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Herbal formula ASHMI suppresses neutrophil predominant airway inflammation in a ragweed sensitized murine asthma model

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Introduction

Asthma is a heterogeneous airway disease which includes eosinophil-predominant, neutrophil-predominant and mixed eosinophil/neutrophil airway inflammation.¹ Increasing evidence shows 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 asthmatics has been shown to be associated with poor lung function and disease outcomes,⁴ as well as sudden asthma fatalities.^{6–8} Presently, there is no effective therapy for neutrophil-predominant airway inflammation.

We previously showed that the herbal medicine intervention, ASHMITM derived from Traditional Chinese Medicine (TCM) was efficacious as a stand-alone therapy in adults with moderate-to-severe allergic asthma. Treatment benefits were associated with increased Th1 responses and reduced Th2 responses.⁹ Further, ASHMITM significantly reduced airway eosinophilic inflammation and AHR in an ovalbumin (OVA) sensitized murine asthma model in which eosinophilic inflammation was dominant.¹⁰ Protective effects in the murine model were attributable to ASHMITM-induced down-regulation of Th2 immune responses

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and increased interferon (IFN)- γ , but not transforming growth factor (TGF)- β production.¹¹ Whether ASHMI is also effective in a neutrophil-predominant asthma is unknown.

Neutrophilic inflammation is driven by TNF- α , IL-8, and IL-17.^{12, 13} Up regulation of these mediators has been shown require activation of the 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 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 co-repressor complex along with CBP/ P300 and RelA and loss of HDAC2 has been shown to cause disruption of the co-repressor 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 (PBMCs) from asthma patients.²⁰

We hypothesized that ASHMITM 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 BAL fluid infiltrate in addition to AHR and mucus hyperproduction. The effects on AHR, cytokine and chemokine production of ASHMITM 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 *Ganoderma 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, ME and housed in pathogen-free facilities at the Mount Sinai vivarium on 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 ASHMITM and ASHMI^{II} HPLC fingerprints

ASHMITM, an extract of 3 TCM herbs- *Ganoderma lucidum* (GL), *Sophora flavescens* (SF) and *Glycyrrhiza uralensis* (GU) and individual herb extracts were prepared by Weifang Zhongshi Pharmaceutical Co., Ltd. (a good manufacturing product (GMP) certified facility), Weifang, China. The method of preparation, composition, quality control and chemical analysis have been described in detail previously.²² Based on the ratio of individual raw herbs in ASHMITM, and the yield of individual herb extracts, the refined formula ASHMI^{II}, that may allow for enhanced ease of product quality control, was made by combining

individual extracts of GL, SF and GU at the following ratios- GL:SF:GU= 3.5: 4.5 :2.0 in our laboratory. The levels of heavy metal, pesticides, and microbial residues all met the standards for botanical products.^{23–25}

Chemical analysis of ASHMITM, ASHMI^{II} and individual herb extracts was performed by HPLC using a Waters Alliance system coupled with a 996 photodiode-array detector (scan from 200~400nm). The separation was carried out on a Waters Sunfire TM C18 column (5µm, 4.6mm×150mm i.d., Waters Corporation, Milford, MA) with a Zorbax ODS guard column (5µm, 4.6mm×12.5mm i.d., Agilent Technologies, Santa Clara, CA) at 30°C. A mixture of 0.1% phosphoric acid (A) and acetonitrile (B) was used as the mobile phase. The gradient started in linear gradient mode (2%–48 % of B for 0~75min) with a flow-rate of 1mL/min. Data were acquired and processed with the Empower® software system. Chromatograms of ASHMITM and ASHMI^{II} are shown in supplement Figure E1.

Endotoxin testing of RW

Endotoxin levels in RW extract (low-endotoxin source material, Greer Laboratories, Lenoir, NC), ASHMITM, and ASHMI^{II} were measured using the Pyrogen Plus Single test kit (Lonza, MA, lot #GL1383). Sensitivity of this kit was 0.06EU/mL (equivalent to 4pg/ml). Based on the Reference Standard Endotoxin/Control Standard Endotoxin (RSE/CSE) ratio of endotoxin provided by the manufacturer for this kit, endotoxin level in RW solution was verified to be 9pg/ml. ASHMITM, ASHMI^{II} endotoxin levels were below the sensitivity of the kit. OVA (Grade V, Sigma, St Louis, MO) endotoxin level was 12 ng/ml.

Antigen sensitization, challenge and treatment protocols

RW is a more clinically relevant antigen than OVA, but OVA is more commonly used in the experimental studies than RW. In our preliminary study, we compared phenotypes and severities of airway inflammation following suboptimal OVA and RW sensitization and challenge protocols. RW induced significantly greater (more than 25 fold) neutrophilic pulmonary inflammation than OVA (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 OVA. We therefore focused on RW as antigen rather than OVA in this study. Mice were sensitized by intraperitoneal (i.p.) injections of RW (100µg) in 0.4ml PBS containing 2 mg alum as adjuvant (Thermo Scientific, Waltham, MA) on days 0 and 7 according to the protocol in Figure 1. Mice then received an intranasal (i.n.) RW challenge (100µg in 50µl PBS) on day 14 (RW mice). One day later, RW mice received either ASHMITM (4.5mg), or ASHMI^{II} (4.5 mg) in 0.5ml of water (RW/ASHMI), or water as sham treatment (RW/sham), twice daily by intragastric administration (i.g.) after two 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 (150µg) in 50µl PBS were administered. Untreated alum (without RW) sensitized/PBS challenged (sham sensitized/sham challenged) mice (alum control group) as well as naive mice (naïve group) 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, MO). Dexamethasone treatment protocol was administered as described by Ito et al¹⁴ and shown in supplement figure E2. Briefly mice were given oral dexamethasone (20µg/mouse)¹⁴ dissolved in 0.5ml water at 24 hours and 2 hours prior to RW challenge. ASHMI^{II} treatment was given as described above.

Measurement of airway hyperreactivity (AHR)

Airway reactivity to acetylcholine (ACh) provocation was measured 48 hours following the final challenge (day 46), using an invasive method of measuring airway pressure time index (APTI), that measures airway pressure changes, as previously described.^{11, 27} Mice anesthetized with a pentobarbital (80mg/kg body wt i.p.)/xylazine (12mg/kg body wt i.p.) were ventilated via a tracheal cannula (18 gauge) at the rate of 120 breaths/min and a constant tidal volume of air (0.2 ml) with a RSP1002 Pressure Controlled Respirator System (Kent Scientific Corporation, Litchfield, CT). Muscle paralysis was induced by injection of decamethonium bromide (25 mg/kg i.v.). 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, ACh was injected (100mg/kg i.v.). Airway pressure changes were recorded for 4 min and respiratory data were acquired and analyzed using DaisyLab software (Kent Scientific Corporation). Airway responsiveness to ACh was expressed as time-integrated changes in peak airway pressure, referred to as APTI (cm H₂O per second).

Evaluation of BAL fluid cells, cytokines and chemokines

Following airway measurements, bronchoalveolar lavage (BAL) fluids were collected by lavaging airways as previously described.²⁷ BAL fluids were centrifuged at 1200 rpm for 15 minutes at 4°C to isolate cells. Supernatants were stored at -80°C for cytokine analysis. After counting, approximately 40×10³ 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 ELISA kits according to the manufacturer's instructions (R&D Systems, MN) for IL-13 and IL-8 (CXCL1/KC); (BD Biosciences, San Diego, CA) for IL-4, IL-5, IL-13, IL17, IFN-γ and TNF-α, IL-10.

Lung histology

After obtaining BAL fluid samples, lungs were removed and fixed in formalin and processed by the Mount Sinai Histology Shared Facilities. 5 µm sections were stained with hematoxylin and eosin (H&E) for the evaluation of inflammation and periodic acid Schiff's (PAS) stain for evaluation of goblet cells. Images were captured using an Axioplan 2 microscope at the Mount Sinai Microscopy Facility. Airway pathology was assessed by two 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 supplementary information (E Methods1)

Determination of GAC1 effects on RW stimulated macrophage TNF- α production

The mouse macrophage cell line RAW264.7 (ATCC, Manassas, VA) was maintained in Dulbecco's Modified Eagles Medium (DMEM, Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 1% penicillin/streptomycin (Mediatech, Manassas, VA). 5×10^5 cells were first cultured with 40 μ g/ml of GAC1 for 24hrs. RW (200 μ g/ml) was then added and cells were cultured for an additional 24hrs. Medium containing of 9 pg/ml 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 ELISA according to the manufacturer's instructions (BD Bioscience, San Diego, CA).

In-Cell Western Blot for measurement of pI κ B- 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 pg/mL) or RW (200 μ g/ml) for 5, 10, 30, and 60 min. In-Cell Western Assay (Li-Cor, Lincoln, Nebraska) was performed according to the manufacturer's instructions utilizing antibodies against phospho-I κ B- α (1/1000) or HDAC2 (1/1000) (Cell Signaling Technology, Danvers MA) with β -actin (1/1000) (Santa Cruz Biotechnology, Birmingham AL) as a loading control. This was followed by incubation with secondary antibodies IRDye800CW donkey anti-goat (1/1000) and IRDye680RD donkey anti-rabbit (1/1000) (Li-Cor). Plates were scanned, fluorescence detected at 700 and 800 nm, and data normalized using an Odyssey CLx Infrared Imaging System.

Statistical analysis

Data were analyzed by SigmaStat 2.03 software (SPSS Inc. Chicago, IL) using analysis of Variance (One way ANOVA). When significant differences were indicated, differences between groups of interest were analyzed by Student's t-test followed by Tukey's post-test. Mann-Whitney Rank Sum Test was applied when data were not normally distributed. P values ≤ 0.05 were considered significant. Power and sample size (Power = 0.8, $\alpha = 0.05$) were calculated based on our preliminary results of ASHMI effects on airway hyperreactivity measurements and minimum of 4 mice per experimental group were needed to demonstrate statistically significant results.

Results

Treatment with ASHMITM or its refined form (ASHMI^{II}) reduced AHR in RW sensitized and challenged mice

RW sensitization and challenge induced marked increases in AHR as indicated by elevated APTI values following ACh provocation when compared to 'alum control' and 'naïve' mice (Figure 2, Pitalic>0.01 RW/Sham vs. alum control; Pbold>0.01 RW/Sham vs. naïve). ASHMITM and ASHMI^{II} treated mice exhibited marked reductions in AHR when compared to RW/sham treated mice (P<0.01 for both, Figure 2).

ASHMITM or ASHMI^{II} treatment suppressed neutrophil-predominant airway inflammation

BAL fluid obtained from RW/sham mice 48 hours after final RW challenge contained significantly increased numbers of total leukocytes compared to BAL fluid from alum control and naïve groups (Figure 3A, $p < 0.01$). BAL fluid from ASHMITM and ASHMI^{II} - treated mice contained significantly fewer total leukocytes ($P < 0.01$ for both, Figure 3A). Differential analysis of BAL fluid cells showed a nearly three-fold higher percent of neutrophils ($32.5 \pm 2.9\%$) compared to eosinophils ($11.9 \pm 2.1\%$) in RW/sham mice, confirming neutrophil predominant airway inflammation (Figure 3B). The percentage of combined granulocytes (neutrophils/eosinophils) in RW/Sham mice ($44.6 \pm 3.7\%$) was significantly reduced by over 90 % by ASHMITM ($7.3 \pm 0.7\%$) and ASHMI^{II} ($6.2 \pm 1.2\%$) treatment ($P < 0.01$ – 0.001 vs. sham, Figure 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 naïve mice, and was nearly eliminated in the BAL fluid collected from ASHMITM and ASHMI^{II} treated mice (Figure 3C). These findings demonstrate that RW induced a predominantly neutrophilic airway inflammation, and that ASHMITM and ASHMI^{II} suppressed neutrophilic as well as eosinophilic inflammation in this murine model.

Treatment with ASHMI formulas reduces pulmonary pathology and mucus hypersecretion

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

ASHMITM and ASHMI^{II} treatment modified the Th2/Th1 immune responses and reduced eotaxin-1 levels

We showed previously that treatment with ASHMI down-regulated Th2 immune responses without global immune-suppression in allergic individuals and murine allergic asthma models.^{9, 11} Similarly, in this study, the Th2 cytokines IL-5 and IL-13 were induced in the BAL fluid following RW sensitization and challenge, but not following negative control conditions ($P < 0.01$, Figure 5A, B). IL-13 (588.4 ± 56.6 pg/ml in RW/Sham) and IL-5 (544.8 ± 57.0 pg/ml in RW/Sham) levels in were significantly reduced reduced by approximately 70 and 90% respectively in BAL fluid of ASHMITM (158.2 ± 18.5 pg/ml for IL-13; 102.6 ± 20.3 pg/ml for IL-5) and ASHMI^{II} (100.5 ± 8.1 pg/ml for IL-13; 35.2 ± 10.3 pg/ml for IL-5) treated mice (Figure 5A, B $P < 0.01$ – 0.001 for all vs. RW/sham). In addition, BAL fluid interferon- γ levels were increased in both RW/ASHMITM (363.4 ± 35.2 pg/ml), RW/ASHMI^{II} (32.1 ± 30.1 pg/ml)-treated mice when compared to the RW/sham treated mice (107.2 ± 29.7 pg/ml), $P < 0.001$ for both, Figure 5C. There were no changes 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 ASHMITM and ASHMI^{II} treated groups ($P < 0.001$, Figure 5D). Consistent with Th1 and Th2 cytokine profiles, serum RW-

specific IgE levels were significantly reduced in ASHMITM and ASHMI^{II} treated mice when compared with RW/sham mice (Figure 5E, $P<0.01$ for both) whereas Th1-associated IgG2a levels were significantly increased in ASHMITM ($P<0.05$, Figure 5F) treated mice and a trend towards increase was observed for mice given ASHMI^{II} ($P=0.06$, Figure 5F).

ASHMITM and ASHMI^{II} treatment markedly reduced TNF- α , IL-17 and IL-8 levels

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 (Figure 6A), IL-8 (Figure 6B) and IL-17 (Figure 6C) in RW/sham mice were elevated when compared to alum control and naïve mice ($P<0.01$ - 0.001 for all). Upon treatment with ASHMITM and ASHMI^{II}, mice exhibited marked and statistically significant reductions in TNF- α (In pg/ml: 323.6 ± 155.7 for RW/Sham, 24.8 ± 16.9 for RW/ASHMI and 34.4 ± 14.4 for RW/ASHMI^{II}), a 4-fold reduction in IL-8 (In pg/ml: 581 ± 107.7 for RW/Sham, 137.8 ± 29.6 for RW/ASHMI and 93.2 ± 31.6 for RW/ASHMI^{II}) and a marked reduction in IL-17 levels (In pg/ml: 85.4 ± 6.9 for RW/Sham, 4.0 ± 4.0 for RW/ASHMI and 1.8 ± 1.8 for RW/ASHMI^{II}) $P<0.01$ - 0.001 for all. For IL-17, reductions on average were similar to that measured among alum control and naïve mice.

GAC1 suppressed RW stimulated macrophage TNF- α production by reducing pI κ B and increasing HDAC2 expression

To facilitate identification of ASHMI herbal constituents that contribute to the formula's suppression of neutrophil-dominant airway inflammation, we employed the mouse macrophage cell line RAW 264.7. Macrophages are an established source of pro-inflammatory 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 *GL*, but not *SF* or *GU*, extracts suppressed TNF- α production in response to LPS stimulation (data not shown). Ganoderic acid C1 (GAC1) isolated from *GL* was the most potent inhibitor of TNF- α production (Liu et al, manuscript in preparation) and was then selected for further analyses in the current study. GAC1 significantly reduced RW stimulated TNF- α production by over 60% (In pg/ml: 3537.2 ± 3.1 for RW, 1424.5 ± 95.5 for RW + GAC1, $p<0.01$ Figure 7A) without cytotoxicity (Figure 7B). In addition, TNF- α levels in medium containing background levels of LPS (9 pg/mL the RW extract) were not different from medium alone. This demonstrated that RW induced TNF- α production is not due to the LPS contamination.

In order 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 ($70.3\pm8.1\%$ of medium for RW+GAC1 vs $137.3\pm14.9\%$ of medium for RW, $P<0.05$, Figure 7C) implying suppression of the NF κ B pathway. Stimulation of RAW264.7 cells with RW led to a significant decrease in HDAC2 ($85.0\pm2.2\%$ of medium), suggestive of impaired transcriptional repression that was reversed with pretreatment with GAC1 ($98.3\pm1.2\%$ of medium, $P<0.05$, Figure 7D).

Treatment with ASHMI^{II} but not dexamethasone decreased AHR and neutrophilic inflammation in response to RW challenge

As neutrophil predominant asthma in humans and murine models has been shown to be steroid resistant^{5, 14} we wished to determine steroid responsiveness of our asthma model and compare effects of steroid treatment to therapy with ASHMI^{II}. Using a dexamethasone (DEX) treatment regimen used previously by Ito et al¹⁴ we found that acute treatment with DEX did not significantly decrease AHR (491.2 ± 43.7 vs 543.6 ± 34.2 cmH₂O/sec for RW/Sham, Figure 8A), and total BAL fluid cellular infiltrate in DEX-treated mice was similar to RW/sham mice ($380,513 \pm 30,893$ cells for RW/Dex vs $433,680 \pm 43,309$ cells for RW/Sham, Figure 8B). Furthermore, while DEX treatment reduced the percentage of eosinophils in BAL fluid ($2.9 \pm 0.5\%$ for RW/DEX vs $14.1 \pm 1.5\%$ for RW/Sham, $P < 0.001$), percentage of neutrophils was slightly increased ($44.4 \pm 2.2\%$ for RW/DEX vs $31.8 \pm 2.5\%$ for RW/Sham, Figure 8C). In contrast, mice given ASHMI^{II} showed markedly and statistically lower AHR (152.4 ± 15.1 cm H₂O/sec) to RW challenge when compared to RW/DEX mice ($P < 0.001$) and RW/Sham mice ($P < 0.001$) as shown in Figure 8A. ASHMI^{II} treated mice also showed significant reductions in total BAL fluid cells ($194,140 \pm 27,009$ cells), eosinophil ($5.12 \pm 1.2\%$) and neutrophil ($8.2 \pm 1.6\%$) percentage in comparison to RW/DEX and RW/Sham animals ($P < 0.01$ to $P < 0.001$ for all, Figure 8B,C). AHR following RW challenge in mice treated with ASHMI and ASHMI^{II} was also significantly reduced compared to RW/DEX mice ($P < 0.001$) and RW/Sham mice ($P < 0.001$) as shown in Figure 8A.

Discussion

Over 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 al recently showed that chronic aerosol OVA exposure of OVA/alum sensitized BALB/c mice induced neutrophil-predominant airway inflammation¹⁴, whereas a model used by Bogaert et al¹⁵ employed aerosol challenges of OVA plus complete Freund's adjuvant (CFA) of OVA sensitized C57BL/6 mice. Liu et al recently developed a neutrophil-predominant but mixed airway inflammation in murine model using a mixture of dust mite, *Aspergillus* and RW extracts.¹⁶ In the present study, we employed 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 OVA-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 significantly higher (more than 10 fold) BALF neutrophil percentage than OVA following suboptimal protocol in our preliminary study, while the RW endotoxin level (9pg/ml) was only 0.001% that of OVA (12ng/ml). The ability of RW to induce neutrophilic inflammation has

previously demonstrated to be attributable to intrinsic NADPH oxidases.³¹ Using this model, we demonstrated that ASHMITM and ASHMI^{II} treatment significantly suppressed both neutrophil and eosinophil airway inflammation via regulation of associated chemokine and cytokines. Additionally we observed significant reductions in AHR, mucus 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, Th2 responses are associated with eosinophil predominant inflammation whereas innate pro-inflammatory responses including TNF- α and IL-8 production are associated with neutrophilic inflammation.^{32, 33} Similar immunological and inflammatory phenotypes also have been recently characterized in murine asthma models.¹⁵ TNF- α contributes to multiple aspects of asthma pathology including AHR, mucus 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 showed that, in addition to suppression of Th2 responses, ASHMITM and ASHMI^{II} treatment resulted in marked reductions in BAL fluid chemokine and cytokine levels involved in neutrophil as well as 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 showed that GAC1, a triterpinoid 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 (ASHMITM) 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 ASHMITM 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 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 and IL-17 and Th1/Th2 cytokine responses. The in vitro models suggested that the compound GAC1 inhibited TNF- α production through suppression of NF- κ B activity due, 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 Th2 transcription factor GATA3^{43, 44} are underway. Because standard asthma therapy including inhaled corticosteroids is less effective for severe neutrophilic asthma⁴, ASHMITM and ASHMI^{II} may be of value for the treatment of neutrophil-predominant asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental Protocol

Mice sensitized with RW (and alum) on days 0 and day 7 received intranasal RW on day 14. Alum control group mice received alum only on days 0 and 7, and intranasal PBS on day 14. Mice in all groups except the naïve group were intranasally challenged with RW on days 43 and 44. Airway reactivity was measured on day 46. D=Day

Figure 2. ASHMI™ and its refined form, ASHMIII reduced AHR in RW-challenged mice
Airway responsiveness to ACh was expressed as Airway Pressure Time Index (APTI). Data are expressed as Mean±SD. N=5 mice/group. **=P<0.01. Cntrl=control

Figure 3. Treatment with ASHMI™ or ASHMI™ decreased neutrophil/eosinophil airway inflammation

A. Total number of BAL fluid leukocytes. B. Percent BAL fluid macrophages (black), eosinophils (red) and lymphocytes (blue) neutrophils (yellow). C. Representative illustration of neutrophil predominance among granulocytes (black arrows point to neutrophils; red arrows point to eosinophils) in BAL fluid from RW/sham mice, which was markedly reduced compared to RW/ASHMI™ and RW/ASHMI™ treated mice. Data for A,B expressed as Mean ± SD. N=5 mice/group. * =P<0.05; **=P<0.01; *** =P<0.001

Figure 4. Airway tissue inflammation and mucus hyperproduction is diminished in asthmatic mice treated with ASHMI formulas

A–E. H&E staining of lung sections of formalin fixed lungs. F–J. Lung sections stained with PAS that stains mucus with a magenta color. Data are representative of 5 mice/group.

Figure 5. Treatment with ASHMITM and ASHMI^{II} inhibited Th2 and increased Th1 responses BAL fluid IL-5 (A), IL-13 (B), IL-IFN- γ (C), Eotaxin (D), Ragweed-specific IgE(E) and IgG2a (F) were measured by ELISA. Data expressed as Mean $\pm$ SD. N=5mice/group. **=P<0.01; ***=P<0.001

Figure 6. Cytokines and chemokines involved in neutrophilic inflammation were decreased following treatment with ASHMI[™] or its refined form

BAL fluid TNF- α (A), IL-8 (B), IL-17 (C) Data expressed as Mean $\pm$ SD. N=5mice/group.

** =P<0.01; *** =P<0.001

Figure 7. GAC1-mediated reduction in TNF-α production in vitro by RW stimulated murine macrophages was associated with decreased pIκB and increased HDAC2 expression
 (A) Effect of GAC1 on TNF-α production by macrophages stimulated with 200μg/ml RW in the presence and absence of GAC1. (B) Cell viability for experiment shown in A. (C) Phospho-IκB expression and (D) HDAC2 levels measured by in-cell western of macrophages stimulated with 200μg/ml RW in the presence and absence of GAC1 treatment. Data for (C) and (D) shown as percentage of medium alone levels. * = p < 0.05

Figure 8. Treatment with ASHMI^{II} but not dexamethasone reduces AHR and neutrophilic inflammation in response to RW challenge

A- Airway responsiveness to ACh was expressed as Airway Pressure Time Index (APTI).

B- Total number of BAL fluid leukocytes. C-Percent BAL fluid macrophages (black), eosinophils (red) and lymphocytes (blue) neutrophils (yellow). Data are expressed as Mean $\pm$ SD. N=5 mice/group. ** =P<0.01; *** =P<0.001 vs RW/Sham; ###, P<.001 vs RW/ASHMI^{II}