCTION

Allergic asthma is clinically characterized by mucus hypersecretion, chronic inflammation of the airways, and airway hyperresponsiveness (AHR). Studies on allergic asthma conducted using human and animal models of asthma have shown that $CD4^+$ type 2 helper T lymphocytes (T_H2 cells) induce an inflammatory cascade via increased cytokine production. This inflammatory cascade comprises eosinophil action, immunoglobulin (Ig)E production, and mast cell activation, consequently producing mediators required for the development of AHR.[1,2] T_H2 cells are the main effector cells involved in airway inflammation, and they cause lung dysfunction via the recruitment and activation of eosinophils. The pathological action of T_H2 cells is mediated by the release of T_H2 cytokines such as interleukin (IL)-4, IL-5, and IL-13.

Caesalpinia sappan L. is used in traditional Chinese medicine as an analgesic and anti-inflammatory agent. In addition, *C. sappan* L. is used to treat emmeniopathy, sprains, and convulsions. Recently, different extracts of this plant were found to have pharmacological activity. For example, an ethanolic *C. sappan* L. extract inhibited inflammation in human chondrocytes and macrophages.[3,4] In addition, the methanol and aqueous extracts of *C. sappan* L. heartwood showed hepatoprotective activity against carbon tetrachloride (CCl_4)-induced toxicity in freshly isolated rat hepatocytes and in a rat model[5] and vasorelaxation activity on the rat aorta and mesenteric artery.[6] Brazilin, or 7,11*b*-dihydrobenz[*b*]indeno-[1,2-*d*]pyran-3,6a,9,10(6*H*)-tetrol, which is the main component of *C. sappan* L. extracts,[7] is a naturally occurring red pigment typically used for histological staining. Brazilin has been reported to exert many pharmacological activities, including a hypoglycemic effect on experimental diabetic animals[8] and vasorelaxation via the activation of nitric oxide synthase (NOS) in human endothelial cells.[9] In addition, it exerts various biological effects, including the aggregation of antiplatelet agents,[10] inhibition of protein kinase C (PKC) and insulin receptor kinase in the rat liver,[11] and protection of cultured hepatocytes against bromotrichloromethane ($BrCCl_3$)-induced toxicity.[12] Due to its anti-inflammatory potential, brazilin can prevent the induction of immunological tolerance caused by ovine red blood cells in vivo,[13] suppressing inducible NOS (iNOS)[14] and anticomplementary activity[15] and initiating the concanavalin A- and lipopolysaccharide (LPS)-induced proliferation of spleen lymphocytes.[16] Brazilin has also been reported to cause anti-T_H1-related inflammation via anti-iNOS activity[17] and LPS-induced spleen lymphocyte proliferation.[16] However, the therapeutic effect of brazilin in T_H2-activated diseases such as allergic diseases is still unclear. Therefore, in this study, we investigated the therapeutic potential of brazilin for allergic asthma and its ability to regulate T_H2 responses in T cells.

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Table 1. Primer Sequences for Real-Time PCR

gene name	sense sequence (5′ → 3′)	antisense sequence (5′ → 3′)
IL-4	CTCATGGAGCTGCAGAGACTCTT	CATTCATGGTGCAGCTTATCGA
IL-5	TGACCGCCAAAAAGAGAAGTG	GAACTCTTGCAGGTAATCCAGGAA
GATA-3	CAGAACCGGCCCCTTATCA	ACAGTTCGCGCAGGATGTC
c-Maf	AGAGGCGGACCCTGAAAAA	GTGTCTCTGCTGCACCCTCTT
T-bet	CTGGATGCGCCAGGAAGT	TGTTGGAAGCCCCCTTGTT-
collagen-I	CCCGAGGTATGCTTGATCTGTATC	CACTCGCCCTCCCGTCTT
MMP-2	CGGTTTATTTGGCGGACAGT	GGCCTCATACACAGCGTCAAT
MMP-9	TGCCGGAAGCGCTCAT	AGAGCCACGACCATACAGATACTG
β-actin	ACTGCCGCATCCTCTTCCT	ACCGCTCGTTGCCAATAGTG

EL4 T cells have been used for several years as a platform to screen potential antiallergic effects.[18,19] Previous studies have shown that antigens can be mimicked by agents such as phorbol myristate acetate (PMA) and calcium ionophores.[20,21] However, under such conditions, these EL4 T cells produce larger quantities of interferon (IFN)-γ than of IL-4 or IL-5, because they behave more like T_H1 cells than T_H2 cells. Therefore, to specifically study the T_H2 response, we used PMA (a PKC activator) combined with dibutyryl cAMP (a protein kinase A [PKA] activator) to activate EL4 T cells to release IL-4 and IL-5[22] but not IFN-γ (IFN-γ production was below the detection level of the kit used; data not shown). In addition, these PMA + cAMP-stimulated EL4 T cells exhibited high-level induction of the master T_H2 transcription factors GATA-binding protein 3 (GATA-3) and c-musculoaponeurotic fibrosarcoma (c-Maf), but not of T box expressed in T cells (T-bet), a T_H1 cell-specific transcription factor. In this study, we found that brazilin exhibited therapeutic potential for allergic disease by its inhibition of T_H2 cell activation in vitro and in vivo.

MATERIALS AND METHODS

Chemicals. Brazilin extracted from *C. sappan* L., which contains 90−100% (weight %) of the compound, was purchased from MP Biomedicals (Solon, OH, USA). PMA, cAMP, and ovalbumin (OVA; grade V) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Brazilin was dissolved in DMSO.

Cell Culture. EL4 murine T lymphoma cells and Jurkat human T lymphoma cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The EL4 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum purchased from Invitrogen (Carlsbad, CA, USA). Confluent cells were subcultured at a ratio of 1:3, and media were changed twice a week.

Cytotoxicity Assay. The EL4 T cells were pretreated with various concentrations of brazilin for 10 min and cultured with or without PMA (5 ng·mL^{-1}) + cAMP (250 μM) for 24 h. At this point, the number of viable cells was determined using trypan blue staining.

Cytokine Assay. EL4 cells (5×10^5) were cultured in 24-well culture plates and treated with different concentrations of brazilin for 10 min and then further cultured in the presence or absence of PMA (5 ng·mL^{-1}) + cAMP (250 μM) for 24 h. The cell culture supernatants were collected 24 h after the different drug treatments and stored at −20 °C before analysis using a commercial enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. Standards were prepared from recombinant mouse IFN-γ, IL-4, IL-5, tumor necrosis factor (TNF)-α, IL-13, and eotaxin-1 (R&D Systems, Minneapolis, MN, USA).

Quantitative Real-Time PCR. RNA was converted into cDNA and subsequently quantified by quantitative real-time polymerase chain reaction (PCR) using an ABI PRISM 7900 sequence detector (Applied Biosystems, Foster City, CA, USA). The PCR conditions used were in accordance with those recommended by Applied Biosystems. The number of partial cycles that resulted in a statistically significant increase in target gene products was determined (threshold cycle; Ct) and normalized to the Ct for β-actin. IL-4, IL-5, GATA-3, c-Maf, T-bet, collagen-1, metalloproteinase (MMP)-2, MMP-9, and β-actin were amplified using a SYBR Green kit (Applied Biosystems). Results normalized to those of β-actin were calculated according to the change in the Ct value (ΔCt) as follows: $\Delta\Delta$Ct = sample ΔCt − reference ΔCt. The primer sequences used are listed in Table 1. All the primers were designed using ABI Primer 3 software, and the specificity was determined using the Basic Local Alignment Search Tool (BLAST).

Animals and Experimental Protocol. Female BALB/c mice, aged 6−8 weeks, were obtained from the Animal Center of the College of Medicine, National Taiwan University, Taiwan. The protocols for animal care and handling were approved by the Animal Committee of China Medical University. The animal study, conducted as described previously,[23] consisted of two treatment protocols. In protocol A (Figure 1A), mice were first sensitized by an intraperitoneal (i.p.) injection of 200 μL of PBS on days 0, 14, and 28. Brazilin was dissolved in PBS containing 1% pure DMSO for all treatment protocols. The mice were intratracheally instilled with 30 μL of PBS containing either 1% pure DMSO (vehicle control), 5 mM brazilin (42.9 μg·mouse^{-1}), or 50 mM brazilin (429 μg·mouse^{-1}) once a day on days 35−39. Mice that were injected intraperitoneally and

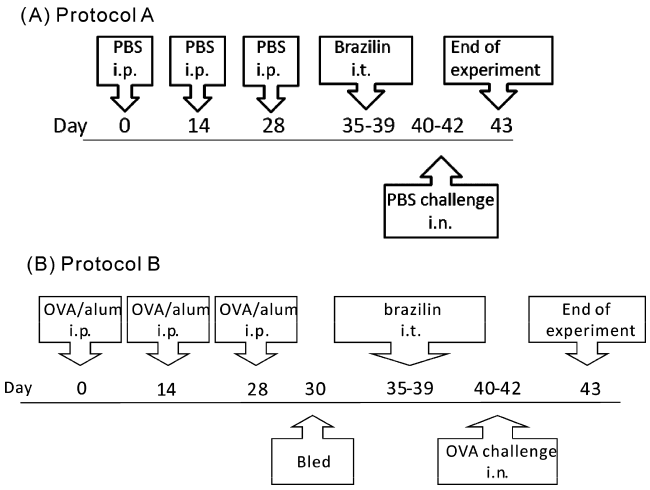

Figure 1. Animal protocols. (A) Brief scheme of animal sensitization and challenge. Mice were divided into four groups as clearly described in the Materials and Methods section: negative control, PBS/vehicle control, 42.9 μg of brazilin, and 429 μg of brazilin. i.p., intraperitoneal; i.n., intranasal; i.t., intratracheal. (B) Brief scheme of animal sensitization and challenge by OVA. Brazilin was dissolved in PBS containing 1% pure DMSO. Mice were intratracheally instilled with 30 μL of PBS containing 1% pure DMSO (OVA group), 5 mM brazilin (42.9 μg·mouse^{-1} group), or 50 mM brazilin (429 μg·mouse^{-1} group), once a day on days 35−39. The PBS group represents mice injected intraperitoneally and intranasally with PBS only.

intranasally with PBS alone represented the negative control group. In protocol B (Figure 1B), mice were first sensitized by an i.p. injection of 50 μg OVA dissolved in 100 μL of PBS and emulsified in 100 μL of PBS containing 2 mg of aluminum hydroxide (AlumImuject; Pierce Chemicals, Rockford, IL, USA), for a total injection volume of 200 μL, to induce a T_H2 response. Mice were injected with 2 booster doses of 25 μg of OVA emulsified in 2 mg of aluminum hydroxide and were challenged 3 times with 100 μg of OVA (in a total volume of 40 μL) by intranasal (i.n.) administration on consecutive days. To confirm that the OVA immunization procedure was successful, mice sera were analyzed for OVA-specific IgE on day 30. The mice were then instilled intratracheally (i.t.) with 30 μL of PBS containing either 1% pure DMSO (OVA group), 5 mM brazilin (42.9 $\mu g \cdot mouse^{-1}$), or 50 mM brazilin (429 $\mu g \cdot mouse^{-1}$), once a day on days 35−39. Mice injected by i.p. and i.n. administration with PBS alone represented the PBS group. There were 8−10 mice per group. All the mice were euthanized using CO_2.

Measurement of Airway Resistance in Anesthetized Mice. Airway resistance was assessed as an increase in pulmonary resistance after challenge with aerosolized methacholine (MCh) in anesthetized mice, using a modification of the techniques described by Wagers et al.[24] Briefly, mice were anesthetized with 70−90 $mg \cdot kg^{-1}$ pentobarbital sodium (Sigma) and tracheostomized, and the trachea was cannulated with an 18 gauge i.t. cannula. Mice were mechanically ventilated using a computer-controlled small animal ventilator (flexiVent; Scireq, Montreal, Canada) at 150 $breaths \cdot min^{-1}$ with a tidal volume of 10 $mL \cdot kg^{-1}$, an inhalation/exhaustion ratio of 2:3, and a positive end-expiratory pressure of 2−3 cm H_2O. Pulmonary resistance was calculated using a software program (flexiVent; Scireq). Data are expressed as the pulmonary resistance (R_L) and represent three independent experiments.

Bronchoalveolar Lavage and Lung Histology. Bronchoalveolar lavage, using 1 mL of Hank's balanced salt solution instilled by syringe, was harvested by gentle aspiration 3 times and subsequently centrifuged.[25] An aliquot of the bronchoalveolar lavage fluid (BALF) cells was used for differential cell count with Liu-stained cytospun cells. In all, 300 cells were counted on at least 4 areas of the slide under a light microscope. BALF supernatants were assayed by ELISA. Lungs were fixed with 10% neutral phosphate-buffered formalin, and sections were prepared and stained with hematoxylin/eosin (H/E), periodic acid−Schiff (PAS), and Masson's trichrome in order to quantify the number of infiltrating inflammatory cells, mucous production, and collagen fibril deposition by microscopy. Quantification of airway inflammation, PAS-positive bronchial epithelial cells, and collagen fibril deposition was performed according to a previous study.[23] Briefly, inflammatory cell infiltration was quantified by counting the number of inflammatory cells per square millimeter in the subepithelial and subendothelial areas. Mucus-producing cells were quantified as the percentage of PAS-positive cells per bronchiole. A score of 0−4 was assigned for collagen fiber deposition (0 = normal lung; 1 = sparse fibrosis with fine fibrils involving <25% of the peribronchiolar area; 2 = mild fibrosis with fine fibrils throughout the peribronchiolar and perivascular areas; 3 = moderate fibrosis with fibrils throughout the peribronchiolar and perivascular areas with fibrils increasing in the submucosal area; 4 = severe fibrosis with fibrils involving the entire peribronchiolar area, throughout the inflammatory infiltrate), and a mean score was derived from the scores of 3 sections from each individual mouse.

OVA-Specific IgE Antibody Assay. Serum samples were collected from the mice on day 30, and the anti-OVA IgE antibody titer was determined by ELISA as described previously.[23]

Lymph Node Preparation and Lung Mononucleocyte Preparation. Mediastinal lymph nodes and lungs were isolated and pooled from each treatment group at the time of sacrifice (day 43). Single-cell suspensions were obtained according to a previous study.[23] Lung mononucleocytes were isolated using Ficoll-Plaque Plus according to the manufacturer's instructions (GE Healthcare, Uppsala, Sweden) and contained 40.2% ± 1.2% CD4+ T cells, as analyzed by flow cytometry (data not shown). Cells were stimulated for 72 h in vitro with anti-CD3 and anti-CD28 (0.5 $\mu g \cdot mL^{-1}$ each). Cell media were collected for cytokine analysis.

Zymography. BALF was mixed with the sample buffer without heating using a reducing agent. The sample was loaded into a sodium dodecyl sulfate (SDS) polyacrylamide gel containing 0.1 $mg \cdot mL^{-1}$gelatin for the detection of MMP-2 and MMP-9 activities and was subjected to electrophoresis with constant voltage. Thereafter, the gel was washed with 2.5% Triton X-100 to remove SDS, rinsed with 50 mM Tris−HCl (pH 7.5), and then incubated overnight at room temperature with the developing buffer (50 mM Tris−HCl (pH 7.5), 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 0.02% thimerosal, and 1% Triton X-100). Data were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis. All experimental data are expressed as the mean (±SEM) and were analyzed using 1-way ANOVA followed by the Newman−Keuls posthoc test. Statistical significance was set at $p < 0.05$.

RESULTS

Brazilin Inhibits Mitogen-Induced Expression of T_H2 Cytokines in EL4 T Cells. First, we evaluated the possible cytotoxic effects of brazilin on EL4 T cells. Treatment with 3, 10, or 30 μM brazilin for 24 h did not result in cytotoxicity to EL4 T cells, as shown by the trypan blue dye exclusion assay (Figure 2A). Next, we investigated the production of IL-4 and IL-5. EL4 T cells treated with different concentrations of brazilin did not exhibit any apparent changes with respect to IL-4 and IL-5 production (Figure 2B). We used a mixture of PMA and cAMP to drive EL4 T cells to behave like T_H2 cells. Brazilin (3, 10, and 30 μM) inhibited the PMA + cAMP-induced expression of IL-4 and IL-5 at both the protein (Figure 2B) and mRNA (Figure 2C) levels in a dose-dependent manner. Compared with control cells, which were treated with PMA + cAMP only, IL-4 production was reduced by 20.2% ± 4.2% and 31.8% ± 2.4% by 10 and 30 μM brazilin, respectively, whereas IL-5 production was reduced by 43.1% ± 10.6% and 59.3% ± 16.7% by 10 and 30 μM brazilin, respectively. Compared with the control group treated with PMA + cAMP only, IL-4 mRNA production was reduced by 31.1% ± 0.5% and 44.5% ± 0.3% by 10 and 30 μM brazilin, respectively, whereas IL-5 mRNA production was reduced by 29% ± 1.0% and 49.9% ± 15.6% by 10 and 30 μM brazilin, respectively. In addition, we analyzed the expression of T_H2- and T_H1-related transcription factors and found that brazilin inhibited the PMA + cAMP-induced mRNA expression of GATA-3 and c-Maf in a dose-dependent manner. However, T-bet was not induced by treatment with PMA + cAMP, and treatment with brazilin did not result in an obvious change in T-bet mRNA expression (Figure 2D).

Inhibition of Allergen-Induced Airway Inflammation by Brazilin. We examined the effects of brazilin in a murine model of asthma; the animal protocols are presented in Figure 1. First, we found that i.t. instillation of brazilin did not affect the BALF cell profile in PBS-sensitized and challenged mice (Figure 3A). Next, for the investigation of the therapeutic potential of brazilin in allergic asthma, OVA-sensitized mice were administered brazilin at 42.9 or 429 $\mu g \cdot mouse^{-1}$ by i.t. instillation, once a day for 5 days, before being challenged with OVA (Figure 2B). No obvious infiltration of inflammatory cells was noted in the BALF from mice sensitized and challenged with PBS only (PBS group). However, after sensitization and challenge with OVA (OVA group), the number of macrophages and eosinophils in the BALF was significantly higher than that in the PBS group (Figure 3A). Challenge of the OVA group

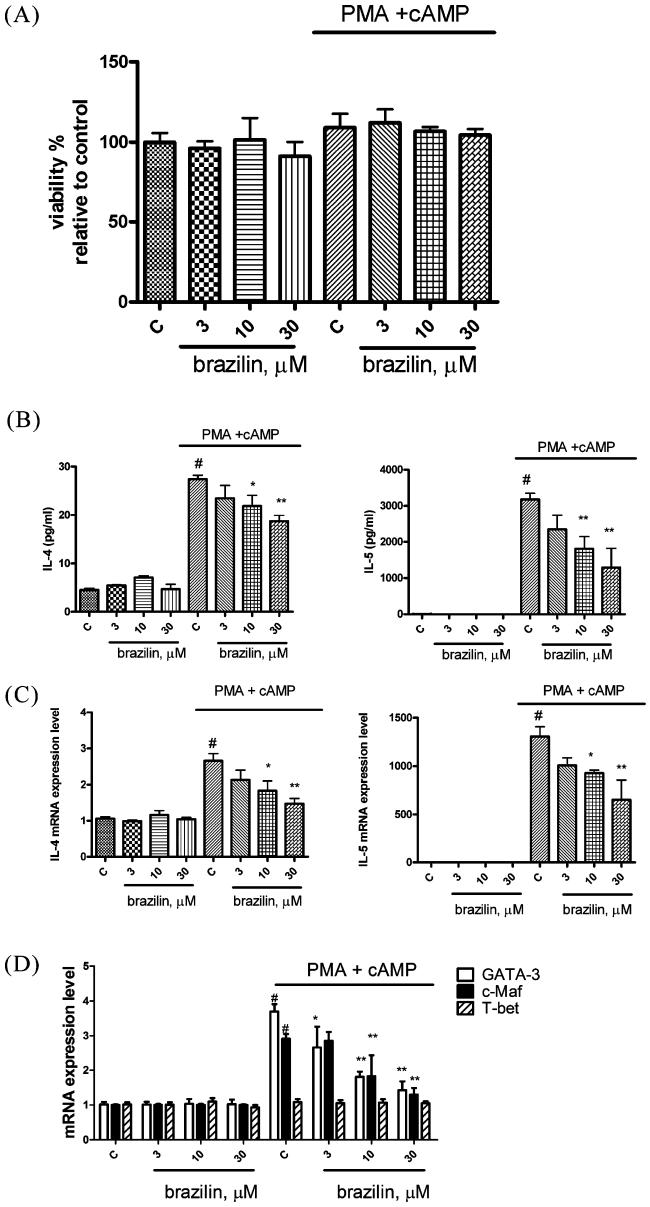

Figure 2. Brazilin inhibited the mitogen-induced expression of T_H2 cytokines in murine EL4 T cells. (A) Brazilin did not exert cytotoxic effects in EL4 T cells. (B) IL-4 and IL-5 production detected by ELISA. Data are expressed as mean ± SEM (n = 6). (C) The mRNA expression levels of IL-4 and IL-5 and (D) of GATA-3, c-Maf, and T-bet, detected by real-time PCR. Data are expressed as mean ± SEM (n = 6). #$p < 0.001$, compared with the control group without PMA plus cAMP (PMA + cAMP) group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with the control group with PMA + cAMP. C represents the control group, consisting of cells cultured in medium containing 0.1% DMSO.

following i.t. instillation of brazilin induced lower numbers of eosinophils in the BALF, but the number of macrophages was not affected.

Histological examination of lung sections from the OVA group revealed a large number of infiltrating inflammatory cells (Figure 3B) and increased mucus formation (Figure 3C) around the airways, compared with the PBS group. Upon treatment with brazilin, the challenged OVA group exhibited substantially lower inflammatory cell infiltration and mucus formation than seen in the absence of brazilin treatment.

Intratracheal Instillation of Brazilin Decreases BALF T_H2 Cytokines Levels and the mRNA Expression of Lung T_H2 Transcription Factors in a Murine Model of Asthma. For further analysis of the mechanisms underlying brazilin-induced inhibition of airway inflammation, cytokine levels in the BALF were measured one day after the final challenge (Figure 1B). The OVA groups exhibited increases in the levels of IL-4, IL-5, IL-13, eotaxin-1, and TNF-α in the BALF compared with the control groups (PBS group) (Figure 4). After treatment with brazilin, the levels of IL-4, IL-5, eotaxin-1, IL-13, and TNF-α in the BALF decreased in a dose-dependent manner. Compared with the levels in the OVA group, the production levels of these five cytokines were reduced in the BALF of the mice treated with brazilin at 42.9 and 429 μg·mouse^{-1} as follows: IL-4, 31.2% ± 9.73% and 59.7% ± 4.1%; IL-5, 20.6% ± 7.3% and 41.5% ± 6.5%; IL-13, 38.5% ± 7.3% and 60.7% ± 8.7%; eotaxin-1, 43.8% ± 6.9% and 64.5% ± 3.7%; and TNF-α, 44.4% ± 14% and 52.2% ± 9.3%, respectively (Figure 4A). The level of IFN-γ in the BALF was below the detection limit of the kit in all the groups studied. With regard to the expression of T_H2 transcription factors in the lungs, we found that OVA-immunized mice showed significantly increased mRNA expression of GATA-3 and c-Maf but not of T-bet (Figure 4B). Treatment with brazilin resulted in a dose-dependent decrease in the mRNA expression levels of GATA-3 and c-Maf.

Intratracheal Instillation of Brazilin Decreases T_H2 Cell Activation in the Lungs in a Murine Model of Asthma. Next, we investigated whether brazilin inhibits T lymphocyte activation in the lungs and mediastinal lymph nodes. As shown in Figure 5, treatment with a mixture of anti-CD3 and anti-CD28 (anti-CD3 + anti-CD28) induced the production of IL-4 and IFN-γ in lung cells isolated from OVA-immunized mice (Figure 5A). Brazilin inhibited anti-CD3 + anti-CD28-induced IL-4 production in a dose-dependent manner, whereas it did not affect IFN-γ production. Because anti-CD3 + anti-CD28 induces the activation of both naïve and effector T cells, the PBS group also showed induction of IL-4 and IFN-γ production. In mediastinal lymph node cells, treatment with a mixture of anti-CD3 + anti-CD28 also induced IL-4 and IFN-γ production in OVA-immunized mice but did not induce IL-4 production in the PBS control (Figure 5B). Because our animal model activated the T_H2 response, the OVA group demonstrated higher IL-4 production than did the PBS group.

Intratracheal Delivery of Brazilin Suppresses the Development of Airway Hyperresponsiveness and Remodeling. We investigated whether brazilin affects the development of airway hyperresponsiveness and remodeling in a murine model of asthma. One day after the final challenge, airway responsiveness was assessed by pulmonary resistance using invasive body plethysmography. The baseline value of lung resistance is 1.25 ± 0.13 cm $H_2O \cdot L^{-1} \cdot s^{-1}$. BALB/c mice sensitized and challenged with OVA exhibited higher airway resistance to MCh inhalation, compared with mice sensitized and challenged with PBS (Figure 6A; OVA and PBS groups). After treatment with brazilin, airway resistance was significantly lower than that in the untreated OVA group. Histological examination of lung sections from the OVA group showed greater fibrosis and formation of collagen fibers around the airway than seen in the PBS group (Figure 6B). After treatment with brazilin, the challenged OVA group showed substantially less fibroblast deposition and collagen fiber formation than seen in untreated OVA mice. Further identification of the

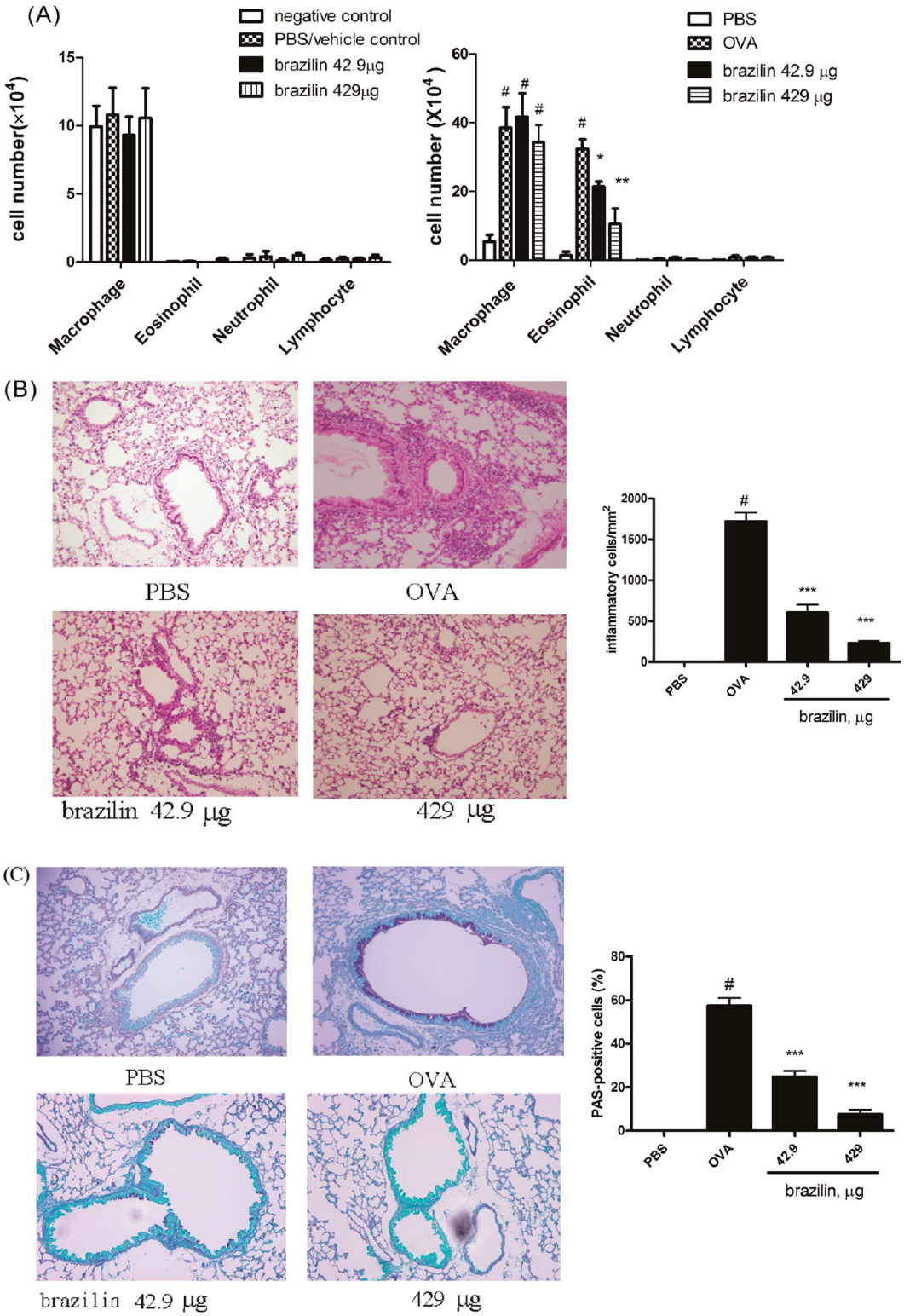

Figure 3. Airway inflammation is inhibited by brazilin. The profile of cells in the bronchial alveolar lavage fluid (BALF) and on histological analysis of lung tissue in mice after brazilin treatment, shown as the daily dose (μg) given by intratracheal instillation for five days, are presented. (A) Total cell counts were determined from 3 mL of BALF, and differential cell counts were assessed by Liu staining. Data are expressed as mean ± SEM (n = 10). #p < 0.001, compared with the PBS group; *p < 0.05, **p < 0.01, compared with the OVA group. (B) Lung sections were stained with hematoxylin/eosin (H/E) for the measurement of inflammatory cells, and the data revealed different extents of cellular infiltration into the periairway region. (C) Lung sections were also subjected to periodic acid−Schiff (PAS) staining for the measurement of mucus production around the airways. Original magnification: 200×. Data are expressed as mean ± SEM (n = 5). #p < 0.001, compared with the PBS group; ***p < 0.001, compared with the OVA group.

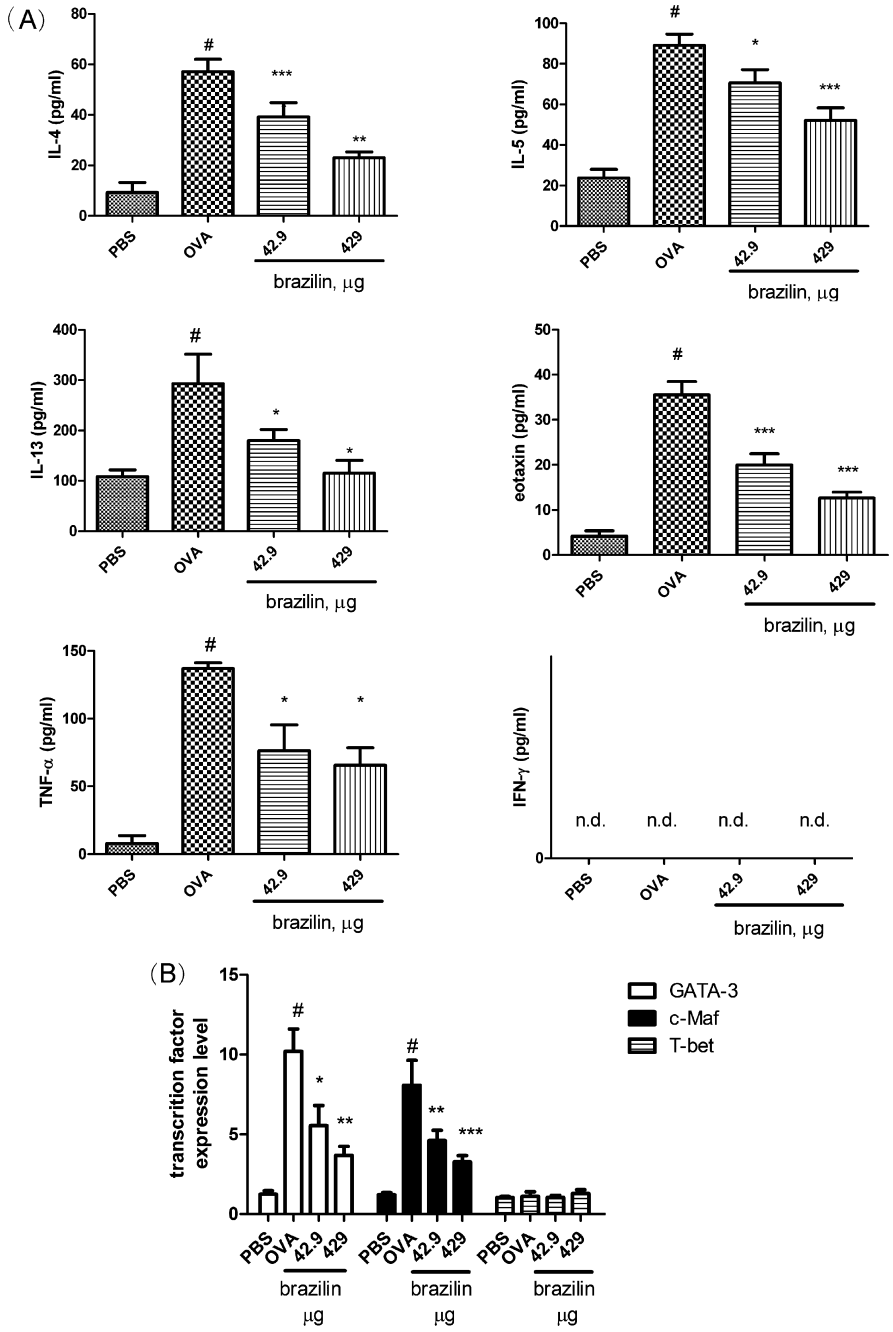

Figure 4. Suppression of cytokine levels in BALF and of transcription factors in lung tissues following brazilin administration in a murine model of asthma. (A) Cytokine levels in the BALF were analyzed by ELISA. Data are expressed as mean ± SEM (n = 10). (B) The mRNA expression levels of GATA-3, c-Maf, and T-bet in lung tissues were detected using real-time PCR. The data are expressed as the mean ± SEM (n = 6). #p < 0.001, compared with the corresponding PBS group; *p < 0.05, **p < 0.01, ***p < 0.001, compared with the OVA group without brazilin treatment.

extracellular matrix deposition-related genes expressed showed that the mRNA levels of collagen-I, MMP-2, and MMP-9 had increased in the OVA group (Figure 6C). Upon treatment with brazilin, the mRNA expression levels of collagen-1, MMP-2, and MMP-9 were lower than those for the untreated OVA group. We also found that the MMP-2 and MMP-9 activities in the BALF of OVA-immunized mice reduced after treatment with 42.9 and 429 μg brazilin·mouse^{-1} (Figure 6C)

■ DISCUSSION

The present study focused on the mechanisms of the antiallergic asthma response induced by brazilin treatment. Brazilin belongs to the class of flavonoid compounds, which are well-known chemical constituents that exert anti-inflammatory activity.[26,27] In this study, we found that brazilin exerted inhibitory effects on allergic asthma responses including the release of T_H2 cytokines in vitro and in vivo, airway inflammation, airway hyperresponsiveness, and airway remodeling in a murine model of asthma. We believe that brazilin possesses therapeutic potential for treating allergic asthma via at least two mechanisms. First, brazilin inhibited the activation of T_H2 cells in vitro and in vivo. In EL-4 T cells, brazilin inhibited PMA + cAMP-induced activation of T_H2 cells in a dose-dependent manner by decreasing the mRNA and protein

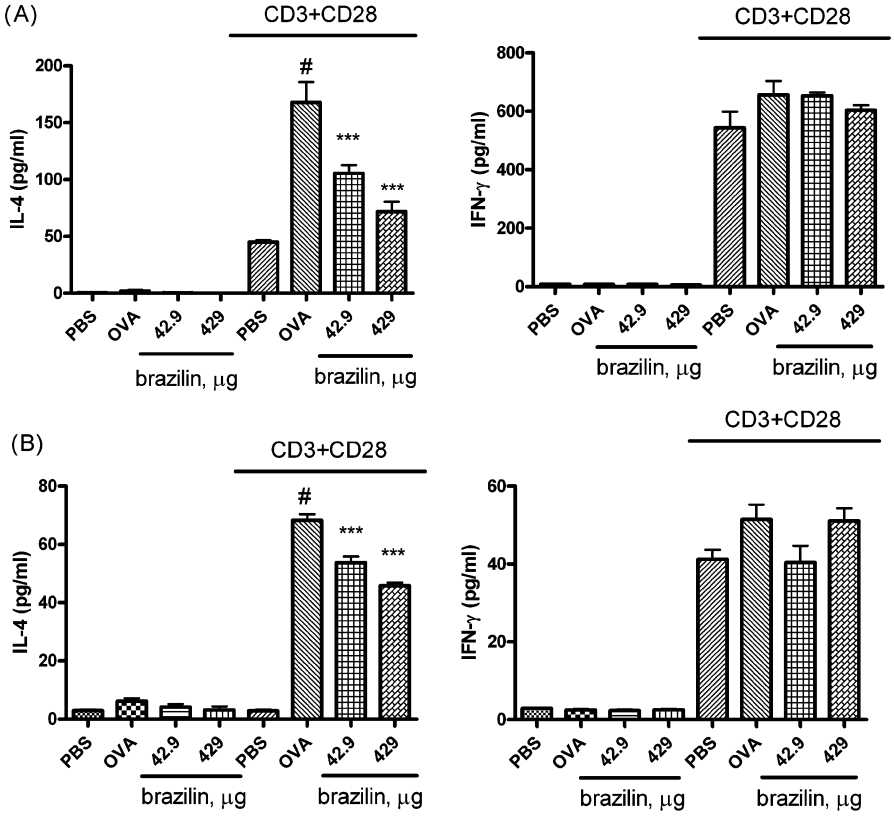

Figure 5. Brazilin suppressed T_H2 cell function in lung cells and mediastinal lymph node cells in a murine model of asthma. Lung cells and mediastinal lymph node cells were isolated from naïve or OVA-immunized mice. Lung cells (A) and mediastinal lymph node cells (B), at 2×10^5 cells each, were treated with 1 $\mu g \cdot mL^{-1}$ anti-CD3 + 1 $\mu g \cdot mL^{-1}$ anti-CD28 for 72 h, and the medium was collected for ELISA investigation of cytokine production. Data are expressed as mean ± SEM ($n = 6$). #$p < 0.001$, compared with the corresponding PBS group; ***$p < 0.001$, compared with the OVA group without brazilin treatment.

expression of IL-4 and IL-5. In vivo, the inhibitory effect of brazilin on airway inflammation and hyperresponsiveness is also due to decreased activation of T_H2 cells. We isolated mononuclear cells from lungs and mediastinal lymph nodes and treated them with anti-CD3 and anti-CD28 antibodies to activate the T cells. In these isolated cells, we observed that brazilin specifically inhibited T_H2 activation in vivo by reducing IL-4 production but not IFN-γ release. The release of T_H2 cytokines (IL-4, IL-5, and IL-13) into the BALF was also attenuated by brazilin treatment.

The gene expression of IL-4, IL-5, and IL-13 is regulated by the transcriptional factor GATA-3,[28] and the IL-4 genes are also regulated by c-Maf.[29] Therefore, the mechanisms underlying the inhibitory effect of brazilin on IL-4, IL-5, and IL-13 expression might involve the inhibition of the mRNA expression of GATA-3 and c-Maf, both in EL-4 T cells and in lung tissue. The signaling pathways involved in PMA + cAMP-induced GATA-3 expression in T cells include pathways regulated by phosphoinositide-dependent kinase 1,[30] PKA,[31] and those regulated by a soluble secreted form of the protein product of the ST2 gene.[32] In our previous study, we also found that the PMA + cAMP-induced expression of GATA-3 and c-Maf in EL-4 T cells involves mitogen activated protein kinases, including extracellular signal-regulated kinase (ERK), Jun N-terminal kinases (JNKs), and p38, as well as nuclear factor κB (NF-κB) activation.[33] However, further research is required to verify whether the aforementioned signaling pathways are involved in the brazilin-induced inhibition of PMA + cAMP-induced GATA-3 and c-Maf expression in EL-4 T cells.

Besides T_H2 cells, a recent clinical and animal study showed that T_H17 cells and T_H17 cell-promoting neutrophils may be important for the pathogenesis of chronic asthma,[34] especially in severe asthma. However, in our acute murine model of asthma, we did not observe neutrophil infiltration into the BALF. Therefore, we believe that T_H17 cells might not play a role in the pathogenic mechanisms in our acute murine model of asthma; however, further investigation is need to determine whether T_H17 cells are involved in the pathological mechanism in our acute murine model of asthma and how brazilin regulates their function.

Second, the in vivo therapeutic potential of brazilin in the inhibition of airway remodeling in a murine asthma model might involve reducing the expression and activity of MMP-2 and MMP-9. Airway remodeling is an important characteristic of asthma, especially in severe cases. Airway remodeling tends to be milder and reversible in acute and mild asthma models.[35] MMP-2 and MMP-9 can regulate airway remodeling by the disruption of the normal basement membrane, by the sequential deposition of collagen types I and III and fibronectin in the reticular layer of the basement membrane,[36] and by increasing airway smooth muscle mass[37] and the induction of TGF-β1 production in the airway epithelium.[38] In our study, we observed that certain characteristics of airway remodeling, such as subepithelial fibrosis, goblet and mucous gland hyperplasia, and extracellular matrix deposition, in OVA-immunized mice were inhibited by brazilin treatment. Meanwhile, we found that brazilin inhibited the mRNA expression and activity of MMP-2 and MMP-9 in a murine model of

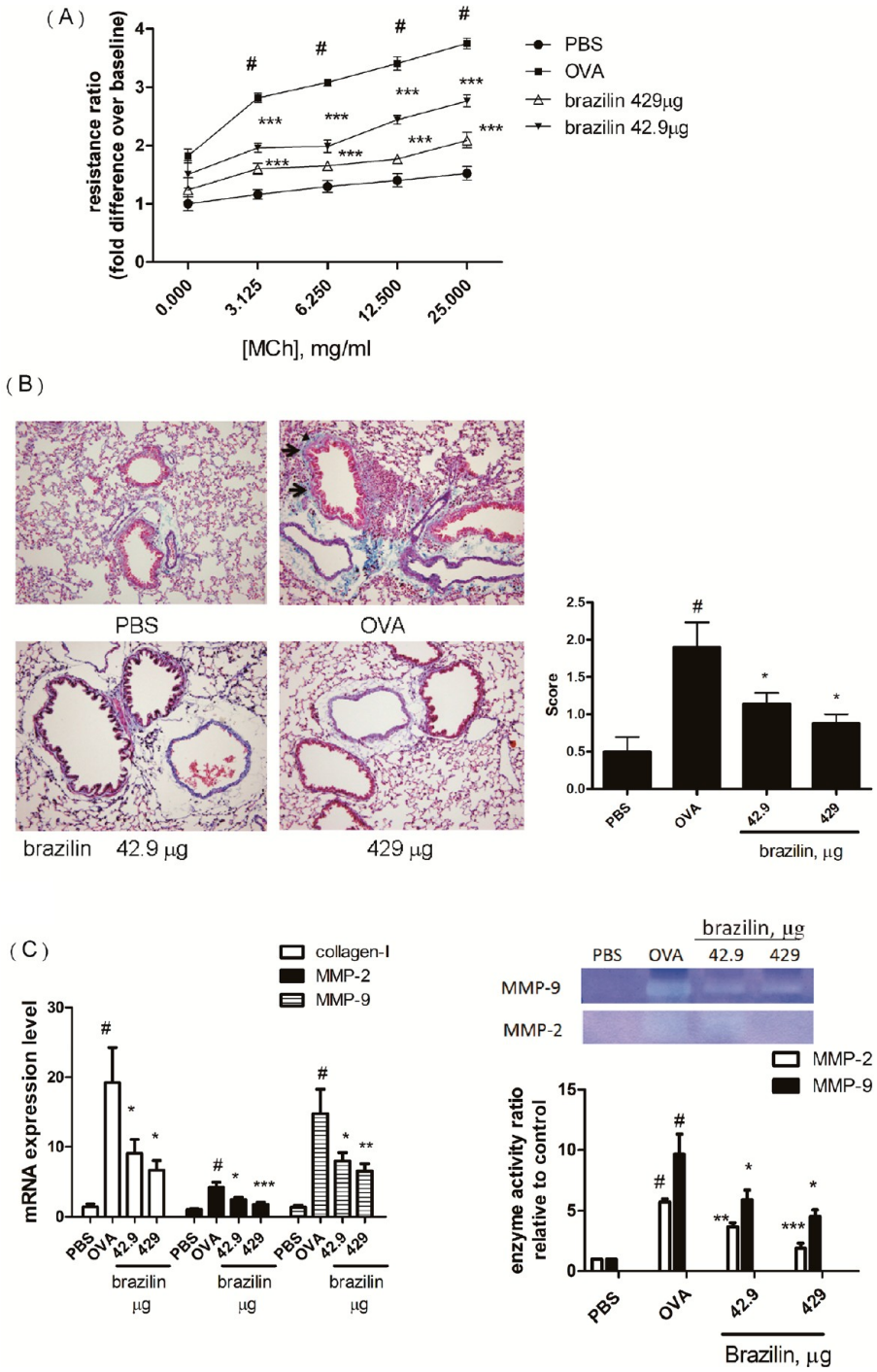

Figure 6. Airway hyperresponsiveness and remodeling are inhibited by brazilin in BALB/c mice. (A) Airway resistance as measured by invasive body plethysmography. Data are expressed as the mean ± SEM of the ratio of airway resistance over the baseline ($n = 5$). (B) Lung sections were stained with Masson's trichrome for the measurement of collagen fiber deposition around the airways and vascular area. Data revealed different extents of collagen fiber deposition in the periairway region. Original magnification: 200×. The fibrosis in lung sections from the OVA group is indicated by arrows. (C) The expression levels of collagen-I, MMP-2, and MMP-9 mRNAs in lung tissues and MMP-2 and MMP-9 activities in the BALF were detected by real-time PCR and zymography. Data are expressed as the mean ± SEM ($n = 5$). #$p < 0.05$, compared with the PBS control. *$p < 0.05$, ***$p < 0.001$, compared with the OVA control without brazilin treatment.

asthma; therefore, we proposed that brazilin-induced inhibition of airway remodeling might be associated with MMP-2 and MMP-9 activation. A previous study found that brazilin inhibits the UVB-induced expression and secretion of MMP-1 and MMP-3 in human dermal fibroblasts;[39] however, whether MMP-1 and MMP-3 contribute to the therapeutic mechanism of brazilin-induced inhibition of airway remodeling remains to be elucidated.

The brazilin-induced reduction in the expression and activities of MMP-2 and MMP-9 might be due to decreased IL-13 production in the BALF because, in a murine model of tobacco-induced lung damage, IL-13 overexpression was found to induce the expression of MMP-2 and MMP-9.[40] Reduced

airway eosinophila by brazilin might contribute to the inhibition of MMP-9 expression because eosinophils are known to express mainly MMP-9.[36] However, the role and mechanism of MMP-2 and MMP-9 in the brazilin-induced inhibition of airway inflammation and remodeling need to be studied further.

In a study by Yodsaoue et al.,[41] brazilin did not show any inhibitory effect on β-hexosaminidase release, a marker of degranulation, in rat basophilic leukemic (RBL-2H3) cells. However, we did not investigate the effects of brazilin on β-hexosaminidase release. Therefore, the inhibitory effect if any of brazilin on basophils and mast cell degranulation remains to be studied. Recently, two studies have compared the pharmacological activities of brazilin and *C. sappan* L. Washiyama et al. identified *C. sappan* L., and not brazilin, as having inhibitory effects on carrageenin-induced mouse paw edema;[42] furthermore, Sasaki et al. found that *C. sappan* L. had higher vasorelaxation activity than did brazilin.[6] These studies indicate that *C. sappan* L. has greater therapeutic potential than does brazilin, likely because *C. sappan* L. contains many other active compounds. However, it remains to be seen whether *C. sappan* L. is also more effective against allergic asthma than brazilin.

We found that brazilin inhibited airway inflammation, airway hyperresponsiveness, and airway remodeling in a murine model of asthma. The mechanism underlying the brazilin-induced inhibitory effect on allergic asthma involves the suppression of T_H2 cell activation, including the secretion of T_H2 cytokines and the expression of T_H2 specific transcription factors both in vitro and in vivo as well as the inhibition of MMP-2 and MMP-9 expression and activity. Brazilin has the potential to be used as a drug for treating allergic asthma.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

PMA,phorbol myristate acetate; GATA-3,GATA-binding protein 3; c-Maf,c-musculoaponeurotic fibrosarcoma; T-bet,T box expressed in T cells; OVA,ovalbumin; BALF,bronchoalveolar lavage fluid; AHR,airway hyperresponsiveness; i.p.,intraperitoneal; i.n.,intranasal; i.t.,intratracheal

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