
The traditional Chinese herbal formula ASHMI inhibits allergic lung inflammation in antigen-sensitized and antigen-challenged aged mice

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Background: Although asthma is typically characterized as a childhood disease, it can develop later in life. Older asthmatic patients may be at increased risk for corticosteroid adverse effects. We developed a novel traditional Chinese medicine to treat asthma called antiasthma simplified herbal medicine intervention (ASHMI). Herbal products may offer safer adjunctive treatment for older asthmatic patients.

Objective: To investigate the effects of ASHMI on characteristics of allergic asthma in an aged mouse model of asthma.

Methods: BALB/c mice (6 weeks old [young] and 6, 12, and 18 months old [aged]) received ASHMI treatment before and during intraperitoneal ovalbumin sensitization and intratracheal challenges. The control groups were untreated, age-matched, ovalbumin-sensitized and ovalbumin-challenged mice (ovalbumin mice) and naive mice. After the final antigen challenge, airway pressure (defined as the time-integrated change in peak airway pressure) after acetylcholine provocation was measured, representing airway hyperresponsiveness, and bronchoalveolar lavage fluid, sera, lung tissues for histologic analysis, messenger RNA, and collagen were collected.

Results: Mean time-integrated change in peak airway pressure values in 6-week-old and 6-, 12-, and 18-month-old ASHMI ovalbumin mice were significantly reduced compared with those of age-matched, nontreated ovalbumin mice. Bronchoalveolar lavage fluid eosinophil numbers were significantly lower in all ASHMI ovalbumin mice. Treatment with ASHMI of young and aged ovalbumin mice resulted in significantly decreased lung inflammation, detected via hematoxylin-eosin staining; airway mucous cell metaplasia, determined by means of periodic acid–Schiff staining; and messenger RNA copy numbers of the mucin gene *MUC5AC*. Levels of ovalbumin specific IgE and the T_H2 cytokines interleukin 4 (IL-4), IL-5, and IL-13 in lung and splenocyte cultures were reduced. Interferon gamma secretion was increased. Treatment with ASHMI reduced collagen production.

Conclusion: Treatment with ASHMI reduces several features of asthma in aged antigen-sensitized and antigen-challenged mice.

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INTRODUCTION

Although asthma is frequently characterized as a chronic inflammatory disease beginning in childhood, as many as 25% of asthmatic patients develop symptoms after the age of 40 years.¹ Airway disease may be more severe in later-onset asthma compared with earlier-onset disease^{2,3}; hospitalization and mortality rates are highest in patients older than 65 years.^{4,5} Despite these findings, the effect of aging on allergic airway and pulmonary inflammation in humans or murine

models has not been well characterized. We⁶ developed a murine model of later-onset asthma that demonstrated that aged mice develop antigen specific IgE, airway hyperresponsiveness (AHR), increased airway mucous cell metaplasia, pulmonary inflammation, and eosinophilia.

Corticosteroids, the cornerstone of persistent asthma therapy, substantially reduce asthma symptoms and improve lung function through anti-inflammatory actions. Systemic corticosteroids have the potential to induce adverse effects (ie, osteoporosis, increased fracture risk, muscle weakness, glucose intolerance, and easy bruising, all particular concerns in older patients), especially when administered for prolonged periods. The potential for these effects is reduced when corticosteroids are inhaled. However, inhaled corticosteroids taken at high doses, generally greater than 1000 μ g/d, have the potential for systemic absorption, producing similar effects as oral corticosteroids.^{7,8} Therefore, it is critical to develop corticosteroid-sparing medications for asthma. Li et al⁹ previously developed an herbal formula from traditional Chinese medicine (TCM) called MSSM-002, an extract of 14 herbs based on an asthma prescription used at the China-

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Japan Friendship Hospital in Beijing. MSSM-002 nearly eliminated AHR; significantly reduced total bronchoalveolar lavage fluid (BALF) cell numbers and eosinophil percentages and antigen specific IgE levels; and inhibited lung mucous cell hyperplasia similar to corticosteroid treatment in a murine model of allergic asthma (6-week-old mice).⁹ Because the formulation of 14 herbs is difficult to standardize to maintain quality, potency, and consistency, we first studied whether a simplified TCM compound could be equally effective in suppressing AHR and airway inflammation in young mice. We subsequently developed a product named antiasthma simplified herbal medicine intervention (ASHMI, original name MSSM-003d) containing 3 of the herbs found in MSSM-002. Next, we evaluated age-dependent effects of this treatment on allergic asthma in a murine model.

METHODS

Mice

Young (6-week-old) BALB/c and AKR mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Aged female BALB/c mice (6, 12, and 18 months old) were obtained from the National Institute on Aging (Bethesda, Maryland). Mice were maintained in the animal facility at Mount Sinai School of Medicine. Standard guidelines for laboratory animal care were followed,¹⁰ and institutional permission for animal handling was approved through the animal facility at Mount Sinai School of Medicine. Mice were housed under pathogen-free conditions.

Preparation of Extracts From Individual Herbs and ASHMI

Individual herbs and simplified formulas derived from MSSM-002 (Table E1, Online Repository) were prepared as previously described.⁹ Briefly, hot water extracts of individual herbs and formulas were prepared by boiling herbs in water twice and lyophilizing the resulting decoctions.¹¹ The ASHMI (Sino-Lion Pharmaceutical Co, Shan Dong, China), composed of 3 herbs, has been described previously¹² and is currently used in our clinical trial. Based on organoleptic and microscopic examination, the raw herbs used in ASHMI were identified as *Ganoderma lucidum*, radix *Sophorae flavescens*, and radix *Glycyrrhiza*. All 3 herbs are of Chinese origin and meet the quality and safety standards of the *Pharmacopoeia of the People's Republic of China*.¹³ Product quality was monitored by means of high-performance liquid chromatography fingerprinting according to the Food and Drug Administration's *Guidance for Industry Botanical Drug Products*,¹⁴ previously described.¹² Endotoxin levels in ASHMI were tested using the Pyrogen Plus assay kit (Lonza, Walkersville, Maryland). No endotoxin was detected (detection limit of <0.03 EU/mL).

Antigen Sensitization and Challenge and Treatment

AKR (n=4-5 per group) and BALB/c (n=5-10 per group) mice were antigen sensitized twice intraperitoneally with 200 μ g of conalbumin (AKR mice) (Fig E1, Online Repository)

or grade VI ovalbumin (, Sigma-Aldrich Corp, St Louis, Missouri) (BALB/c mice) (Fig 1) adsorbed with 2 mg of alum (Pierce Biotechnology Inc, Rockford, Illinois) in 0.4 mL of phosphate-buffered saline 1 week apart. Ten days after the last sensitization, mice were challenged intratracheally 3 times at 7- to 10-day intervals with 100 μ g of conalbumin (AKR mice) or ovalbumin (BALB/c mice) in 0.05 mL of phosphate-buffered saline, determined to be optimal for distribution to both lobes of the lung,¹⁵ as previously described.¹⁶ (Mice sensitized to and challenged with conalbumin or ovalbumin are labeled as conalbumin or ovalbumin mice, respectively). Controls were nontreated, age-matched conalbumin mice (n=4-5 per group) and ovalbumin mice (n=5-10 per group) and age- and strain-matched naive mice (n=5 per group). In the first experiment, AKR conalbumin mice were fed individual herbs from MSSM-002 or the complete formula using the protocol previous reported (Fig E1, Online Repository).⁹ Based on AHR and lung inflammation results, as well as TCM formulation concept, 3 individual herbs were selected to create the simplified formula (ASHMI). The AKR conalbumin mice were then fed MSSM-002 (73.4 mg/d) or ASHMI (9.5 mg/d) using the same protocol (Fig E1), and AHR and BALF were measured. In the aging mouse protocol, BALB/c mice were fed ASHMI (10 mg/d) for 4 consecutive weeks before antigen sensitization and challenge (Fig 1). The doses of individual herbs and formulas (Fig E1) were calculated based on dry yields from raw herb decoctions according to the prescribed human dose, including a dose-escalation safety trial,¹² and normalizing for mice using human to animal dose ratios.¹⁷ Treatment was delivered via intragastric feeding using a 25-gauge stainless steel blunt feeding needle (Fine Science Tool, Foster City, California). Mice were fed the ASHMI dissolved in 1 mL of water per day in 2 divided doses.

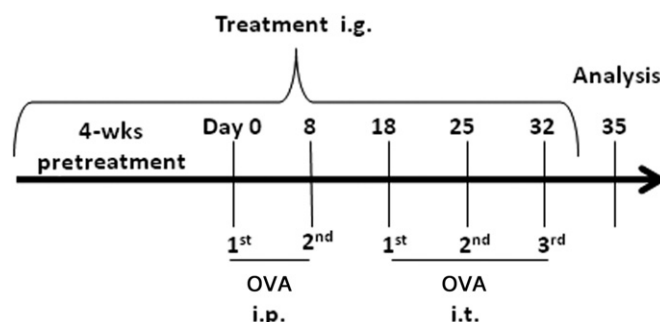


Figure 1. Mice (aged 6 weeks and 6, 12, and 18 months) were sensitized intraperitoneally (i.p.) with ovalbumin (OVA) (200 μ g per mouse) twice at weekly intervals and then were challenged intratracheally (i.t.) on days 18, 25, and 32 with OVA (100 μ g per mouse). One set of mice from each age group was pretreated with intragastric (i.g.) antiasthma simplified herbal medicine intervention (ASHMI) (10 mg/d per mouse) in a twice-daily divided dose starting 4 weeks before sensitization. Treatment was continued through the sensitization and challenge periods. Seventy-two hours after the final OVA challenge, the mice were analyzed. Age-matched naive mice served as additional controls.

Airway Pressure Recording and BALF Preparation

Seventy-two hours after the last antigen challenge, airway responsiveness to acetylcholine was measured. The chest was opened and BALF was collected. Procedures are described in the “Methods” section of this article’s Online Repository.

Lung Histologic Analysis

Lung tissue was surgically removed and separated from the trachea after BALF collection (BALB/c mice) for assessment of mucous cell hyperplasia, as described in the “Methods” section of this article’s Online Repository.

RNA Extraction, Reverse Transcription and Quantitative Real-time Polymerase Chain Reaction

After BALF collection (BALB/c mice), the left upper and middle lobes were placed in reagent (TRIzol; Invitrogen, Carlsbad, California) and were frozen at -80°C . Samples were thawed at 4°C and homogenized, and RNA was extracted according to the manufacturer’s directions. Real-time reverse transcription quantitative polymerase chain reaction (qPCR) was performed as described in the “Methods” section of this article’s Online Repository.

Collagen Assay

Frozen nonlabeled right lungs (BALB/c mice) were homogenized in 1 mL of ice-cold phosphate-buffered saline with a protease inhibitor (Roche Diagnostics, Indianapolis, Indiana). The lung homogenates were gently shaken for 12 hours at 4°C and were centrifuged at $15,000g$ for 60 minutes to remove debris. The clear upper supernatant fluid was used for the Sircol assay (Biocolor Ltd, Carrickfergus, England) according to the manufacturer’s instructions, and optical density readings were performed at 540 nm. Results are expressed as micrograms of collagen per milligram of total protein, determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, California).

Spleen Cell Culture

Immediately after time-integrated change in peak airway pressure (APTI) measurements (BALB/c mice), pooled spleen cells from 4 or 5 mice were isolated and cultured with ovalbumin ($200\text{ }\mu\text{g/mL}$), concanavalin A (2.5 mg/mL , positive control), or medium alone for 72 hours as previously described.⁹ Levels of interleukin 4 (IL-4), IL-5, IL-13, and interferon gamma (IFN- γ) in spleen culture supernatants (in duplicate or triplicate) were determined by means of enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota).

Statistical Analysis

Statistical analyses were performed using the *t* test for comparisons between 2 groups. Pearson correlation coefficients were used to determine correlation values. A *P* < .05 was considered statistically significant. All statistical analyses were performed using a software program (Sig-

maPlot/Stat; Systat Software Inc, Point Richmond, California).

RESULTS

Selection of Individual Herbs for Preparation of ASHMI

We first determined the effect of the individual herbs found in MSSM-002 on APTI and BALF eosinophil percentage in antigen-sensitized and antigen-challenged young mice. No individual herb alone had the same suppressive effect as the MSSM-002 preparation on APTI and eosinophil lung infiltration. However, some individual herbs were more effective than were others in decreasing AHR, and some were more effective in attenuating airway inflammation. Other herbs had little or no effect on either variable (Table E2, Online Repository). To prepare ASHMI, we selected 3 herbs at specific percentages of dry weight: Ling-Zhi (*Ganoderma lucidum*, 62.5%); Ku-Shen (radix *Sophora flavescens*, 28.1%), which had higher percentage reductions of APTI and BALF eosinophilia; and Gan-Cao (radix *Glycyrrhiza uralensis*, 9.4%), which is included in many TCM formulas at low doses as a detoxifier.^{11,18} Next, APTI and BALF eosinophilia were determined in AKR mice that were not treated or that were treated with ASHMI. Treatment with ASHMI caused a comparable reduction in APTI and BALF eosinophils as MSSM-002 use (Table E2, Online Repository).

Reduction of AHR in ASHMI-Treated Aged Ovalbumin Mice

The APTI values were significantly higher in all nontreated antigen-sensitized and antigen-challenged mice compared with age-matched naive controls, indicating induction of AHR (Fig 2). Mean (SEM) APTIs were greater in 6-week-old nontreated ovalbumin mice ($725.9\text{ [}87.58\text{] cm H}_2\text{O/sec}$) than in each group of aged nontreated ovalbumin mice (6 months old: $330.0\text{ [}36.46\text{] cm H}_2\text{O/s}$, *P* = .009; 12 months old: $356.1\text{ [}50.47\text{] cm H}_2\text{O/s}$, *P* = .009; and 18 months old: $318.4\text{ [}33.1\text{] cm H}_2\text{O/s}$, *P* = .004). There were no significant differences in APTI between naive groups. Treatment with ASHMI significantly reduced the APTI in all aged and young ovalbumin mice compared with age-matched nontreated ovalbumin mice. The effect was most pronounced in 6-week-old mice.

ASHMI Treatment Reduces Antigen-Induced Pulmonary Inflammation

To determine whether AHR suppression was associated with reduced airway inflammation, we measured total BALF cell numbers and percentages of eosinophils immediately after APTI. All the age groups of nontreated ovalbumin mice developed significantly (*P* < .001) elevated mean total BALF leukocyte numbers compared with age-matched naive mice (Fig 3A-D). Mean (SEM) BALF leukocyte numbers were increased significantly in all aged nontreated ovalbumin mice (6 months old: $110.16\text{ [}6.013\text{]} \times 10^4$, *P* < .01; 12 months old: $113.19\text{ [}3.46\text{]} \times 10^4$, *P* < .001; and 18 months old: $116.32\text{ [}5.34\text{]} \times 10^4$, *P* < .01) compared with 6-week-old nontreated

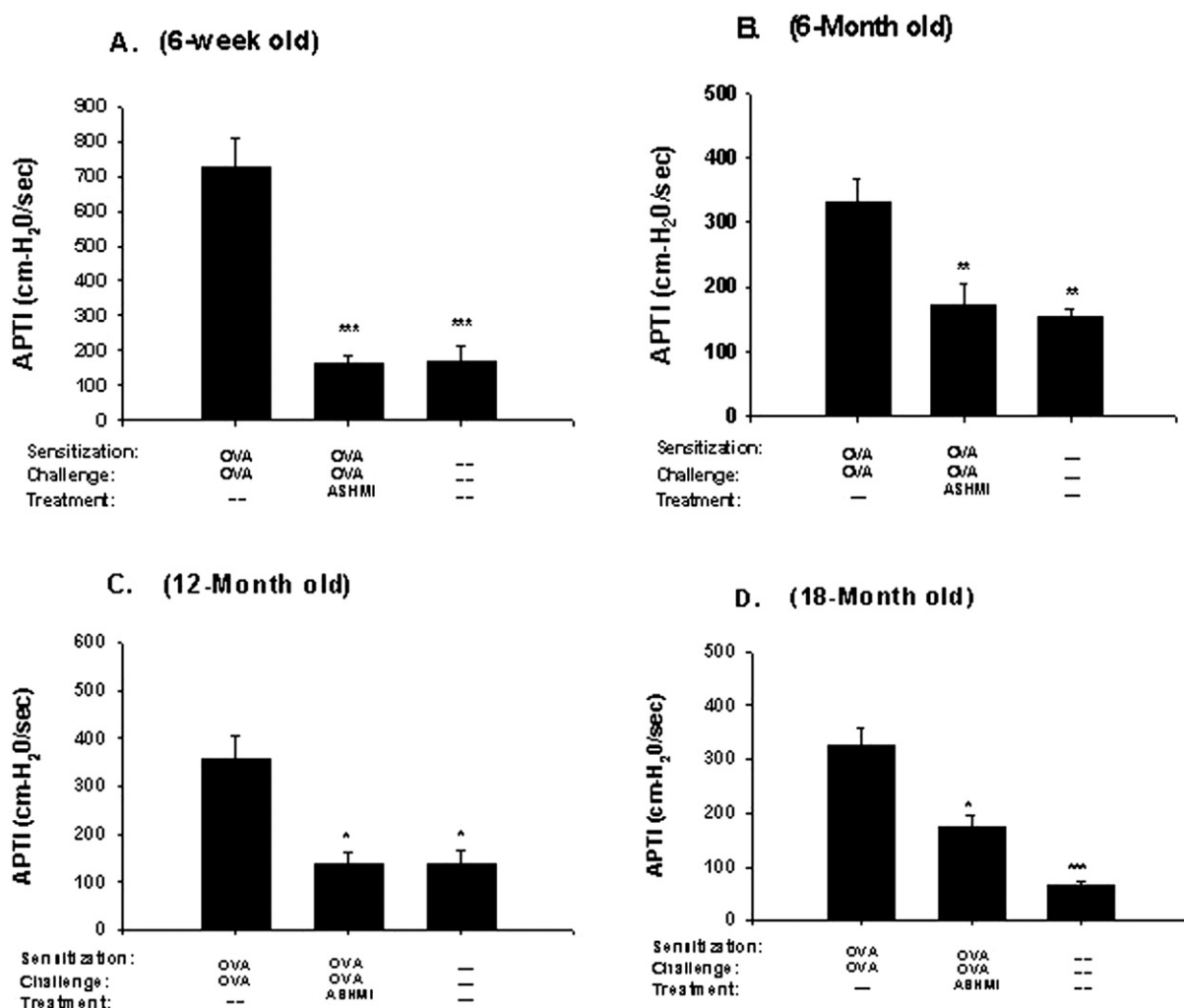


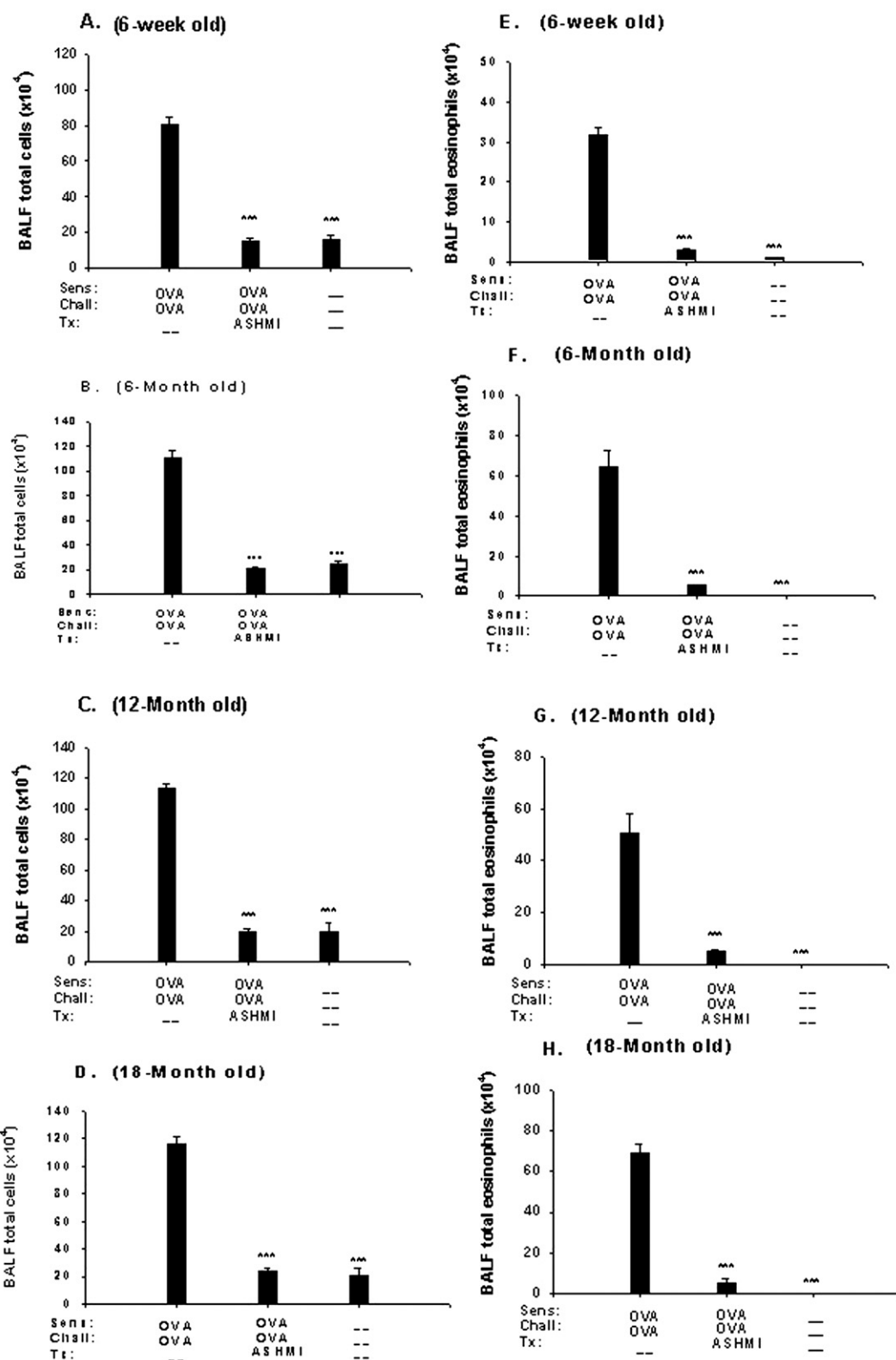
Figure 2. Effect of antiasthma simplified herbal medicine intervention (ASHMI) on antigen-induced airway hyperresponsiveness in mice aged 6 weeks (A) 6 months (B), 12 months (C), and 18 months (D). Three days after the last antigen challenge, the airway reactivity of mice in each group was determined by measuring the airway pressure change after acetylcholine challenge. The time-integrated changes in peak airway pressure (APTI) were calculated. Data are given as mean (SEM) for each group. * $P < .05$, † $P < .01$, ‡ $P < .001$ compared with age-matched nontreated ovalbumin mice.

ovalbumin mice ($80.24 [4.4] \times 10^4$). Use of ASHMI significantly decreased the leukocyte count in all ovalbumin mice age groups compared with age-matched nontreated ovalbumin mice (Fig 3A-D). There were no significant differences in total BALF leukocyte counts between ASHMI-treated ovalbumin mice compared with age-matched naive mice for

all ages. There were no significant differences in total cell counts among all ages of naive groups.

Mean BALF eosinophil numbers were significantly elevated in all nontreated ovalbumin mice compared with their age-matched naive controls ($P < .001$) (Fig 3E-H). Mean (SEM) eosinophil numbers were significantly ele-

Figure 3. Effect of antiasthma simplified herbal medicine intervention (ASHMI) on bronchoalveolar lavage fluid (BALF) total cell counts (A-D) and eosinophil numbers (E-H). After airway response measurement, mice were humanely killed, and the lungs were lavaged. Total leukocytes were counted, and BALF was cytocentrifuged on slides, fixed, and stained for leukocyte differential. A-D, Mean BALF total cell counts in nontreated ovalbumin (OVA) mice, ASHMI-treated OVA mice, and naive mice aged 6 weeks (A), 6 months (B), 12 months (C), and 18 months (D). E-H, Mean BALF absolute eosinophil counts in the same mouse groups aged 6 weeks (E), 6 months (F), 12 months (G), and 18 months (H). Error bars represent SEM. * $P < .05$, ** $P < .01$, *** $P < .001$ compared with age-matched nontreated OVA mice.



vated in the 6-, 12-, and 18-month-old nontreated ovalbumin mice ($64.32 [8.66] \times 10^4$, $P < .001$; $50.63 [7.2] \times 10^4$, $P < .01$; and $69.38 [4.8] \times 10^4$, $P < .001$, respectively) compared with the nontreated 6-week-old ovalbumin mice ($31.57 [1.86] \times 10^4$). Use of ASHMI in all the ovalbumin mice significantly decreased BALF eosinophil numbers compared with those in age-matched nontreated ovalbumin mice (Fig 3E-H).

To correlate BALF total cell counts with lung tissue inflammation, lung sections collected 72 hours after the final ovalbumin challenge were stained with hematoxylin-eosin. All nontreated ovalbumin mice demonstrated peribronchial and perivascular inflammation, not seen in naive control mice. Mean (SEM) perivascular and peribronchial inflammation grades, respectively, were greatest in aged ovalbumin mice (6 months old: $2.12 [0.07]$, $P < .001$, and $1.89 [0.16]$, $P < .01$; 12 months old: $1.67 [0.11]$, $P < .01$, and $1.62 [0.18]$, $P = .10$; and 18 months old: $1.98 [0.19]$, $P < .001$, and $1.87 [0.19]$, $P < .01$) compared with 6-week-old ovalbumin mice ($0.18 [0.09]$ and $1.43 [0.09]$). Treatment with ASHMI of ovalbumin mice significantly decreased perivascular lung tissue inflammation in the 6-week-old, 6-month-old, and 18-month-old ovalbumin mice (Fig 4). Perivascular inflammation was decreased with ASHMI in 12-month-old ovalbumin mice, but not significantly (Fig 4). Treatment with ASHMI significantly decreased peribronchial inflammation in all age

groups of ovalbumin mice, but not to the extent in 18-month-old ovalbumin mice.

Mucous Cell Metaplasia Was Suppressed by ASHMI Treatment

We previously reported that TCM treatment of young antigen-sensitized and antigen-challenged AKR/J mice decreases mucous cell metaplasia, as determined by periodic acid-Schiff (PAS) staining.⁹ In the present study, numbers of positively staining PAS epithelial cells in bronchioles were significantly increased in all nontreated ovalbumin mice compared with naive mice, in which there was no PAS epithelial cell staining. There was significantly increased mean (SEM) PAS staining in the airways of 12-month-old nontreated ovalbumin mice ($82.05\% [1.29\%]$) compared with 6-week-old nontreated ovalbumin mice ($61.14\% [3.40\%]$) ($P < .05$). There were no significant differences in PAS staining among other aged mice (6 months old: $62.64\% [3.4\%]$ ($P = .87$) and 18 months old: $71.33 \pm 5.18\% [5.18\%]$) ($P = .32$) and 6-week-old nontreated ovalbumin mice. Treatment with ASHMI significantly decreased mucous cell numbers in all age groups (Fig 5A). We performed real-time PCR analysis of expression of the major airway mucus-producing gene *MUC5AC*¹⁹ in lung tissue. Results are expressed as the fold change in messenger RNA (mRNA) copy number of *MUC5AC* in ovalbumin mice compared with age-matched naive controls. *MUC5AC* mRNA expression was significantly increased in the lungs of 12- and 18-month-old nontreated ovalbumin mice ($P = .003$ and $P = .02$, respectively) compared with young nontreated ovalbumin mice. Treatment with ASHMI significantly decreased PAS mRNA expression in all age groups of ovalbumin mice (Fig 5B). Figure 5C-H shows representative histologic analyses from the different groups: naive mice, nontreated ovalbumin mice, and ASHMI-treated ovalbumin mice. The PAS staining was increased in lungs from mice of both ages in the nontreated ovalbumin groups, which was prevented by ASHMI.

ASHMI Treatment Reduced Antigen Specific IgE Levels in OVA Mice of All Ages

To assess the degree of sensitization in ovalbumin mice and to determine whether ASHMI alters sensitization, we measured ovalbumin specific serum IgE. All the mice developed significantly elevated levels of serum specific IgE to ovalbumin in response to ovalbumin sensitization and challenge (Fig 6). Aged ovalbumin mice exhibited significantly higher levels of ovalbumin specific serum IgE than did 6-week-old ovalbumin mice (Fig 6). Ovalbumin specific IgE was undetectable in all naive mice. Use of ASHMI significantly decreased ovalbumin specific IgE levels in all ages of ovalbumin mice (Fig 6).

ASHMI Treatment Decreased Lung Collagen Formation

Several murine models of asthma and studies in patients with asthma have demonstrated increased airway collagen depo-

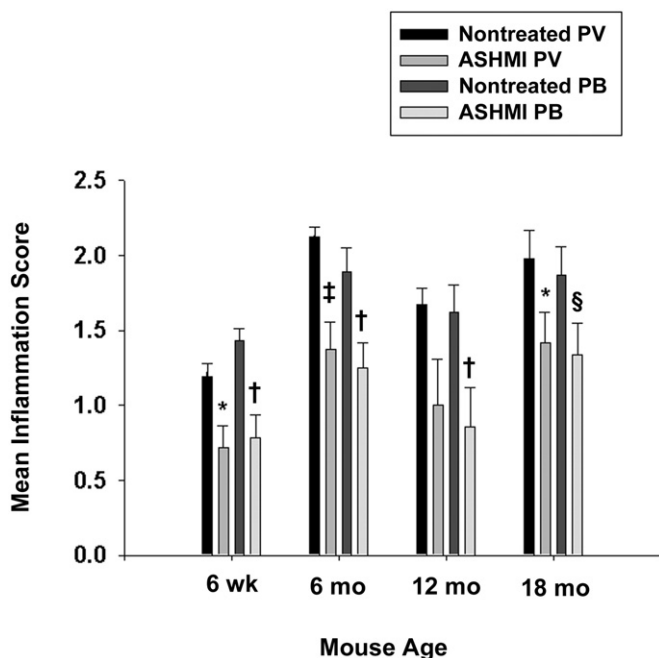


Figure 4. Effect of antiasthma simplified herbal medicine intervention (ASHMI) on pulmonary inflammation. Perivascular (PV) and peribronchial (PB) inflammation of lung tissues stained with hematoxylin-eosin was graded as described in the "Methods" section of the article. Error bars represent SEM. * $P < 0.05$ (PV), ‡ $P < 0.01$ (PV), § $P < 0.05$ (PB), † $P < 0.01$ (PB) compared with age-matched nontreated ovalbumin mice.

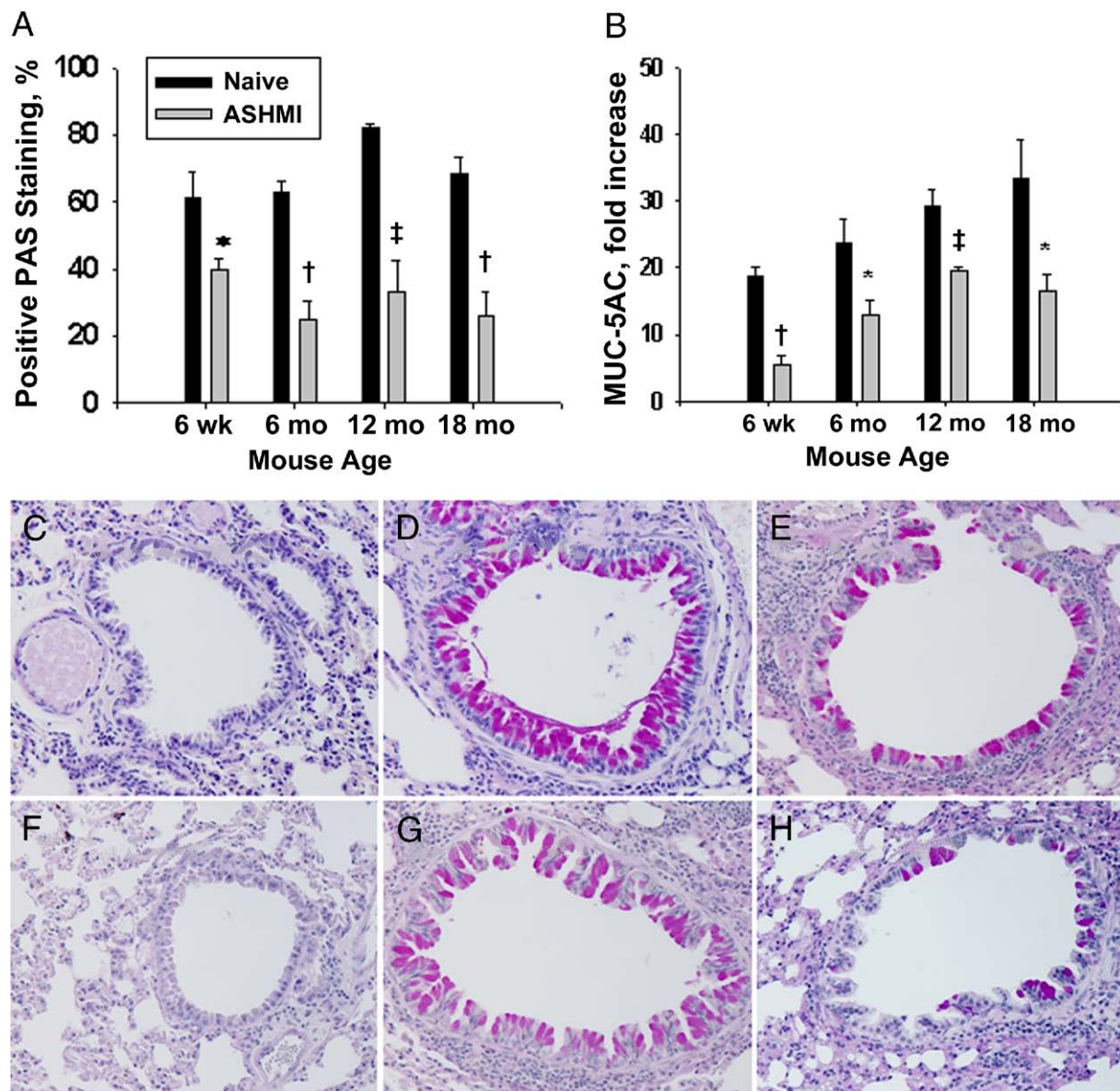


Figure 5. Goblet cell metaplasia. A, Mean left lower lung periodic acid–Schiff (PAS) grading for ASHMI ovalbumin mice and age-matched naive mice. B, Quantitative polymerase chain reaction for *MUC5AC* expressed as mean fold increase in copy number. Error bars represent SEM. * $P < .05$, † $P < .001$, ‡ $P < .01$ compared with age-matched nontreated OVA mice. C–H, The PAS-positive (deep purple) staining in 6-week-old naive mice (C), 6-week-old naive mice (D), 6-week-old antiasthma simplified herbal medicine intervention (ASHMI) ovalbumin mice (E), 18-month-old naive mice (F), 18-month-old untreated OVA mice (G), and 18-month-old ASHMI ovalbumin mice (H).

sition.^{20,21} We determined, using a colormetric assay, the relative amounts of collagen in the experimental groups. Collagen synthesis was increased in all nontreated ovalbumin mice compared with naive age-matched mice but significantly only in 6-week-old ($P = .009$), 6-month-old ($P < .05$), and 18-month-old ($P < .001$) mice (Fig 7). Use of ASHMI reduced collagen synthesis in all the ovalbumin mice; however, this reduction reached statistical significance only in 6-week-old and 18-month-old mice (Fig 7).

Lung and Systemic Cytokine Production

The Center for Chinese Herbal Therapy for Allergy and Asthma, Pediatric Allergy and Immunology, Mount Sinai School of Medicine previously demonstrated in young ovalbumin mice that ASHMI reduction of lung inflammation is due, at least in part, to reduced lung and systemic T_H2 cytokine production and increased $IFN-\gamma$ production.^{22a} To determine whether ASHMI treatment of aged ovalbumin

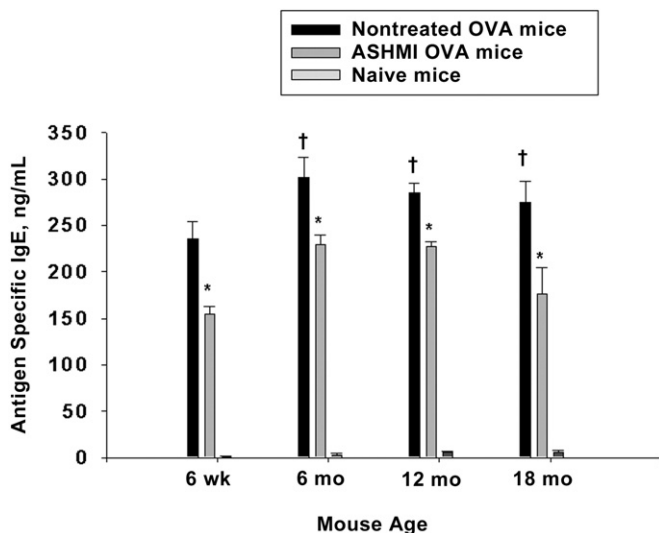


Figure 6. Mean serum ovalbumin (OVA) specific IgE levels determined by means of enzyme-linked immunosorbent assay from sera collected 72 hours after the final OVA challenge. Error bars represent SEM. * $P < .05$ compared with age-matched nontreated OVA mice. † $P < .05$ compared with 6-week-old nontreated OVA mice.

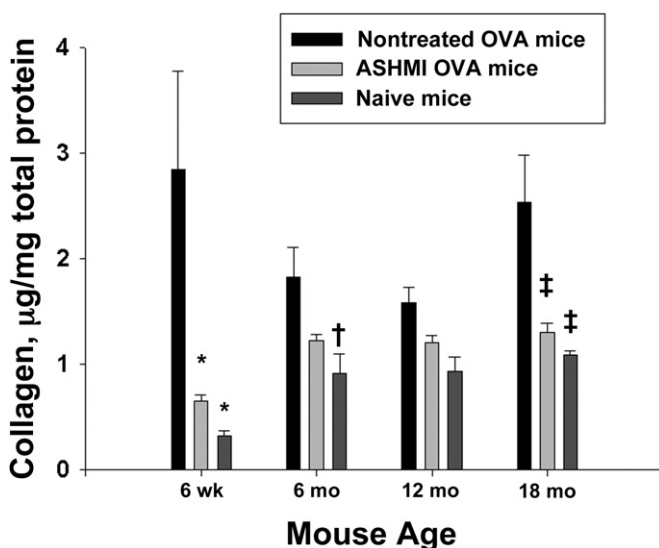


Figure 7. Mean collagen deposition in the lungs measured quantitatively and normalized to total protein in the lung for nontreated and antiasthma simplified herbal medicine intervention (ASHMI)-treated ovalbumin (OVA) mice and age-matched naive mice. Error bars represent SEM. † $P < .05$, * $P < .01$, ‡ $P < .001$ compared with age-matched nontreated OVA mice.

mice altered cytokine expression, we measured IL-4, IL-5, IL-13, and IFN- γ mRNA expression in lung tissue using qPCR. (Results are expressed in fold increase in mRNA copy number from ovalbumin mice compared with mRNA copy number from age-matched naive mice.) Treatment with ASHMI significantly decreased IL-4, IL-5, and IL-13 expres-

sion in 6-week-old ovalbumin mice compared with nontreated age-matched ovalbumin mice (Fig 8A-C). Use of ASHMI also significantly reduced the expression of IL-4 in 12-month-old ($P < .01$), IL-5 in all ages ($P < .01$), and IL-13 in 6-month-old ($P < .01$) ovalbumin mice (Fig 8A-C). Expression of IFN- γ was increased in all ages of ovalbumin mice treated with ASHMI, but it reached statistical significance only in 6-month-old ovalbumin mice (Fig 8D).

To examine systemic cytokine protein levels, we stimulated pooled spleen cells with ovalbumin. Levels of cytokines IL-4, IL-5, IL-13, and IFN- γ were elevated in all nontreated ovalbumin mice compared with age-matched naive controls (Fig 8E-H). Treatment with ASHMI had the greatest effect on IL-4 protein expression in spleen cell culture. Levels of IL-4 were decreased approximately 85%, 65%, 60%, and 35% in 6-week-old and 6-, 12-, and 18-month-old ovalbumin mice, respectively (Fig 8E). The IL-5 level was reduced approximately 40% to 50% in all full-dose ovalbumin mice compared with cultures from nontreated ovalbumin mice (Fig 8F). Use of ASHMI reduced IL-13 levels in spleen cell cultures approximately 64% in 6-week-old ovalbumin mice and approximately 30% in 6- and 18-month-old ovalbumin mice (Fig 8G). The IL-13 spleen cell levels were decreased by less than 10% in 12-month-old ovalbumin mice treated with ASHMI compared with nontreated ovalbumin controls (Fig 8G). Spleen cell culture IFN- γ levels were increased in nontreated aged ovalbumin mice compared with in 6-week-old nontreated ovalbumin mice (Fig 8H). Similar to previous data, treatment with ASHMI increased IFN- γ expression by spleen cells in 6-week-old ovalbumin mice, and in this study, we noted that it also increased it in aged ovalbumin mice (Fig 8H). Concanavalin A stimulation (positive control) of splenocyte cultures produced increased expression of all the cytokines (data not shown), and culture in media alone (negative control) produced minimal cytokine expression (data not shown).

DISCUSSION

Asthma treatment of older patients is not widely investigated. Asthma is a heterogeneous disease that typically presents in the pediatric and young adult populations; however, it can affect individuals of all ages. Corticosteroids are the mainstay of treatment for persistent disease.²² When administered systemically or in high doses via inhalation, corticosteroids may produce several adverse effects. These effects may be particularly detrimental for older patients and include accelerated bone loss and osteoporosis, an increased risk of fracture, and glucose intolerance.^{23–25} In addition, the inhalation technique may be more challenging for older patients.^{26,27} Therefore, the development of corticosteroid-sparing medications, in a form that is easy to administer, for the prevention or treatment of asthma in older patients is critical, especially as the population ages.

We originally developed a novel formula for asthma, MSSM-002. This compound was an extract of 14 herbs that nearly eliminated AHR, significantly reduced BALF total cell

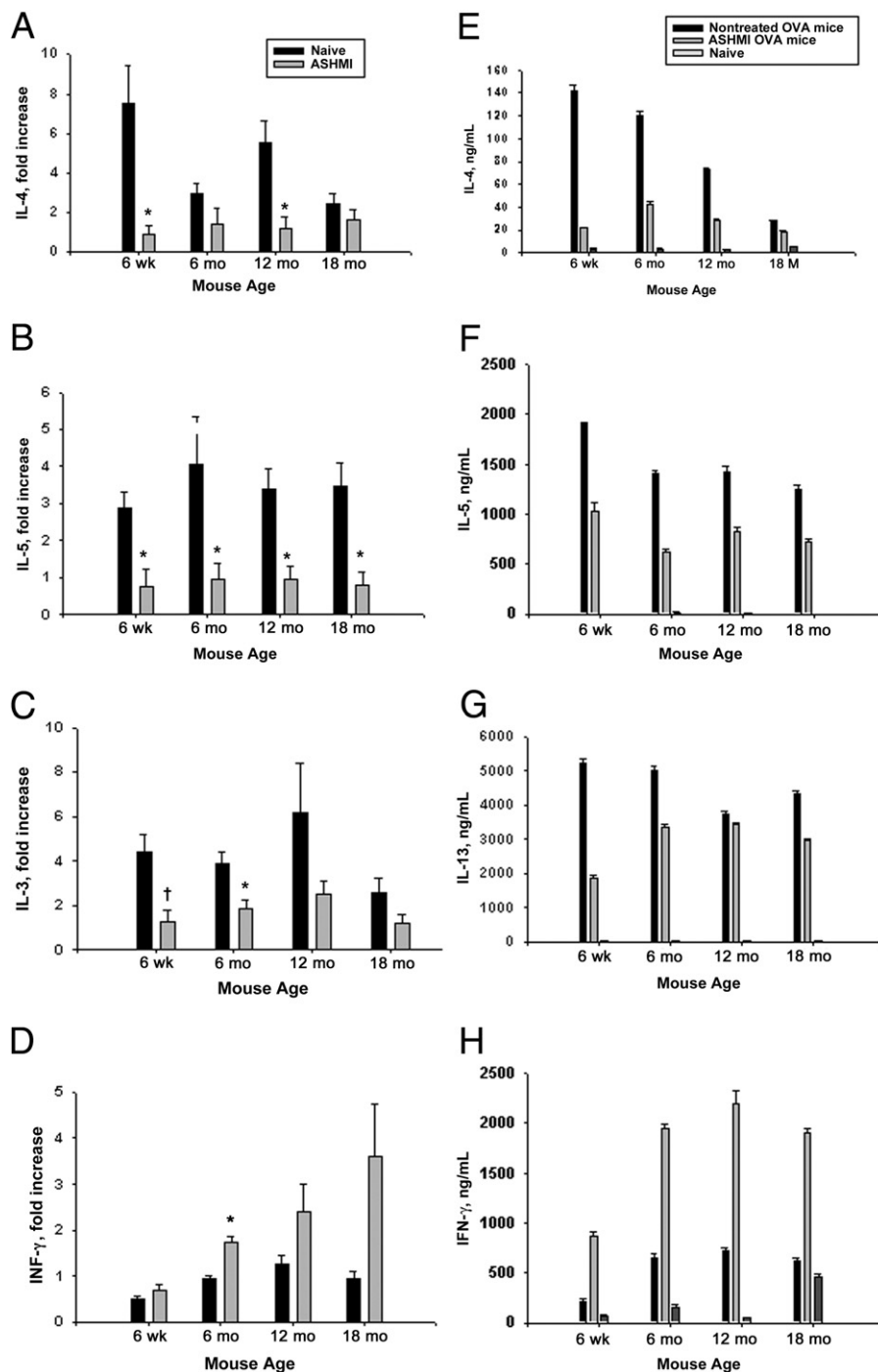


Figure 8. Pulmonary tissue and spleen cell culture cytokines. A-D, Quantitative polymerase chain reaction for interleukin 4 (IL-4) (A), IL-5 (B), IL-13 (C), and interferon gamma (INF- γ) (D) from lung tissue from young and aged mice. Results are expressed as mean fold increase in copy number compared with age-matched naive mice. E-H, Pooled spleen cells from 4 mice per group were isolated and cultured in complete medium in the presence of ovalbumin, concanavalin A (data not shown), or medium alone (data not shown). Supernatants were collected after 72 hours of culture. Levels of IL-4 (E), IL-5 (F), IL-13 (G), and INF- γ (H) were determined by means of enzyme-linked immunosorbent assay. Results are mean values for duplicate or triplicate assays. Error bars represent SEM. * $P < .01$, † $P < .05$, compared with age-matched nontreated ovalbumin mice.

numbers and eosinophil percentages and antigen specific IgE levels, and inhibited mucous cell hyperplasia of the lungs comparable with corticosteroid treatment in a murine model of allergic asthma using 6-week-old mice.⁹ To simplify this formula, we tested the effects of individual herbs in MSSM-002 on the reduction of AHR and BALF eosinophilia and found that none alone had effects comparable with MSSM-002. However, a combination of 3 herbs (Ling-Zhi, Ku-Shen, and Gan-Cao), named ASHMI, decreased AHR and BALF eosinophilia similar to MSSM-002.

ASHMI has an excellent safety profile; administration of 12 times the dose did not produce any abnormalities in the liver, kidneys, and complete blood cell counts of mice (Wen et al, unpublished data). In a study²⁸ conducted in China, asthmatic adults (mean age, 44.6 years) treated with ASHMI exhibited improved asthma symptom scores and forced expiratory volume in 1 second and decreased serum IgE levels and peripheral blood eosinophil numbers similar to prednisone. A subsequent dose-escalation phase 1 trial¹² in the United States in patients with asthma (aged 18-40 years) using ASHMI demonstrated an excellent safety profile. These trials, however, did not address the use of ASHMI in older asthmatic patients.

In the present study, we demonstrated that treatment of young and aged mice with 10 mg of ASHM daily before and during antigen sensitization and challenge prevented several features of allergic airway inflammation (AHR, increased total BALF cell counts and eosinophil percentages, mucous cell metaplasia, and lung tissue inflammation). In addition, treatment with ASHMI decreased the level of antigen specific IgE. We previously characterized a murine model of allergic asthma in aged mice.⁶ Antigen sensitization and airway challenge of aged BALB/c mice increased airway inflammation (BALF total cell number and eosinophil percentage, hematoxylin-eosin staining, and mucous cell metaplasia) compared with similarly treated 6-week-old mice. The aged antigen-sensitized/challenged mice also developed AHR after acetylcholine challenge. However, despite greater airway inflammation in aged mice compared with 6-week-old mice, AHR was significantly higher in the latter. The reasons for the discord between increased airway inflammation in aged antigen-treated mice and AHR are not entirely clear but may include alteration in eosinophil²⁹ or smooth airway function with age. Use of ASHMI effectively prevented AHR in all ages of ovalbumin mice. There are several potential mechanisms by which ASHMI reduces AHR. It decreased BALF total cell numbers to the level of age-matched naive mice and significantly reduced BALF eosinophil numbers in all age groups. Furthermore, ASHMI diminished the inflammation score on airway histologic analysis. However, the potential differences in inflammatory cell function with increasing age need to be considered. Furthermore, although a causal relationship has been proposed between airway inflammation, specifically, eosinophilia, and AHR in asthma, this hypothesis is controversial.³⁰⁻³²

Treatment with ASHMI also prevented several other pathologic features of the asthmatic airway that may increase AHR. Use of ASHMI reduced airway mucous cell metaplasia, measured by means of PAS staining, and expression of the major mucin gene *MUC5AC*. The effect of mucus hypersecretion on AHR is not clearly established^{33,34} and may differ in an aged population. In addition, ASHMI treatment decreased lung collagen deposition to near naive levels. Increased airway collagen deposition, a component of airway remodeling, may affect AHR.³⁵

Administration of ASHMI lowers serum antigen specific IgE levels. IgE mediates the allergic response by triggering mast cell and basophil degranulation after antigen exposure. Therefore, reduction in antigen specific IgE levels may attenuate AHR. However, a direct relationship between AHR and IgE is not straightforward. The use of anti-IgE for asthma has greater effects on the reduction of asthma symptoms and rescue medication/corticosteroid use than on airway function.³⁶

The mechanisms for the anti-inflammatory properties of ASHMI are not completely understood but involve T_H2 suppression. We previously demonstrated that the original 14-herb TCM preparation MSSM-002 and the present simplified formula ASHMI suppress T_H2 responses^{9,16} and the T_H2 transcription factor GATA-3.³⁷ Corticosteroid treatment also decreases the T_H2 response. However, the TCM formulation, unlike corticosteroids, increases IFN- γ expression, which may play a role in its protective airway effects.^{9,16} In this study, ASHMI suppressed mRNA expression in the lung tissue of the T_H2 cytokines IL-4, IL-5, and IL-13 and its systemic level from spleen cell culture. Similar to previous data, ASHMI treatment increased IFN- γ expression, which may attenuate AHR.

We administered ASHMI to mice before antigen exposure, suggesting that it may prevent asthma in aged people. We recently showed that ASHMI reversed the established allergic airway response in young adult mice and that this effect is long lasting (Srivastava et al manuscript submitted). Investigation of ASHMI in established asthma in aged antigen-sensitized mice is under way. In conclusion, ASHMI treatment of young and aged murine models of allergic asthma inhibited the development of several key features of antigen-induced airway inflammation, including antigen-induced AHR, mucous cell metaplasia, increased lung inflammation, and BALF total cell number and eosinophilia.

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SUPPLEMENTARY DATA

Additional photos and tables accompany the on-line version of this article (available at <http://www.annallergy.org>).

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Airway Pressure Recording

Mice were anesthetized intraperitoneally with pentobarbital (80 mg/kg), and a 16-gauge cannula was placed in the trachea. Mice were ventilated with a constant tidal volume of 0.2 mL at 120 breaths/min. Decamethonium bromide (25 mg/kg) was administered into the inferior vena cava to eliminate muscle movements. Airway pressure was measured using a pressure transducer via a port in the tracheal cannula. A stable baseline was recorded for at least 2 minutes, acetylcholine (50 μ g/kg) was injected into the inferior vena cava, and the recording continued for at least 4 minutes. Airway responsiveness was defined as the time-integrated change in peak airway pressure.¹

Bronchoalveolar Lavage Fluid Preparation and Cell Differential Counts

The left mainstem bronchus was occluded using forceps, the right lung was lavaged with 0.5 mL of cold Hanks balanced salt solution (without calcium or magnesium), and the bronchoalveolar lavage fluid was collected into chilled tubes. The fluid was centrifuged, and the supernatant was transferred and stored at -80° for cytokine measurement by means of enzyme-linked immunosorbent assay. The cell pellet was resuspended in Hanks balanced salt solution, and slides were prepared by means of centrifugation of samples at 700 rpm for 7 minutes using a cytospin. Total cell numbers were determined using a hemocytometer. The slides were then stained with Diff-Quick (Dade Diagnostics of PR, Aguada, Puerto Rico), and cell differential counts were obtained by counting at least 500 cells per slide by means of light microscopy.

Lung Histologic Analysis

The left lower lobe was fixed in neutral-buffered formaldehyde, and 5- μ m paraffin sections were stained with periodic acid-Schiff (PAS) for evaluation of goblet cells. Randomly chosen lung sections were examined in a blinded manner without preview. Mucus expression was calculated, adapting a previously published method,² by determining the percentage of PAS-positive epithelial cells (number of PAS-positive cells divided by the total epithelial cell number) in at least 6 randomly selected bronchioles per animal. Results are expressed as the mean percentage of PAS-positive cells per bronchiole for each group of mice. In addition, lung sections were stained with hematoxylin-eosin. Perivascular and peribronchial inflammation were graded using a standard scoring method (0=normal, 1=mild, 2=intermediate, and 3=severe).³

Quantitative Real-time Polymerase Chain Reaction

Real-time reverse transcription quantitative polymerase chain reaction was performed using a previously published SYBR green protocol using ABI7900 HT (Applied Biosystems, Foster City, California).⁴ Each transcript in each sample was assayed 3 times, and the median threshold cycle was used to calculate the fold change values and control changes for each gene. Three housekeeping genes were used for global normalization in each experiment (β -actin, RPS11, and α -tubulin). Data validity by modeling of reaction efficiency and analysis of measurement precision was described previously.⁵ Primer sequences for these reactions have been previously described.⁶

Table E1. Individual Herbs in MSSM-002 and Doses Used^a

Material Medica (Ping Yin)	Pharmaceutical name	Human daily dosage of raw herb (g)	Human daily dosage of extract (g)	Mouse daily dosage of extract (mg)
Bai Shao (BS)	Radix Paeoniae Lactiflorae	9	1.20	6.0
Da Zao (DZ)	Fructus Zizyphi Jujubae	6	2.22	8.5
Dang Gui (DG)	Radix Angelicae sinensis	9	4.62	17.9
Gan Cao (GC)	Radix Glycyrrhizae Uralensis	3	0.56	2.2
Ge Gen (GG)	Radix Puerariae	9	1.89	7.3
Huang Qin (HQ)	Radix Scutellariae baicalensis	9	2.05	7.9
Jie Geng (JG)	Radix Platycodi grandiflori	6	3.16	12.2
Ku Shen (KS)	Radix Sophorae Flavescentis	9	1.38	5.4
Ling Zhi (LZ)	Ganoderma	20	4.2	4.2
Sheng Jiang (SJ)	Rhizoma Zingiberis	6	0.39	1.5
Ting Li Zi (TLZ)	Semen Descurainiae sealepidii	9	0.82	3.2
Xing Ren (XR)	Semen Pruni armeniaca	9	1.03	3.9
Zi Su	Folium Perillae Frutescentis	9	4.63	9.0
Zhen Zhu Mu (ZZM)	Concha Margaritifera	15	0.25	0.5
ASHMI		32	3.6	9.5
MSSM-002		128	19.4	73.4

^a MSSM-002 is composed of 14 herbs, which are listed in Ping Yin and by pharmaceutical name. The doses of formulas for MSSM-002 and ASHMI are also listed.

Table E2. Effects of Individual Herbs of MSSM-002 on AHR and Eosinophilic Lung Inflammation

Herbs	% of APTI reduction Vs Sham*	% of Eosinophil reduction Vs Sham*
DZ	−43.97	−58.69
ZZM	−30.65	23.07
GC	−13.5%	17.3
DG	−10.10	58.26
SZ	0.90	−1.12
XR	25.69	33.27
SJ	19.57	31.17
BG	20.62	51
LZ	26.00	63.76
HQ	30.6	22.12
TLZ	31.55	4.27
JG	39.95	30.88
GG	43.43	41.94
KS	52.12	31.12
MSSM-002	58.14	47.65
ASHMI	60	63

Abbreviations: AHR, airway hyperresponsiveness; APTI, time-integrated change in peak airway pressure; BG, Bai-Sao and Gan-Cao (commonly used as a pair in traditional Chinese medicine).

Antigen-sensitized and antigen-challenged mice were treated with individual herbs of the MSSM-002 formula or with the entire formula. Percentage reduction in APTI (a measure of AHR) and bronchoalveolar lavage fluid eosinophils were calculated based on the values of nontreated antigen-sensitized and antigen-challenged mice (conalbumin mice). A minimum of 4 mice were included in each group. Minus indicates an increase in APTI or eosinophil percentage vs nontreated conalbumin mice. Complete herbal names are listed in Table E1.

* APTI values in treated conalbumin mice were not normalized by subtracting the APTI values in naive mice.

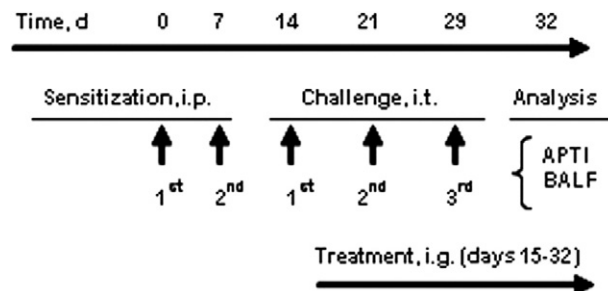


Figure E1. Experimental protocol. AKR mice (6 weeks old) were sensitized intraperitoneally (i.p) with concanavalin A (200 μ g per mouse) twice at weekly intervals and then were challenged intratracheally (i.t.) on days 14, 21, and 29 with concanavalin A (100 μ g per mouse). Twenty-four hours after the first challenge, mice were treated with 1 of the 14 herbs in MSSM-002 or with complete MSSM-002, all delivered at a dose of 1 mL per mouse intragastrically (i.g.) twice daily for 18 days. Nontreated antigen-sensitized and antigen-challenged mice and naive mice served as controls. Seventy-two hours after the final ovalbumin challenge, mice were humanely killed for analysis. APTI indicates time-integrated change in peak airway pressure; and BALF, bronchoalveolar lavage fluid.

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