

Effect of Antiasthma Simplified Herbal Medicine Intervention on neutrophil predominant airway inflammation in a ragweed sensitized murine asthma model

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ABSTRACT

Background: Neutrophil-predominant asthma is less responsive to steroids and associated with poorer disease control. The effects of Antiasthma Simplified Herbal Medicine Intervention (ASHMI), a traditional Chinese medicine formula reported to be efficacious in asthmatic patients and murine asthma models, on neutrophil predominant asthma are unknown.

Objective: To determine the effects of standard ASHMI and refined formula ASHMI (ASHMI^{II}) in a neutrophil-predominant murine model of ragweed (RW) asthma and explore underlying mechanisms.

Methods: BALB/c mice were systemically sensitized, intranasally challenged with RW extract, and orally treated with ASHMI, ASHMI^{II}, or vehicle (water). In a separate experiment, some RW sensitized mice were treated with dexamethasone before challenge. After RW challenge, airway hyperreactivity (AHR), total and differential bronchoalveolar lavage fluid leukocyte counts, lung histologic features, and bronchoalveolar lavage fluid cytokine and chemokine levels were assessed. RW stimulation of the murine macrophage cell line RAW264.7 was used to determine effects of ASHMI active compound ganoderic acid C1 (GAC1) on tumor necrosis factor α (TNF- α) production and regulation of phosphorylated I κ B and histone deacetylase 2 (HDAC2) levels.

Results: ASHMI and ASHMI^{II} markedly reduced AHR, mucous production, neutrophilic inflammation, and TNF- α , interleukin 8, and interleukin 17 levels and decreased eosinophilic inflammation and T_H2 responses in vivo ($P < .01$ –.001 for all). GAC1 inhibited TNF- α production in RW-stimulated RAW264.7 cells in association with suppression of phosphorylated I κ B and increased HDAC2 expression. Dexamethasone failed to reduce AHR and neutrophilic inflammation.

Conclusion: ASHMI treatment was efficacious in a murine model of neutrophil-predominant asthma via modulation of innate chemokines, T_H2 responses, nuclear factor- κ B, and HDAC2. ASHMI, and/or its constituent GAC1, may be a valuable option for treating neutrophil-predominant asthma.

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Introduction

Asthma is a heterogeneous airway disease that includes eosinophil-predominant, neutrophil-predominant, and mixed

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eosinophil and neutrophil airway inflammation.¹ Increasing evidence indicates that severe and steroid refractory asthma are often characterized by mixed granulocytic airway inflammation.^{2–5} The presence of neutrophils or neutrophil predominance in sputum of asthmatic patients has been reported to be associated with poor lung function and disease outcomes,⁴ as well as sudden asthma fatalities.^{6–8} Currently, there is no effective therapy for neutrophil-predominant airway inflammation.

We previously found that the Antiasthma Simplified Herbal Medicine Intervention (ASHMI) derived from traditional Chinese medicine was efficacious as a stand-alone therapy in adults with moderate-to-severe allergic asthma. Treatment benefits were associated with increased T_H1 responses and reduced T_H2 responses.⁹ Further, ASHMI significantly reduced airway eosinophilic inflammation and airway hyperreactivity (AHR) in an ovalbumin sensitized murine asthma model in which eosinophilic inflammation was

dominant.¹⁰ Protective effects in the murine model were attributable to ASHMI-induced down-regulation of T_H2 immune responses and increased interferon γ (IFN- γ) but not transforming growth factor β (TGF- β) production.¹¹ Whether ASHMI is also effective in a neutrophil-predominant asthma is unknown.

Neutrophilic inflammation is driven by tumor necrosis factor α (TNF- α), interleukin (IL) 8, and IL-17.^{12,13} Up-regulation of these mediators has been found to require activation of the nuclear factor- κ B (NF- κ B) signaling pathway.^{14–16} Nuclear translocation of NF- κ B requires phosphorylation of I κ B- α and subsequent degradation of I κ B- α by the 26S proteasome. Thus, increased levels of the phosphorylated I κ B- α (pI κ B) protein indicates heightened NF- κ B activation. In contrast, histone deacetylase 2 (HDAC2) suppresses NF- κ B activation by participation in a corepressor complex along with CBP/P300 and RelA, and loss of HDAC2 has been reported to cause disruption of the corepressor complex, leading to sustained activation of NF- κ B.^{17–19} Decreased HDAC2 expression has been found in alveolar macrophages and peripheral blood mononuclear cells from asthma patients.²⁰

We hypothesized that ASHMI and some of its active compounds may suppress neutrophil predominant airway inflammation in vivo. Further, we hypothesized that TNF- α , IL-8, and IL-17, critical to underlying mechanisms of neutrophilic inflammation, would be regulated by administration of ASHMI. Our approach was to generate a murine model of ragweed asthma in which intranasal RW challenge of RW sensitized mice resulted in predominantly neutrophilic bronchoalveolar lavage (BAL) fluid infiltrate in addition to AHR and mucous hyperproduction. The effects on AHR, cytokine, and chemokine production of ASHMI therapy and a refined ASHMI (ASHMI^{II}) treatment group were studied. The refined formula was prepared by combining individual ASHMI herb extracts (extracts of *Ganoderma lucidum*, *Sophora flavescens*, and *Glycyrrhiza uralensis*). Finally, to explore mechanisms of neutrophilic suppression, we studied in vitro the effects of ganoderic acid C1 (GAC1), an active compound isolated from the ASHMI constituent *G lucidum*, in a murine macrophage cell line on TNF- α production, phosphorylation of I κ B, and HDAC2 expression.

Methods

Animals

Female BALB/c mice were purchased from Jackson Laboratory, Bar Harbor, Maine, and housed in pathogen-free facilities at the Mount Sinai vivarium according to standard guidelines for the care and use of animals.²¹ All animal procedures were approved by the Institutional Animal Care and Use Committee (animal protocol 04-0206PE).

Generation of ASHMI and ASHMI^{II} High-Performance Liquid Chromatography Fingerprints

ASHMI, an extract of 3 traditional Chinese medicine herbs (*G lucidum*, *S flavescens*, and *G uralensis*) and individual herb extracts

were prepared by Weifang Zhongshi Pharmaceutical Co Ltd (a good manufacturing product certified facility), Weifang, China. The methods of preparation, composition, quality control, and chemical analysis have been described in detail previously.²² On the basis of the ratio of individual raw herbs in ASHMI and the yield of individual herb extracts, the refined formula ASHMI^{II}, which may allow for enhanced ease of product quality control, was made by combining individual extracts of *G lucidum*, *S flavescens*, and *G uralensis* at the ratios of 3.5, 4.5, and 2.0, respectively, in our laboratory. The levels of heavy metal, pesticides, and microbial residues all met the standards for botanical products.^{23–25}

Chemical analysis of ASHMI, ASHMI^{II}, and individual herb extracts was performed by high-performance liquid chromatography using a system coupled with a 996 photodiode-array detector (scan from 200 to 400 nm). The separation was performed on a Waters Sunfire TM C18 column (5 μ m, 4.6 $\times$ 150-mm internal diameter; Waters Corporation, Milford, Massachusetts) with a Zorbax ODS guard column (5 μ m, 4.6 $\times$ 12.5-mm internal diameter; Agilent Technologies, Santa Clara, California) at 30°C. A mixture of 0.1% phosphoric acid and acetonitrile was used as the mobile phase. The gradient started in linear gradient mode (2%–48% of acetonitrile for 0 to 75 minutes) with a flow rate of 1 mL/min. Data were acquired and processed with the Empower software system (Waters Corporation, Milford, Massachusetts). Chromatograms of ASHMI and ASHMI^{II} are shown in eFigure 1.

Endotoxin levels in RW extract (low-endotoxin source material; Greer Laboratories, Lenoir, North Carolina), ASHMI, and ASHMI^{II} were measured using the Pyrogen Plus Single test kit (lot GL1383; Lonza, Hopkinton, Massachusetts). Sensitivity of this kit was 0.06 EU/mL (equivalent to 4 pg/mL). On the basis of the reference standard endotoxin/control standard endotoxin ratio of endotoxin provided by the manufacturer for this kit, the endotoxin level in the RW solution was verified to be 9 pg/mL. ASHMI, ASHMI^{II}, and endotoxin levels were below the sensitivity of the kit. The ovalbumin (grade V; Sigma, St Louis, Missouri) endotoxin level was 12 ng/mL.

Antigen Sensitization, Challenge, and Treatment Protocols

RW is a more clinically relevant antigen than ovalbumin, but ovalbumin is more commonly used in the experimental studies than RW. In our preliminary study, we compared phenotypes and severities of airway inflammation after suboptimal ovalbumin and RW sensitization and challenge protocols. RW induced significantly greater (>25-fold) neutrophilic pulmonary inflammation than ovalbumin (data not shown). In addition, although both RW and OVA were of high purity, as certified by the vendor, and our testing indicated that RW endotoxin level was only 0.001% that of ovalbumin. We therefore focused on RW as the antigen rather than ovalbumin in this study. Mice were sensitized by intraperitoneal injections of RW (100 μ g) in 0.4 mL of phosphate-buffered saline (PBS) containing 2 mg of alum as adjuvant (Thermo Scientific, Waltham, Massachusetts) on days 0 and 7 according to the protocol in Figure 1. Mice then received

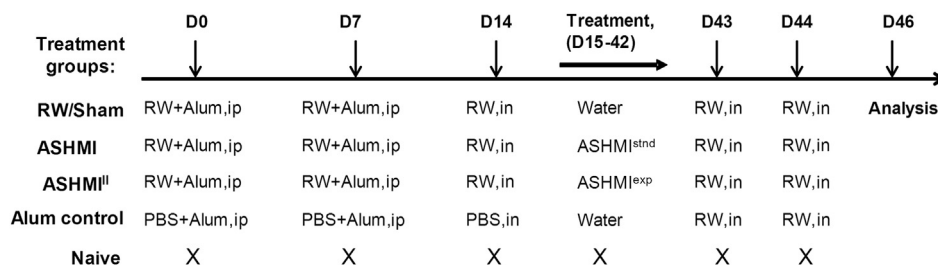


Figure 1. Experimental protocol mice sensitized with ragweed (RW) (and alum) on day (D) 0 and D7 received intranasal RW on D14. Alum control group mice received alum only on D0 and D7 and intranasal phosphate-buffered saline (PBS) on D14. Mice in all groups except the naive group were intranasally challenged with RW on D43 and D44. Airway reactivity was measured on day 46. ASHMI indicates antiasthma simplified herbal medicine intervention; in, intranasally; ip, intraperitoneally.

an intranasal RW challenge (100 μ g in 50 μ L of phosphate-buffered saline) on day 14 (RW mice). One day later, RW mice received either ASHMI (4.5 mg) or ASHMI^{II} (4.5 mg) in 0.5 mL of water (RW/ASHMI) or water as sham treatment (RW/sham) twice daily by intragastric administration after 2 hours of fasting. The daily dose for ASHMI and ASHMI^{II} was determined by a conversion table of equivalent human to animal dose ratios based on body surface area described previously.²⁶ Treatment duration was 4 weeks. After completing treatment, a second set of intranasal challenges consisting of 2 consecutive daily doses (days 43 and 44) with RW (150 μ g) in 50 μ L of PBS were administered. Untreated alum (without RW) sensitized or PBS challenged (sham sensitized or sham challenged) mice (alum control group) and naive mice served as negative controls. In a separate experiment mice were sensitized with RW as described above and then given either ASHMI^{II} or dexamethasone (Sigma-Aldrich, St. Louis, Missouri). Dexamethasone treatment protocol was administered as described by Ito et al.¹⁴ (eFig 2). Briefly, mice were given oral dexamethasone (20 μ g per mouse)¹⁴ dissolved in 0.5 mL of water at 24 hours and 2 hours before RW challenge. ASHMI^{II} treatment was given as described above.

Measurement of AHR

Airway reactivity to acetylcholine provocation was measured 48 hours after the final challenge (day 46), using an invasive method of measuring airway pressure time index (APTI), which measures airway pressure changes, as previously described.^{11,27} Mice anesthetized with a pentobarbital (80 mg/kg intraperitoneally)-xylazine (12 mg/kg intraperitoneally) mixture received ventilation via a tracheal cannula (18 gauge) at the rate of 120/min and a constant tidal volume of air (0.2 mL) with a RSP1002 Pressure Controlled Respirator System (Kent Scientific Corporation, Litchfield, Connecticut). Muscle paralysis was induced by injection of decamethonium bromide (25 mg/kg intravenously). Airway pressure was measured with a pressure transducer via a port linked to a tracheal cannula. Two minutes after establishing a stable airway pressure recording, acetylcholine was injected (100 mg/kg intravenously). Airway pressure changes were recorded for 4 minutes, and respiratory data were acquired and analyzed using DaisyLab software (Kent Scientific Corporation). Airway responsiveness to acetylcholine was expressed as time-integrated changes in peak airway pressure, referred to as APTI.

Evaluation of BAL Fluid Cells, Cytokines, and Chemokines

After airway measurements, BAL fluids were collected by lavaging airways as previously described.²⁷ BAL fluids were centrifuged at 1,200 rpm for 15 minutes at 4°C to isolate cells. Supernatants were stored at –80°C for cytokine analysis. After counting, approximately 40×10^3 cells were cytospun onto glass slides for differential analysis by HEMA-3 staining. Eosinophils, neutrophils, lymphocytes, and macrophage numbers per 500 cells were counted using standard criteria.²⁷ Cytokine and chemokine levels in BAL fluid supernatants were assayed by commercial enzyme-linked immunosorbent assay kits according to the manufacturer's instructions for IL-13 and IL-8 (CXCL1/KC) (R&D Systems, Minneapolis, Minnesota) and for IL-4, IL-5, IL-10, IL-13, IL-17, IFN- γ , and TNF- α (BD Biosciences).

Lung Histologic Analysis

After obtaining BAL fluid samples, lungs were removed and fixed in formalin and processed by the Mount Sinai Histology Shared Facilities. Then 5- μ m sections were stained with hematoxylin-eosin for the evaluation of inflammation and periodic acid–Schiff stain for evaluation of goblet cells. Images were

captured using an Axioplan 2 microscope at the Mount Sinai Microscopy Facility. Airway pathologic findings were assessed by 2 independent observers.

Measurement of Serum RW Specific IgE and IgG2a Levels

Serum was isolated from blood drawn from vena cava by syringe immediately after APTI measurements. RW specific IgE and IgG2a was measured as described in the eMethods.

Determination of GAC1 Effects on RW-Stimulated Macrophage TNF- α Production

The mouse macrophage cell line RAW264.7 (American Type Culture Collection, Manassas, Virginia) was maintained in Dulbecco modified Eagle medium (Mediatech, Manassas, Virginia) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, Georgia) and 1% penicillin-streptomycin (Mediatech). Then 5×10^5 cells were first cultured with 40 μ g/mL of GAC1 for 24 hours. RW (200 μ g/mL) was then added and cells were cultured for an additional 24 hours. Medium containing 9 μ g/mL of lipopolysaccharide (LPS) (background levels of LPS present in the RW extract) and medium alone served as negative controls. Culture with RW alone was the positive control. TNF- α levels were measured in the supernatants by enzyme-linked immunosorbent assay according to the manufacturer's instructions (BD Biosciences).

In-Cell Western Blot for Measurement of plkB- α and HDAC2 Expression

RAW264.7 cells (5×10^5) were cultured in a 96-well plate overnight in serum free medium with or without GAC1 (40 μ g/mL). Cells were stimulated with background levels of LPS (9 μ g/mL) or RW (200 μ g/mL) for 5, 10, 30, and 60 minutes. In-Cell Western Assay (Li-Cor, Lincoln, Nebraska) was performed according to the manufacturer's instructions using antibodies against plkB- α (1/1,000) or HDAC2 (1/1,000) (Cell Signaling Technology, Danvers, Massachusetts) with β -actin (1/1,000) (Santa Cruz Biotechnology, Birmingham Alabama) as a loading control. This was followed by incubation with secondary antibodies IRDye800CW donkey anti-goat (1/1,000) and IRDye680RD donkey anti-rabbit (1/1,000) (Li-Cor). Plates were scanned, fluorescence detected at 700 and 800 nm, and data normalized using an Odyssey CLx Infrared Imaging System (Li-Cor).

Statistical Analysis

Data were analyzed by SigmaStat 2.03 software (SPSS Inc, Chicago, Illinois) using 1-way analysis of variance. When significant differences were indicated, differences between groups of interest were analyzed by the *t* test followed by the Tukey posttest. The Mann-Whitney Rank Sum Test was applied when data were not normally distributed. $P \leq .05$ was considered significant. Power and sample size (power = 0.8, $\alpha = .05$) were calculated based on our preliminary results of the ASHMI effects on AHR measurements, and a minimum of 4 mice per experimental group were needed to demonstrate statistically significant results.

Results

AHR Reduction With ASHMI or ASHMI^{II} Treatment in RW Sensitized and Challenged Mice

RW sensitization and challenge induced marked increases in AHR as indicated by elevated APTI values after acetylcholine provocation when compared with alum control and naive mice ($P < .01$ RW/sham vs alum control; $P < .01$ RW/sham vs naive; Fig 2). ASHMI and ASHMI^{II} treated mice exhibited marked reductions in AHR when compared with RW/sham treated mice ($P < .01$ for both; Fig 2).

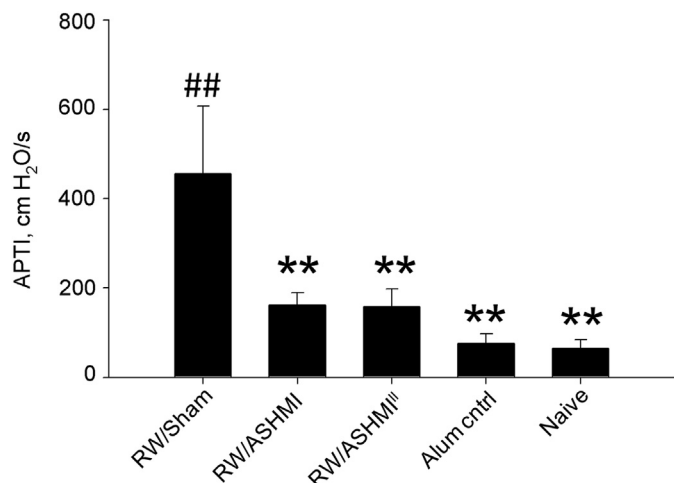


Figure 2. The Antiasthma Simplified Herbal Medicine Intervention (ASHMI) and its refined form, ASHMI^{II}, reduced airway hyperreactivity in ragweed-challenged mice. Airway responsiveness to acetylcholine was expressed as Airway Pressure Time Index (APTI). Data are expressed as mean (SD). $n = 5$ mice per group. ** $P < .01$. Cntrl indicates control.

Suppression of Neutrophil-Predominant Airway Inflammation by ASHMI or ASHMI^{II}

BAL fluid obtained from RW/sham mice 48 hours after final RW challenge contained significantly increased numbers of total leukocytes compared with BAL fluid from alum control and naive groups ($P < .01$; Fig 3A). BAL fluid from ASHMI and ASHMI^{II} treated mice contained significantly fewer total leukocytes ($P < .01$ for both; Fig 3A). Differential analysis of BAL fluid cells revealed a nearly 3-fold higher percentage of neutrophils (32.5%

$\pm 2.9\%$) compared with eosinophils (11.9% $\pm 2.1\%$) in RW/sham mice, confirming neutrophil-predominant airway inflammation (Fig 3B). The percentage of combined granulocytes (neutrophils and eosinophils) in RW/sham mice (44.6% $\pm 3.7\%$) was significantly reduced by more than 90% by ASHMI (7.3% $\pm 0.7\%$) and ASHMI^{II} (6.2% $\pm 1.2\%$) treatment ($P < .01$ –.001 vs sham; Fig 3B). Slight but not statistically significant reductions in lymphocyte and macrophage numbers also were observed (Fig 3B). Images from cytospin slides illustrate the predominance of neutrophils mixed with eosinophils in a BAL fluid from RW/sham mice, which was absent in alum controls and naive mice, and was nearly eliminated in the BAL fluid collected from ASHMI and ASHMI^{II} treated mice (Fig 3C). These findings demonstrate that RW induced a predominantly neutrophilic airway inflammation and that ASHMI and ASHMI^{II} suppressed neutrophilic and eosinophilic inflammation in this murine model.

Reduction of Pulmonary Disease and Mucous Hypersecretion With ASHMI Treatment

Histologic analysis revealed that lungs from RW/sham mice contained extensive peribronchial and perivascular inflammation (Fig 4A), numerous airway goblet cells with positive staining for mucus (Fig 4B), and luminal mucous plugs (Fig 4C). Inflammation was markedly reduced and goblet cells were virtually absent in airways of ASHMI and ASHMI^{II} treated mice (Fig 4, D–G). These were similar in appearance to naive and alum control group lungs (Fig 4, H–K).

Modification of the T_H2/T_H1 Immune Responses and Reduction of Eotaxin 1 Levels by ASHMI and ASHMI^{II} Treatment

We previously found that treatment with ASHMI down-regulated T_H2 immune responses without global immunosuppression in allergic individuals and murine allergic asthma

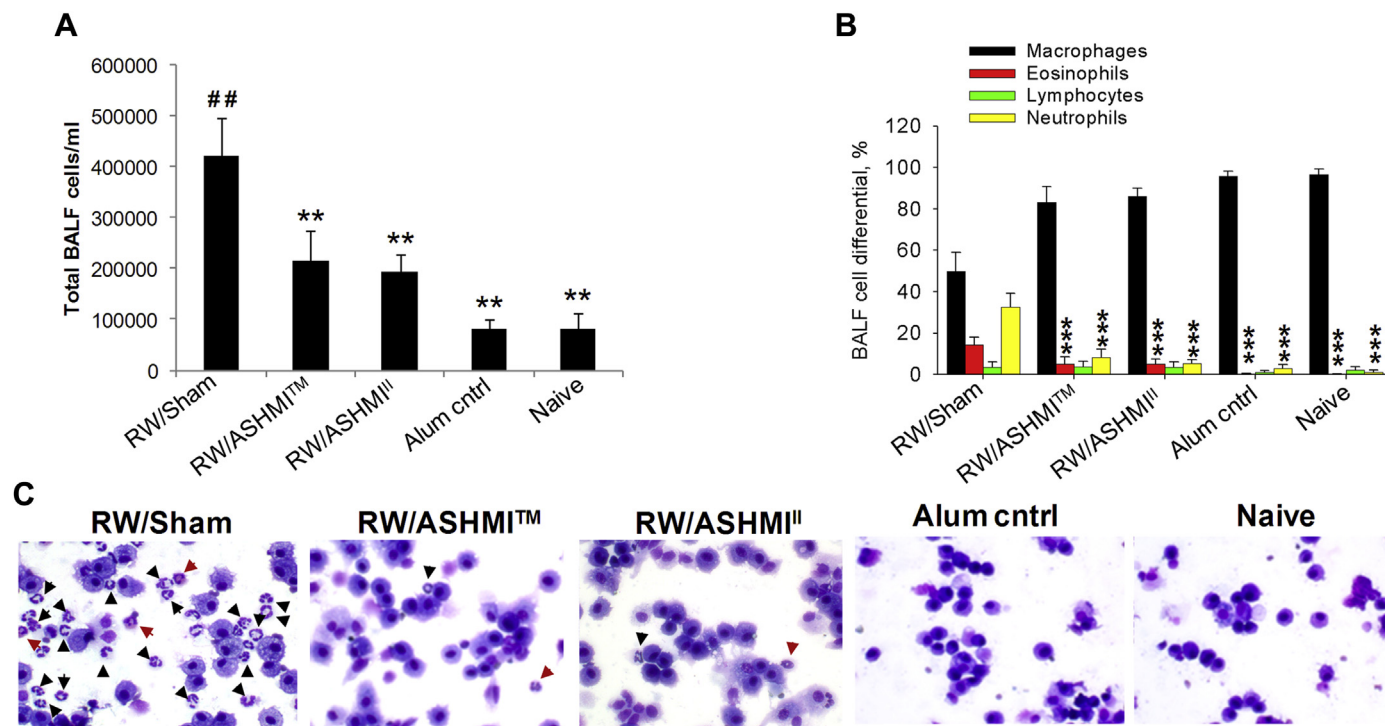


Figure 3. Treatment with antiasthma simplified herbal medicine intervention (ASHMI) or refined ASHMI (ASHMI^{II}) decreased neutrophil-eosinophil airway inflammation. A, Total number of bronchoalveolar lavage fluid (BALF) leukocytes. B, Percentage of BALF macrophages (black), eosinophils (red), lymphocytes (blue), and neutrophils (yellow). C, Representative illustration of neutrophil predominance among granulocytes (black arrows point to neutrophils; red arrows point to eosinophils) in BALF from ragweed (RW)/sham mice, which was markedly reduced compared with RW/ASHMI and RW/ASHMI^{II} treated mice. A and B, Data expressed as mean (SD). $n = 5$ mice per group. * $P < .05$, ** $P < .01$, and *** $P < .001$.

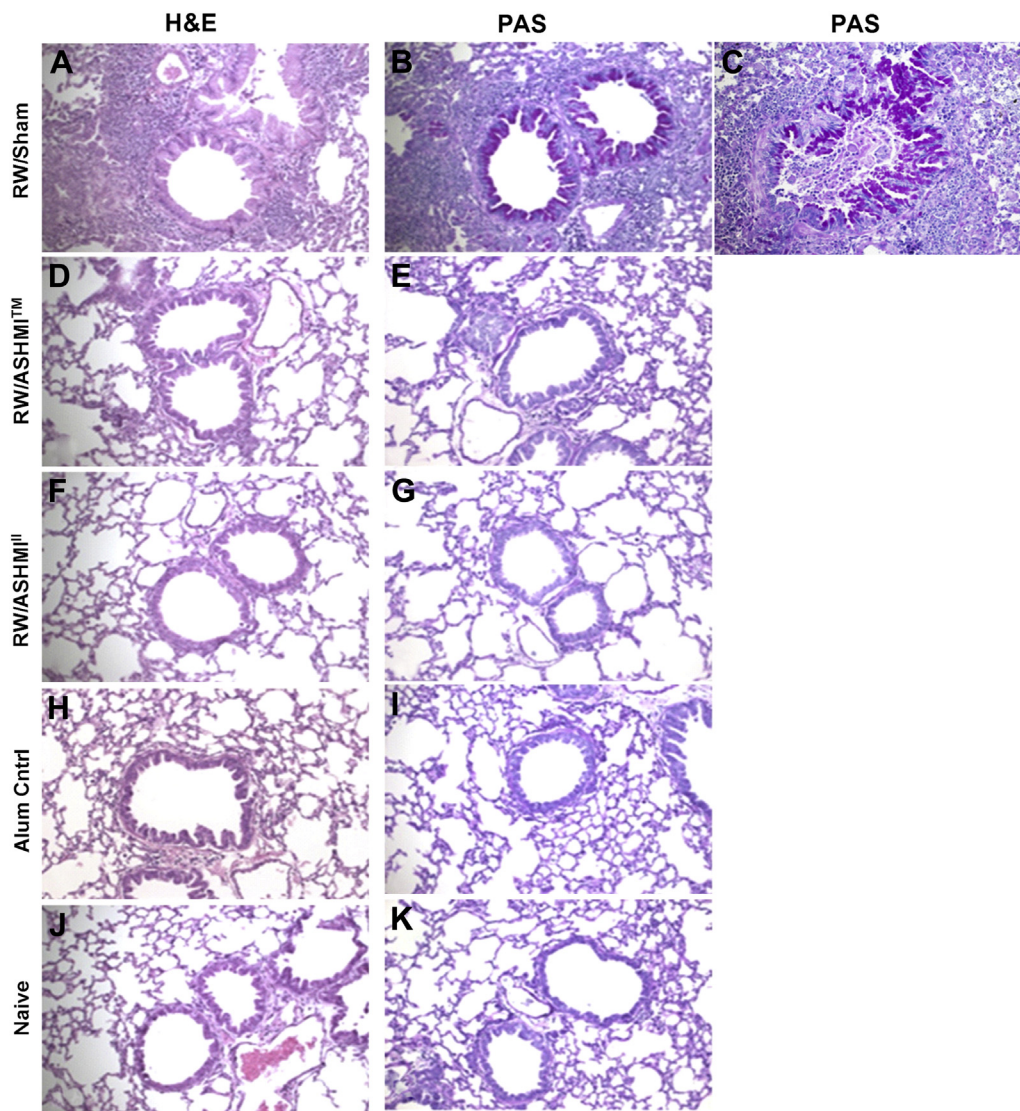


Figure 4. Airway tissue inflammation and mucous hyperproduction is diminished in asthmatic mice treated with antiasthma simplified herbal medicine intervention (ASHMI) formulas. A–E, Hematoxylin–eosin (H&E) staining of lung sections of formalin-fixed lungs. F–J, Lung sections stained with periodic acid–Schiff (PAS) that stains mucus with a magenta color. Data are representative of 5 mice per group. Cntrl indicate control; RW, ragweed.

models.^{9,11} Similarly, in this study, the T_H2 cytokines IL-5 and IL-13 were induced in the BAL fluid after RW sensitization and challenge but not after negative control conditions ($P < .01$; Fig 5, A and B). IL-13 (588.4 ± 56.6 pg/mL in RW/sham mice) and IL-5 (544.8 ± 57.0 pg/mL in RW/sham mice) levels were significantly reduced by approximately 70% and 90%, respectively, in BAL fluid of ASHMI (158.2 ± 18.5 pg/mL for IL-13 and 102.6 ± 20.3 pg/mL for IL-5) and ASHMI^{II} (100.5 ± 8.1 pg/mL for IL-13 and 35.2 ± 10.3 pg/mL for IL-5) treated mice ($P < .01$ – $.001$ for all vs RW/sham mice; Fig 5A and B). In addition, BAL fluid IFN- γ levels were increased in both RW/ASHMI (363.4 ± 35.2 pg/mL) and RW/ASHMI^{II} (32.1 ± 30.1 pg/mL) treated mice when compared with the RW/sham treated mice (107.2 ± 29.7 pg/mL) ($P < .001$ for both; Fig 5C). No changes were found in BAL fluid IL-10 levels (data not shown). Levels of the eosinophil chemoattractant eotaxin 1 in BAL fluid of RW/sham treated mice also were reduced in the ASHMI and ASHMI^{II} treated groups ($P < .001$; Fig 5D). Consistent with T_H1 and T_H2 cytokine profiles, serum RW specific IgE levels were significantly reduced in ASHMI and ASHMI^{II} treated mice when compared with RW/sham mice ($P < .01$ for both; Fig 5E), whereas T_H1 -associated IgG2a levels

were significantly increased in ASHMI ($P < .05$; Fig 5F) treated mice, and a statistically insignificant increase was observed for mice given ASHMI^{II} ($P = .06$; Fig 5F).

Marked Reduction in TNF- α , IL-17, and IL-8 Levels After ASHMI and ASHMI^{II} Treatment

In light of the preponderance of neutrophils in BAL fluid from RW/sham mice, we determined whether ASHMI treatment would affect the production of cytokines and chemokines involved in neutrophil recruitment. Levels of TNF- α (Fig 6A), IL-8 (Fig 6B), and IL-17 (Fig 6C) in RW/sham mice were elevated when compared with alum control and naive mice ($P < .01$ – $.001$ for all). On treatment with ASHMI and ASHMI^{II}, mice exhibited marked and statistically significant reductions in TNF- α (323.6 ± 155.7 pg/mL for RW/sham mice, 24.8 ± 16.9 pg/mL for RW/ASHMI mice, and 34.4 ± 14.4 pg/mL for RW/ASHMI^{II} mice), a 4-fold reduction in IL-8 (581 ± 107.7 pg/mL for RW/sham mice, 137.8 ± 29.6 pg/mL for RW/ASHMI mice, and 93.2 ± 31.6 pg/mL for RW/ASHMI^{II} mice), and a marked reduction in IL-17 levels (85.4 ± 6.9 pg/mL for RW/sham mice, 4.0 ± 4.0 for RW/ASHMI

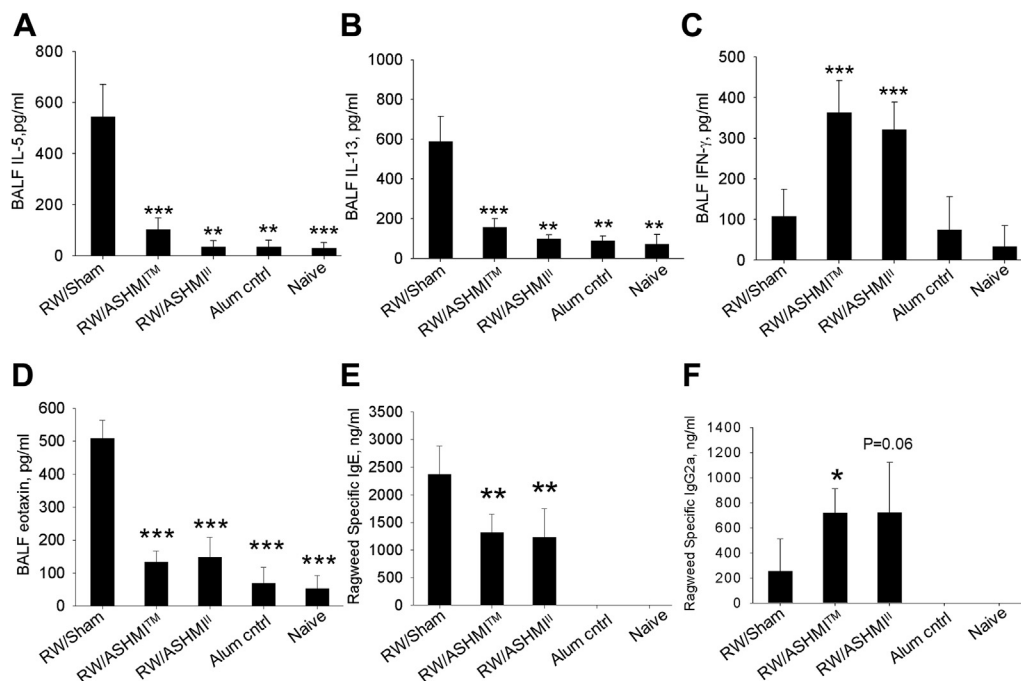


Figure 5. Treatment with antiasthma simplified herbal medicine intervention (ASHMI) and refined ASHMI (ASHMI^{II}) inhibited T_H2 and increased T_H1 responses. Bronchoalveolar lavage fluid (BALF) interleukin (IL) 5 (A), IL-13 (B), IL and interferon γ (IFN- γ) (C), eotaxin (D), ragweed (RW) specific IgE (E), and IgG2a (F) were measured by enzyme-linked immunosorbent assay. Data are expressed as mean (SD). n = 5 mice per group. **P < .01, ***P < .001. Cntrl indicates control.

mice, and 1.8 ± 1.8 for RW/ASHMI^{II} mice) ($P < .01$ –.001 for all). For IL-17, reductions on average were similar to that measured among alum control and naive mice.

Suppression of RW-Stimulated Macrophage TNF- α Production by GAC1

To facilitate identification of ASHMI herbal constituents that contribute to the formula's suppression of neutrophil-dominant airway inflammation, we used the mouse macrophage cell line RAW264.7. Macrophages are an established source of proinflammatory cytokines in the airway, such as TNF- α , and their role in severe asthma is receiving attention.²⁸ Furthermore, TNF- α is associated with neutrophil recruitment.²⁹ Initial screening revealed that ASHMI and *G. lucidum* but not *S. flavescens* or *G. uralensis* suppressed TNF- α production in response to LPS stimulation (data not shown). GAC1 isolated from *G. lucidum* was the most potent inhibitor of TNF- α production (Liu et al, unpublished data) and was then selected for further analyses in the current study. GAC1 significantly reduced RW-stimulated TNF- α production by more than 60% ($3,537.2 \pm 3.1$ pg/mL for RW and $1,424.5 \pm 95.5$ pg/mL for

RW and GAC1; $P < .01$; Fig 7A) without cytotoxicity (Fig 7B). In addition, TNF- α levels in medium that contained background levels of LPS (9 pg/mL in the RW extract) were not different from medium alone. This demonstrated that RW-induced TNF- α production is not due to the LPS contamination.

To understand how GAC1 blocks production of TNF- α , we next assessed levels of pI κ B as a biomarker of TNF- α -induced NF- κ B activation. GAC1 significantly suppressed levels of pI κ B ($70.3\% \pm 8.1\%$ of medium for RW and GAC1 vs $137.3\% \pm 14.9\%$ of medium for RW; $P < .05$; Fig 7C), implying suppression of the NF- κ B pathway. Stimulation of RAW264.7 cells with RW led to a significant decrease in HDAC2 ($85.0\% \pm 2.2\%$ of medium), suggestive of impaired transcriptional repression that was reversed with pretreatment with GAC1 ($98.3\% \pm 1.2\%$ of medium; $P < .05$; Fig 7D).

Decreased AHR and Neutrophilic Inflammation in Response to RW Challenge With ASHMI^{II} Treatment

Because neutrophil-predominant asthma in humans and murine models has been reported to be steroid resistant,^{5,14} we wished to determine steroid responsiveness of our asthma model and

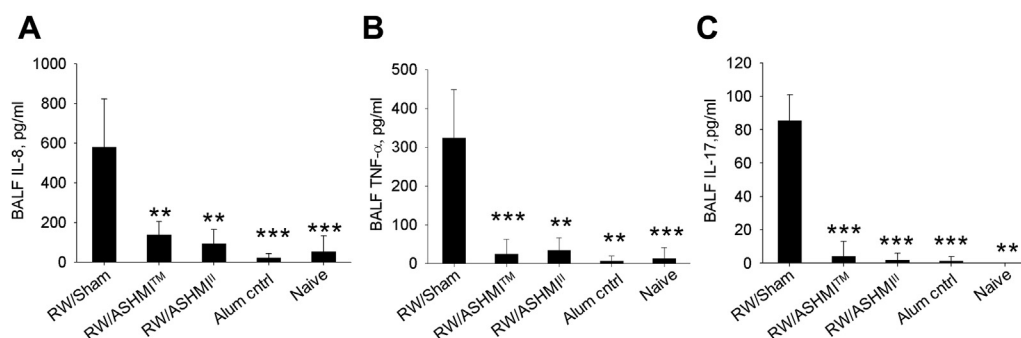


Figure 6. Cytokines and chemokines involved in neutrophilic inflammation were decreased after treatment with antiasthma simplified herbal medicine intervention (ASHMI) or its refined form (ASHMI^{II}). Bronchoalveolar lavage fluid (BALF) tumor necrosis factor α (TNF- α) (A), interleukin (IL) 8 (B), and IL-17 (C) were measured. Data are expressed as mean (SD). n = 5 mice per group. **P < .01, ***P < .001. Cntrl indicates control.

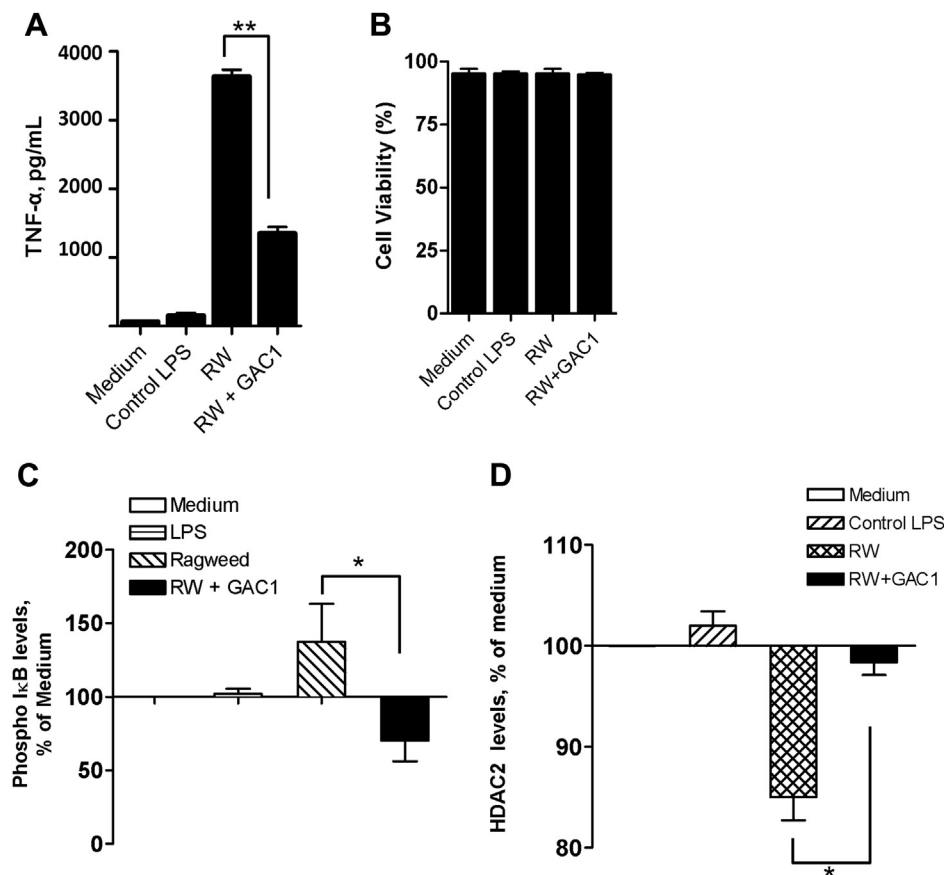


Figure 7. Ganoderic acid C1 (GAC1)–mediated reduction in tumor necrosis factor α (TNF- α) production in vitro by ragweed (RW)–stimulated murine macrophages was associated with decreased phosphorylated (phospho) I κ B and increased histone deacetylase 2 (HDAC2) expression. A, Effect of GAC1 on TNF- α production by macrophages stimulated with 200 μ g/mL of RW in the presence and absence of GAC1. B, Cell viability for experiment shown in A. Phospho-I κ B expression (C) and HDAC2 levels (D) measured by in-cell Western blotting of macrophages stimulated with 200 μ g/mL of RW in the presence and absence of GAC1 treatment. C and D, Data are percentage of medium alone levels. * $P < .05$. LPS indicate lipopolysaccharide.

compare effects of steroid treatment to therapy with ASHMI^{II}. Using a dexamethasone treatment regimen used previously by Ito et al,¹⁴ we found that short-term treatment with dexamethasone did not significantly decrease AHR (491.2 ± 43.7 vs 543.6 ± 34.2 cm H₂O/s for RW/sham; Fig 8A), and total BAL fluid cellular infiltrate in dexamethasone-treated mice was similar to RW/sham mice ($380,513 \pm 30,893$ cells for RW/dexamethasone mice vs $433,680 \pm 43,309$ cells for RW/sham mice; Fig 8B). Furthermore, although dexamethasone treatment reduced the percentage of eosinophils in BAL fluid ($2.9\% \pm 0.5\%$ for RW/dexamethasone mice vs $14.1\% \pm 1.5\%$ for RW/sham mice; $P < .001$), the percentage of neutrophils was slightly increased ($44.4\% \pm 2.2\%$ for RW/dexamethasone mice vs $31.8\% \pm 2.5\%$ for RW/sham mice; Fig 8C). In contrast, mice given ASHMI^{II} had markedly and statistically lower AHR (152.4 ± 15.1 cm H₂O/s) to RW challenge when compared with RW/dexamethasone mice ($P < .001$) and RW/sham mice ($P < .001$) (Fig 8A). ASHMI^{II} treated mice also had significant reductions in total BAL fluid cells ($194,140 \pm 27,009$ cells), eosinophils ($5.12\% \pm 1.2\%$), and neutrophils ($8.2\% \pm 1.6\%$) compared with RW/dexamethasone and RW/sham animals ($P < .01$ – $< .001$ for all; Fig 8, B and C). AHR after RW challenge in mice treated with ASHMI and ASHMI^{II} was also significantly reduced compared with RW/dexamethasone mice ($P < .001$) and RW/sham mice ($P < .001$) (Fig 8A).

Discussion

During the past decades, most experimental asthma studies have used murine asthma models characterized by eosinophilic

inflammation.³⁰ Given the findings that neutrophil-predominant inflammation is associated with more severe and refractory asthma,⁵ increasing attention is being paid to neutrophil-predominant asthma. However, our understanding of the immunopathogenesis of neutrophil-predominant asthma and its treatment is still limited.

Neutrophil-predominant murine asthma models are limited. Ito et al¹⁴ reported that long-term aerosol ovalbumin exposure of ovalbumin/alum sensitized BALB/c mice induced neutrophil-predominant airway inflammation, whereas a model used by Bogaert et al¹⁵ used aerosol challenges of ovalbumin plus complete Freund adjuvant of ovalbumin sensitized C57BL/6 mice. Liu et al¹⁶ recently developed a neutrophil-predominant but mixed airway inflammation in a murine model that used a mixture of dust mite, *Aspergillus*, and RW extracts.¹⁶ In the present study, we used a RW asthma model characterized by neutrophil-predominant, mixed granulocytic airway inflammation. RW is a clinically relevant antigen, and our model does not require prolonged daily aerosolized exposures or use of an adjuvant for airway challenge of antigens, all of which represent advantages over ovalbumin-based models of neutrophil-predominant asthma discussed above. In addition, RW-induced neutrophilic inflammation in this model was not due to endotoxin because RW induced a significantly higher (>10 -fold) BALF neutrophil percentage than ovalbumin following a suboptimal protocol in our preliminary study, whereas the RW endotoxin level (9 pg/mL) was only 0.001% that of ovalbumin (12 ng/mL). The ability of RW to induce neutrophilic inflammation has been previously demonstrated to be attributable to intrinsic NADPH oxidases.³¹ Using

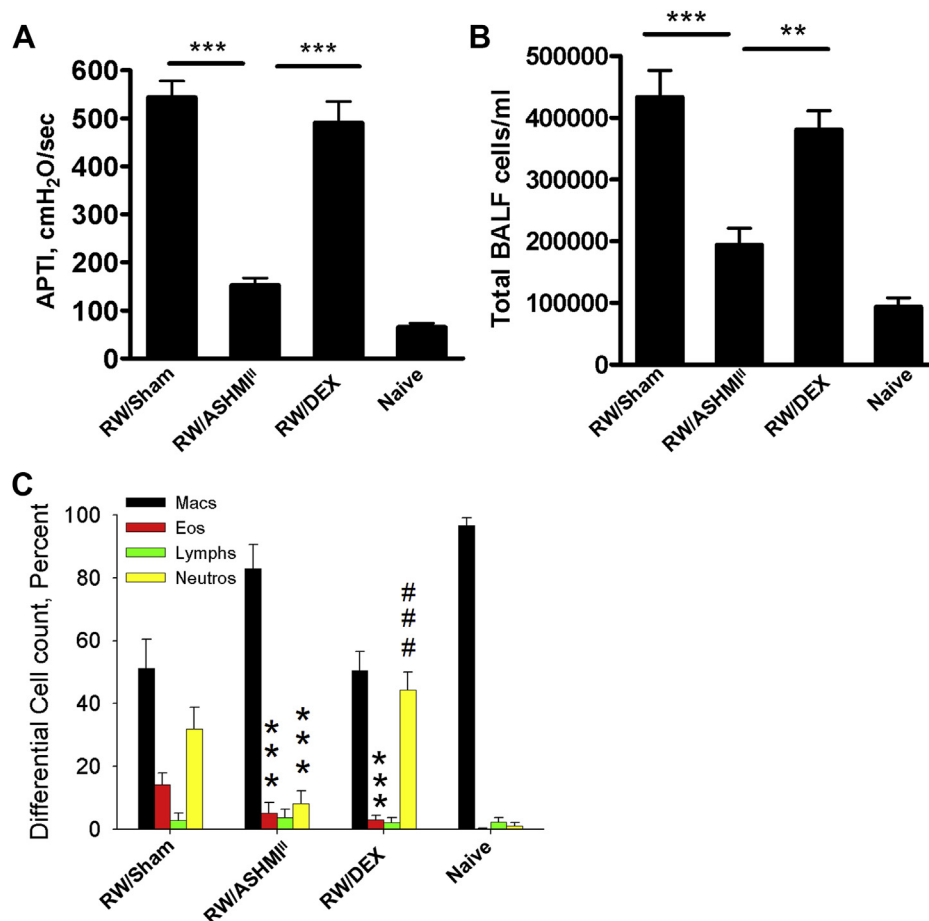


Figure 8. Treatment with refined antiasthma simplified herbal medicine intervention (ASHMI^{II}) but not dexamethasone (DEX) reduces airway hyperreactivity and neutrophilic inflammation in response to ragweed (RW) challenge. A, Airway responsiveness to acetylcholine was expressed as Airway Pressure Time Index (APTI). B, Total number of bronchoalveolar lavage fluid (BALF) leukocytes. C, Percentage of BALF macrophages (macs; black), eosinophils (eos; red), lymphocytes (lymphs; blue), and neutrophils (neutros; yellow). Data are expressed as mean (SD). $n = 5$ mice per group. ** $P < .01$, *** $P < .001$ vs RW/sham mice, ### $P < .001$ vs RW/ASHMI^{II} mice.

this model, we demonstrated that ASHMI and ASHMI^{II} treatment significantly suppressed both neutrophil and eosinophil airway inflammation via regulation of associated chemokine and cytokines. In addition, we observed significant reductions in AHR, mucous production, and RW specific IgE. Consistent with previous findings using neutrophil-predominant murine asthma models by others,^{14,16} we found that steroid treatment reduced BAL fluid eosinophil percentage but failed to decrease neutrophil numbers or decrease AHR in our RW asthma model.

Different patterns of airway inflammation are associated with distinct cytokine profiles. In humans, T_H2 responses are associated with eosinophil predominant inflammation, whereas innate proinflammatory responses, including TNF- α and IL-8 production, are associated with neutrophilic inflammation.^{32,33} Similar immunologic and inflammatory phenotypes also have been recently characterized in murine asthma models.¹⁵ TNF- α contributes to multiple aspects of asthma symptoms, including AHR, mucous hyperproduction, and proliferation of airway smooth muscle cells.^{34–36} Eotaxin 1 facilitates recruitment of eosinophils and other inflammatory cells, including neutrophils.³⁷ IL-17 also is associated with neutrophil inflammation.¹³ The current study found that, in addition to suppression of T_H2 responses, ASHMI and ASHMI^{II} treatment resulted in marked reductions in BAL fluid chemokine and cytokine levels involved in neutrophil and eosinophil recruitment.^{38,39} These findings demonstrate that ASHMI suppressive effects are associated with immunomodulation of both adaptive and innate responses.

HDAC2 negatively regulates NF- κ B, a master transcriptional activator of TNF- α production. In the present study, we found that GAC1, a triterpenoid compound in ASHMI, significantly suppressed RW-stimulated TNF- α production in murine macrophages, and this effect was associated with significantly reduced pI κ B and significantly increased HDAC2 expression. Reduced HDAC2 expression has been found in macrophages of asthma patients and is believed to contribute to steroid resistance.^{40,41} Therefore, increasing macrophage HDAC2 expression may be a novel therapeutic mechanism by which to down-regulate NF- κ B activation in asthma.

Chinese herbal medicines are commonly prepared by aqueous extraction of a mixture of herbs.⁴² Simultaneous extraction of multiple herbs has the potential to generate novel compounds. For the first time, we compared the effects of ASHMI prepared by the simultaneous extraction method (ASHMI) to ASHMI^{II}, which was made by extracting individual herbs separately and then combining extracts to prepare the whole formula. That ASHMI^{II} replicated the effects of ASHMI in all parameters suggests that the pharmaceutically active compounds in ASHMI are compounds present in individual component herbs rather than novel compounds generated by simultaneous extraction. Furthermore, methods used to prepare ASHMI^{II} have the advantage of being more efficient for standardization and scaled-up isolation of active compounds.

In summary, we demonstrated for the first time, to our knowledge, that ASHMI inhibits neutrophil-predominant airway inflammation in a RW asthma murine model. Effects on eosinophilic inflammation also were observed, a new finding in the case of

ASHMI^{II} treatment. Therapeutic effects were associated with ASHMI-induced differences in TNF- α , IL-8, IL-17, and T_H1/T_H2 cytokine responses. The in vitro models suggested that the compound GAC1 inhibited TNF- α production through suppression of NF- κ B activity at least partly due to up-regulation of HDAC2. Studies to determine the effects of in vivo treatment with GAC1 alone and in combination with other biologically active ASHMI compounds, such as 7,4'-dihydroxyflavone isolated from *G uralensis*, which we reported to suppress IL-4 and IL-5 production, and the T_H2 transcription factor GATA3,^{43,44} are under way. Because standard asthma therapy, including inhaled corticosteroids, is less effective for severe neutrophilic asthma,⁴⁵ ASHMI and ASHMI^{II} may be of value for the treatment of neutrophil-predominant asthma.

Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2014.01.021>.

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eMethods

Measurement of Serum RW Specific IgE and IgG2a Levels

Serum was isolated from blood drawn from vena cava by syringe immediately after Airway Pressure Time Index (APTI) measurements. Ragweed (RW) specific IgE and IgG2a was measured as described in the eMethods previously by Shibata et al with some modifications.¹ Briefly, 96-well microplates were incubated overnight at 4°C with 50 µg/mL of RW extract in sodium acetate buffer, pH 6. Reference wells were coated with anti-mouse IgE. After 3 washes, plates were blocked with 2% bovine serum albumin–phosphate-buffered saline for 1 hour at room temperature. After washing, 1:10 dilution of serum was added to RW-coated wells, and purified IgE was added to reference wells and incubated overnight at 4°C. Plates

were subsequently washed and incubated with a 1:1,000 dilution of 1 mg/mL of avidin-horseradish peroxidase (Sigma-Aldrich, St. Louis, Missouri) for 45 minutes. Plates were then washed and developed with ABTS substrate (KPL, Minneapolis, Minnesota) and absorbance was read at 405 nm by a spectrophotometer. RW specific IgG2a was measured using microtiter plates coated with 2 µg/mL of RW extract, and all other steps have been described previously in detail.²

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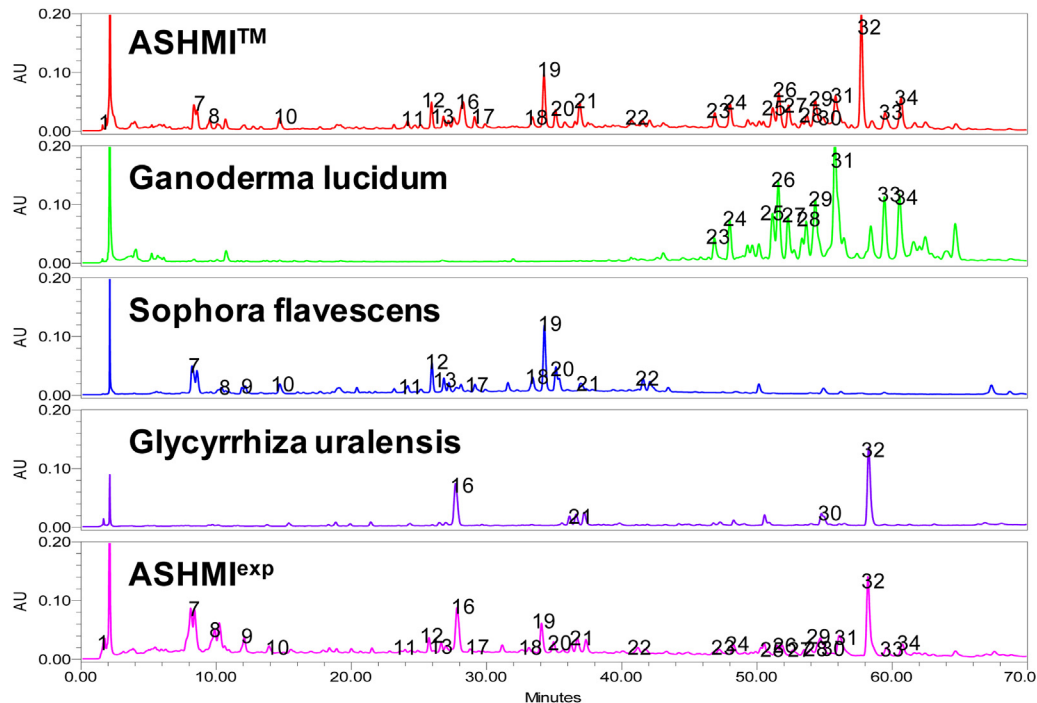


Figure 1. High-performance liquid chromatograms of antiasthma simplified herbal medicine intervention (ASHMI) and refined ASHMI (ASHMI^{II}), *Ganoderma lucidum*, *Sophora flavescens*, and *Glycyrrhiza uralensis*. Numbers indicate major peaks identifying the major compound(s). Peaks 16 and 32 correspond to liquiritin and glycyrrhizin from *G uralensis*. Peaks 7 and 19 correspond to matrine and kushenol O from *S flavescens*. Peaks 26 and 27 correspond to ganoderic acid D and ganoderic acid A from *G lucidum*.

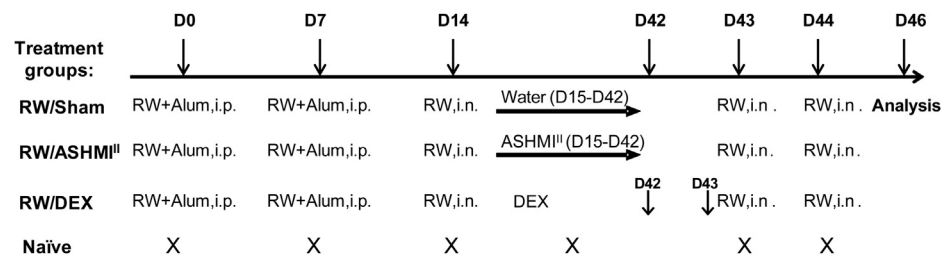


Figure 2. Experimental protocol. Mice sensitized with ragweed (RW) (and alum) on day (D) 0 and D7 received intranasal RW on D14. RW/refined antiasthma simplified herbal medicine intervention (ASHMI^{II}) mice received oral ASHMI treatment for 4 weeks starting on D15. Mice in the RW/dexamethasone (DEX) group received oral DEX on D42 and 2 hours before RW challenge on D43. Mice in all groups except the naïve group were intranasally challenged with RW on D43 and D44. Airway reactivity was measured on D46.