

ORIGINAL ARTICLE

Anti-Asthma Simplified Herbal Medicine Intervention-induced long-lasting tolerance to allergen exposure in an asthma model is interferon- γ , but not transforming growth factor- β dependent

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Summary

Background Chronic allergic asthma is the result of a T-helper type 2 (Th2)-biased immune status. Current asthma therapies control symptoms in some patients, but a long-lasting therapy has not been established. Anti-Asthma Simplified Herbal Medicine Intervention (ASHMI™), a Chinese herbal formula improved symptoms and lung function, and reduced Th2 responses in a controlled trial of patients with persistent moderate to severe asthma.

Objective We evaluated the persistence of ASHMI™ beneficial effects following therapy in a murine model of chronic asthma and the immunological mechanisms underlying such effects.

Methods BALB/c mice sensitized intraperitoneally with ovalbumin (OVA) received 3 weekly intratracheal OVA challenges to induce airway hyper-reactivity (AHR) and inflammation (OVA mice). Additionally, OVA mice were treated with ASHMI™ (OVA/ASHMI™) or water (OVA/sham) for 4 weeks, and then challenged immediately and 8 weeks post-therapy. In other experiments, OVA mice received ASHMI™ treatment with concomitant neutralization of IFN- γ or TGF- β . Effects on airway responses, cytokine- and OVA-specific IgE levels were determined 8 weeks post-therapy.

Results Before treatment, OVA mice exhibited AHR and pulmonary eosinophilic inflammation following OVA challenge, which was almost completely resolved immediately after completing treatment with ASHMI™ and did not re-occur following OVA re-challenge up to 8 weeks post-therapy. Decreased allergen-specific IgE and Th2 cytokine levels, and increased IFN- γ levels also persisted at least 8 weeks post-therapy. ASHMI™ effects were eliminated by the neutralization of IFN- γ , but not TGF- β , during therapy.

Conclusion ASHMI™ induced long-lasting post-therapy tolerance to antigen-induced inflammation and AHR. IFN- γ is a critical factor in ASHMI™ effects.

Keywords allergic asthma, IgE, IFN- γ , mice, Th2 cytokines, traditional Chinese medicine

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Introduction

Allergic asthma is an increasingly common disease in western societies [1]. Standard therapy for asthma using a combination of steroids and β_2 -adrenergic agonists controls symptoms in most but not all asthmatics [2, 3]. Relapse after therapy withdrawal is common [4], and the adverse effects of steroids on growth in children, steroid-induced immunosuppression and HPA-axis disturbances are of some concern [5–7]. Anti-IgE therapy is beneficial for some asthmatics; however, its effects are transient and cysteinyl leukotriene inhibitors have shown only limited efficacy [8, 9]. Recently,

safety concerns regarding both of these therapies have been raised [10, 11]. Thus, a safe, effective and long-lasting therapy remains to be established.

In recent years, a small, but increasing number, of well-controlled clinical trials provide evidence that some traditional Chinese medicines may have potential for the development of asthma medications [12]. In pre-clinical studies, we showed that the Anti-Asthma Simplified Herbal Medicine Intervention (ASHMI™), an extract of three Chinese medicinal herbs, exerted broad-based protective effects on asthma pathology and skewed T-helper type 2 (Th2)-dominant responses to Th1 in mice. We also found

that *Glycyrrhiza uralensis*, a key herbal component of ASHMI™-reduced eotaxin production by human pulmonary fibroblasts [13, 14]. In humans, ASHMI™ was effective in a controlled trial of subjects with moderate to severe asthma [15]. ASHMI™ suppressed Th2 cytokines while increasing IFN- γ . This beneficial immunomodulatory effect was also reported in children receiving ASHMI™ treatment as a complementary therapy to inhaled steroid [12]. ASHMI™ is currently in clinical trials in the United States and early reports indicate that it is well tolerated [16]. However, whether this treatment has a long-lasting effect, and whether INF- γ or perhaps other cytokines such as IL-10 or TGF- β , are essential in ASHMI™-mediated clinical effect and Th2 suppression is unknown.

Using a well-established murine model of asthma, our current study seeks to test the persistence of ASHMI™-mediated protection from allergic asthma. Furthermore, we sought to determine the functional contribution of ASHMI™-induced elevation of IFN- γ to the persistent benefits of ASHMI™ therapy. We found that 4-week ASHMI™ treatment completely reversed asthmatic characteristics. ASHMI™-treated mice were tolerant to ovalbumin (OVA) challenges for at least 8 weeks post-therapy. Lung Th2 cytokine and OVA-specific IgE levels were also significantly lower, but IFN- γ levels were higher 8 weeks post-therapy. In additional experiments, mice were treated with concomitant neutralization of IFN- γ or TGF- β . ASHMI™-induced therapeutic effects were virtually eliminated by the neutralization of IFN- γ , but not TGF- β during therapy, indicating that increased IFN- γ is essential for persistent benefits of ASHMI™ treatment in mice.

Methods

Anti-Asthma Simplified Herbal Medicine Intervention preparation and quality control

ASHMI™ is an extract of three TCM herbs – *Ganoderma lucidum* (Ling-Zhi), *Sophora flavescens* Ait (Ku-Shen) and *Glycyrrhiza uralensis* Fischer (Gan-Gao). ASHMI™ was prepared by the Sino-Lion Pharmaceutical Company (a GMP-certified facility; Weifang, China) as described previously [16]. Composition, quality control and chemistry analysis have been described in extensive detail previously [13, 16]. Endotoxin levels in ASHMI™ were tested using the Pyrogen Plus assay kit (Lonza, Hopkinton, MA, USA). No endotoxin was detected in ASHMI™ (<0.03 EU/mL, the limit of sensitivity for this kit).

Protocol for induction of asthma, Anti-Asthma Simplified Herbal Medicine Intervention treatment and post-therapy evaluation

Six-week-old BALB/c (Jackson Laboratory, Bar Harbor, ME, USA) female mice sensitized twice with weekly

intraperitoneal (i.p.) injections of 200 μ g, OVA (Type V; Sigma-Aldrich, St Louis, MO, USA) and 2 mg of alum (Thermo Scientific, Rockland, IL, USA) in 0.4 mL of phosphate buffered saline (PBS), were challenged intratracheally with 100 μ g of OVA in 50 μ L of PBS weekly for 3 weeks (Fig. 1a). Forty-eight hours after the third challenge, airway pressure time index (APTI) measurements and bronchoalveolar lavage (BAL) were performed in a group of OVA mice compared with naïve mice ($n = 5$ per group). Additional groups of OVA mice were given 9 mg of ASHMI™ in 0.5 mL of drinking water twice daily (OVA/ASHMI™) or water (OVA/sham) by oral gavage for 4 weeks. The ASHMI™ dose was determined by a conversion table of equivalent human to animal dose ratios based on body surface area [17]. Naïve mice served as normal controls. Post-therapy evaluations for airway hyper-reactivity (AHR) and airway inflammation were performed at 1 day, 4 weeks and 8 weeks post-therapy on separate groups of mice (Fig. 1a).

Protocol for examining the role of interferon- γ or transforming growth factor- β in Anti-Asthma Simplified Herbal Medicine Intervention long-lasting post-therapy effect

ASHMI™ was administered to OVA mice as above for 6 weeks in additional experiments (Fig. 4a). Anti-IFN- γ (clone H22, 100 μ g/week, i.p.) or anti-TGF- β (clone ID11, 100 μ g/week, i.p.) antibodies (Abs), both purchased from R&D Systems (Minneapolis, MN, USA) or appropriate isotype control Abs (Hamster IgG1 for anti-IFN- γ and mouse IgG1 for anti-TGF- β , 100 μ g/week, BD Biosciences, San Diego, CA, USA) were administered i.p. to ASHMI™ or sham-treated mice, 1 day before treatment and then weekly for 6 weeks. All mice (except naïve mice) were re-challenged as above up to 8 weeks post-therapy. Forty-eight hours after the final OVA challenge, airway responses and cytokine profiles were determined in all mice.

Airway hyper-reactivity measurement and bronchoalveolar lavage fluid cell differential counts

AHR to acetylcholine provocation was measured following a challenge at time-points indicated in Figs 1a and 4a by measuring APTI, a classical invasive measure of AHR [18, 19]. Mice were killed immediately following APTI measurements and bronchoalveolar lavage fluid (BALF) and lung tissues were collected. Cytospin slides of BALF cells were stained with HEMA 3 stain (Fisher Scientific, Fair Lawn, NJ, USA) for differential cell counting [20].

Lung histology

Formalin-fixed lung samples were processed at the Mount Sinai Histology Shared Facilities. Sections were stained

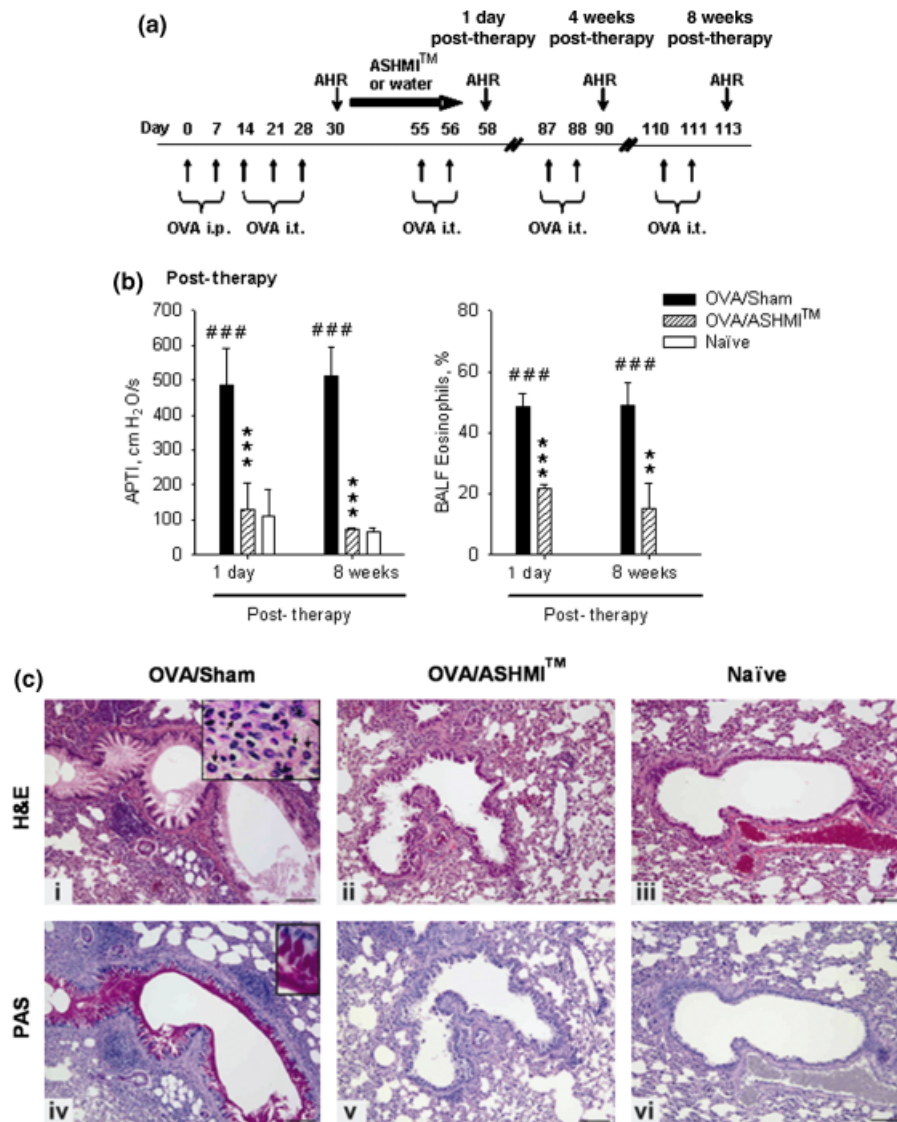


Fig. 1. (a) Protocol: Mice were sensitized and challenged with ovalbumin (OVA) at times indicated. Daily oral ASHMITM treatment lasted for 4 weeks. Mice were subsequently challenged 1 day, 4 and 8 weeks post-therapy. (b) Airway hyper-reactivity (left panel) and % of bronchoalveolar lavage eosinophils (right panel) were measured 48 h after the 5th OVA challenge (day 58) and the 9th OVA challenge (day 113). Data shown as mean ± SD in individual samples ($N = 4-5$ mice/group). ** $P < 0.01$; *** $P < 0.001$ vs. Sham; ###, $P < 0.001$ vs. Naïve. (c) panels i-iii – Haematoxylin and eosin staining of lung sections from formalin-fixed unaltered left lung. Inset in panel i shows eosinophils indicated by arrows. (c) panels iv-vi and inset shows lung sections stained with periodic acid Schiff that stains mucin with a magenta colour. Bar indicates 100 µm.

with haematoxylin and eosin for the evaluation of inflammation. Periodic acid Schiff (PAS) stain was used for the assessment of mucus secretion and percentage of PAS-positive cells/bronchiole was calculated. Three to five bronchioles were counted per slide (12–20/group). Collagen deposition was stained using trichrome staining, and peribronchial collagen deposition was analysed by morphometric methods used previously [21]. The area of peribronchial trichrome staining was outlined and quantified on high-resolution digital images captured using an Olympus B × 60 microscope and analysed using Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA). Results

are expressed as the area of trichrome staining per micron length of basement membrane of bronchioles. Three bronchioles per slide were analysed (12/group).

Splenocyte culture and measurement of splenocyte and bronchoalveolar lavage fluid cytokines

Following APTI measurements, splenocytes from each animal were prepared and BALF was collected. Splenocytes were cultured in the presence or absence of 50 µg/mL OVA or 2.5 µg/mL Concanavalin A (Con A) (data not shown) for 72 h. Cytokine levels (IL-4, IL-5, IL-10, IL-13, IFN-γ and

TGF- β) in splenocyte or BALF supernatants were assayed using commercial ELISA kits according to the manufacturer's instructions (R&D Systems for IL-13 and TGF- β and BD Biosciences for all others) as described previously [22].

Measurements of Ag-specific Immunoglobulin E levels

Serum was harvested from blood collected immediately after APTI measurements. OVA-specific IgE was measured as described previously [19]. Serum samples were evaluated using OVA-coated plates and detected using sheep anti-mouse IgE (The Binding Site, San Diego, CA, USA) followed by biotinylated donkey anti-sheep IgG (Accurate Chemical and Scientific Co., Westbury, NY, USA). Avidin-horseradish peroxidase and ABTS substrate were used for colour development.

Statistical methods

Data were analysed by SigmaStat 2.03 software (SPSS Inc, Chicago, IL, USA) using two-way ANOVA for time course IgE data and one-way ANOVA for all other data, followed by pair-wise testing using Bonferroni's adjustment. For data that failed tests for normality and equal variance, differences between groups were analysed by the Kruskal–Wallis one-way ANOVA on Ranks followed by all pair-wise comparisons. *P*-values ≤ 0.05 were considered significant.

Results

Anti-Asthma Simplified Herbal Medicine Intervention produced long-term post-therapy suppression of allergic airway responses

ASHMITM treatment commenced after the third OVA challenge (Fig. 1a). At this time, asthmatic responses were fully established (supporting information Fig. S2). Immediately after completing treatment, OVA/sham mice showed sustained AHR following OVA re-challenge, evidenced by significantly higher APTI levels than naïve mice ($P < 0.001$, Fig. 1b, left panel). In contrast, OVA/ASHMITM mice showed complete resolution of AHR evidenced by normal APTI levels. Importantly, OVA/ASHMITM mice showed no AHR in response to subsequent OVA re-challenges up to 8 weeks post-therapy, whereas OVA/sham mice showed sustained AHR. Consistently, immediately after completing treatment, eosinophil numbers were significantly reduced in OVA/ASHMITM mice as compared with the OVA/sham mice ($P < 0.01$ – 0.001 , Fig. 1b, right panel), and similar results were observed 8 weeks post-therapy 48 h following the final antigen challenge ($P < 0.01$ – 0.001 vs. sham).

Histological analysis also showed near normal features in OVA/ASHMITM mice, similar to naïve mice immediately (data not shown) and 8 weeks post-therapy (Fig. 1c). Consistent with the BAL data, OVA/sham mice exhibited

pronounced peribronchial inflammation containing numerous eosinophils (Fig. 1c, panel i and indicated by arrows in inset). As illustrated in Fig. 1c, panel ii, inflammation was dramatically reduced in the lungs from OVA/ASHMITM mice. Numerous goblet cells were present in airways of OVA/sham mice as shown by PAS-positive staining (Fig. 1c, panel iv and inset). Goblet cells were almost absent in airways of OVA/ASHMITM mice (Fig. 1c, panel v), which were similar in appearance to those of naïve mice (Fig. 1c, panel vi). Taken together, these findings show that ASHMITM was effective in reversing established allergic airway changes in an established asthma model, and that this effect lasted at least 8 weeks post-therapy during which time six OVA re-challenges were administered.

Anti-Asthma Simplified Herbal Medicine Intervention induced persistent suppression of T-helper type 2 immune responses

Th2 cytokines, IL-4, IL-5 and IL-13 were significantly lower in BALF from OVA/ASHMITM mice, compared with BAL from OVA/sham mice at 8 weeks post-therapy ($P < 0.01$ – 0.001 , Fig. 2a). However, the Th-1 cytokine

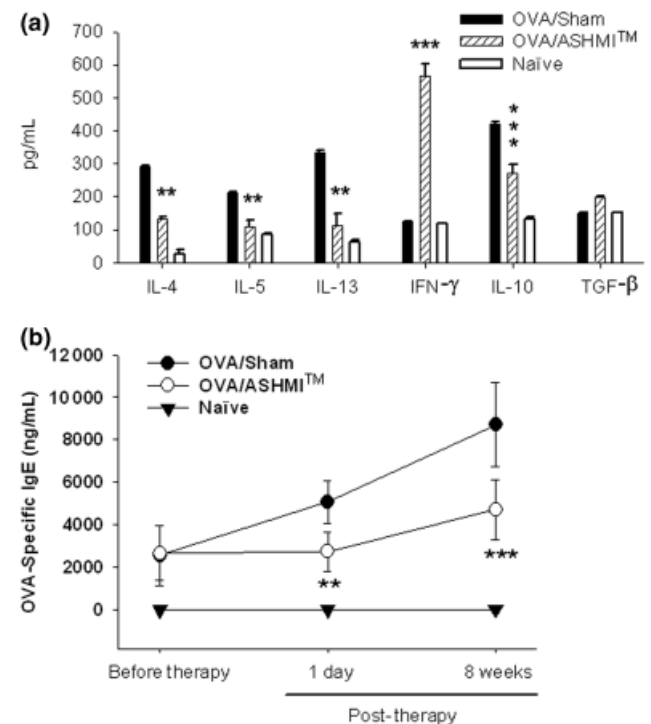


Fig. 2. (a) Cytokines in bronchoalveolar lavage fluid harvested 48 h after the final set of ovalbumin (OVA) challenges were determined by ELISA. (b) OVA-specific IgE in serum that was collected from mice one day before ASHMITM therapy, 4 weeks and 8 weeks after stopping ASHMITM therapy was measured by ELISA. Results are expressed as mean $\pm$ SD of pooled samples in triplicate for cytokines and individual samples for IgE. ($n = 4$ – 5 /group). Results were replicated at least once in a separate experiment. ** $P < 0.01$ and *** $P < 0.001$ vs. Sham.

IFN- γ was markedly elevated in BALF from OVA/ASHMITM mice ($P < 0.001$ vs. sham). In addition, IL-10 production was significantly reduced in BALF ($P < 0.001$ vs. sham). TGF- β levels were slightly but not significantly increased in BALF from OVA/ASHMITM mice compared with BAL from OVA/sham mice. These results were similar to those observed immediately following challenge (data not shown), and demonstrate that the therapeutic effect of ASHMITM was associated with persistent, specific suppression of Th2 responses and increased IFN- γ levels.

In addition, elevated OVA-specific IgE levels of OVA mice were significantly lower in OVA/ASHMITM mice than OVA/sham mice immediately after completing the treatment ($P < 0.001$). Following the stoppage of treatment, a trend towards increased IgE was observed in both sham and ASHMITM-treated mice likely due to additional challenges. However, at all post-therapy time-points, OVA-specific IgE levels were significantly reduced in OVA/ASHMITM-treated, compared with OVA/sham mice ($P < 0.001$, Fig. 2b).

Anti-Asthma Simplified Herbal Medicine Intervention protection against airway hyper-reactivity and inflammation was eliminated by interferon- γ , but not transforming growth factor- β neutralization

Using an antibody neutralization protocol described in Fig. 3a, ASHMITM suppression of AHR was abolished with the co-administration of anti-IFN- γ but not isotype control Abs during treatment (Fig. 3b ASHMITM vs. ASHMITM + anti-IFN- γ , $P < 0.05$), when evaluated 8 weeks post-therapy. ASHMITM suppression of AHR was not affected by anti-TGF- β or isotype control Abs. Administration of anti-IFN- γ or anti-TGF- β Abs during treatment had no effect on APTI of OVA-sham mice. Similarly, ASHMITM suppression of eosinophilic inflammation was eliminated by the co-administration of anti-IFN- γ Abs (Fig. 3c, ASHMITM vs. ASHMITM + anti-IFN- γ , $P < 0.001$), but not isotype control Abs. ASHMITM suppression of eosinophils was not affected by anti-TGF- β or isotype control Abs. Administration of anti-IFN- γ or anti-TGF- β Abs during treatment had no effect on the percentage of eosinophils in the BAL of OVA/sham mice. Histological analysis also showed that ASHMITM + anti-IFN- γ Abs, but not ASHMITM + anti-TGF- β Abs restored peribronchial inflammatory infiltration, similar to the OVA/sham (Fig. 3d).

Interferon- γ neutralization abrogates Anti-Asthma Simplified Herbal Medicine Intervention-induced reduction of mucus cell generation and collagen deposition

Mucus-positive goblet cells (stained by PAS) were abundant in airways of sham-treated OVA mice but dramatically decreased in those of ASHMITM-treated OVA mice (Fig. 4a, left panel and Fig. 4b, $P < 0.05$ vs. sham). The

number of goblet cells in ASHMITM-treated mice given IFN- γ -neutralizing Abs was similar to that of sham-treated animals and significantly higher than ASHMITM only-treated mice (Fig. 4a, left panel and Fig. 4b, $P < 0.05$ vs. ASHMITM alone). Also, we observed strong positive peribronchial and perivascular staining for collagen in lung sections of sham-treated mice (Fig. 4, right panel). This was considerably reduced in ASHMITM-treated mice ($P < 0.05$ vs. sham, Fig. 4c) but not in ASHMITM-treated, IFN- γ -neutralized animals ($P < 0.05$ vs. ASHMITM alone). TGF- β neutralization or isotype control Abs did not alter ASHMITM effect on mucus hyperplasia or collagen deposition.

Anti-Asthma Simplified Herbal Medicine Intervention suppression of T-helper type 2 cytokines is largely interferon- γ dependent

BALF IL-4, IL-5, IL-13 and IL-10 levels from ASHMITM-treated OVA mice (second bars) were significantly lower than those from sham-treated mice (first bars) ($P < 0.05$ – 0.001 , Figs 5 a–c and e). IFN- γ and TGF- β levels were higher ($P < 0.001$ and 0.01 , respectively, Figs 5d and f). Neutralization of IFN- γ (third bars) completely attenuated ASHMITM reduction of IL-4 and IL-5 ($P < 0.05$ – 0.001 vs. ASHMITM alone), where levels of these cytokines were similar to those observed in the sham group. ASHMITM suppression of IL-10 and IL-13 was dramatically but not completely reversed by IFN- γ neutralization because IL-10 and IL-13 levels in this group were still lower than sham ($P < 0.001$ for IL-10 and $P = 0.055$ for IL-13, ASHMITM + anti-IFN- γ vs. sham). As expected, ASHMITM + anti-IFN- γ treatment reduced IFN- γ ($P < 0.001$), but it had no effect on TGF- β , implying that ASHMITM-mediated reduction in Th2 cytokines is largely IFN- γ dependent. TGF- β neutralization had minimal effect on ASHMITM reduction of IL-4 and IL-13, but significantly increased IL-5 levels ($P < 0.05$ ASHMITM + anti-TGF- β Ab vs. ASHMITM). Anti-IFN- γ or TGF- β isotype-controlled Abs did not affect ASHMITM effects. In addition, anti-IFN- γ and TGF- β Abs had no effects on cytokine production in the sham-treated OVA mice. This might be attributable to anti-IFN- γ Abs being administered when Th2-mediated allergic airway responses were already established, and IFN- γ levels were negligible. These results were similar to those observed in splenocyte culture supernatants to recall OVA stimulation 8 weeks post-therapy (Fig. S3).

Anti-Asthma Simplified Herbal Medicine Intervention persistently suppressed ovalbumin-specific Immunoglobulin E production in an interferon- γ -dependent manner

OVA-specific IgE levels in ASHMITM-treated mice were considerably reduced 8 weeks post-therapy, compared

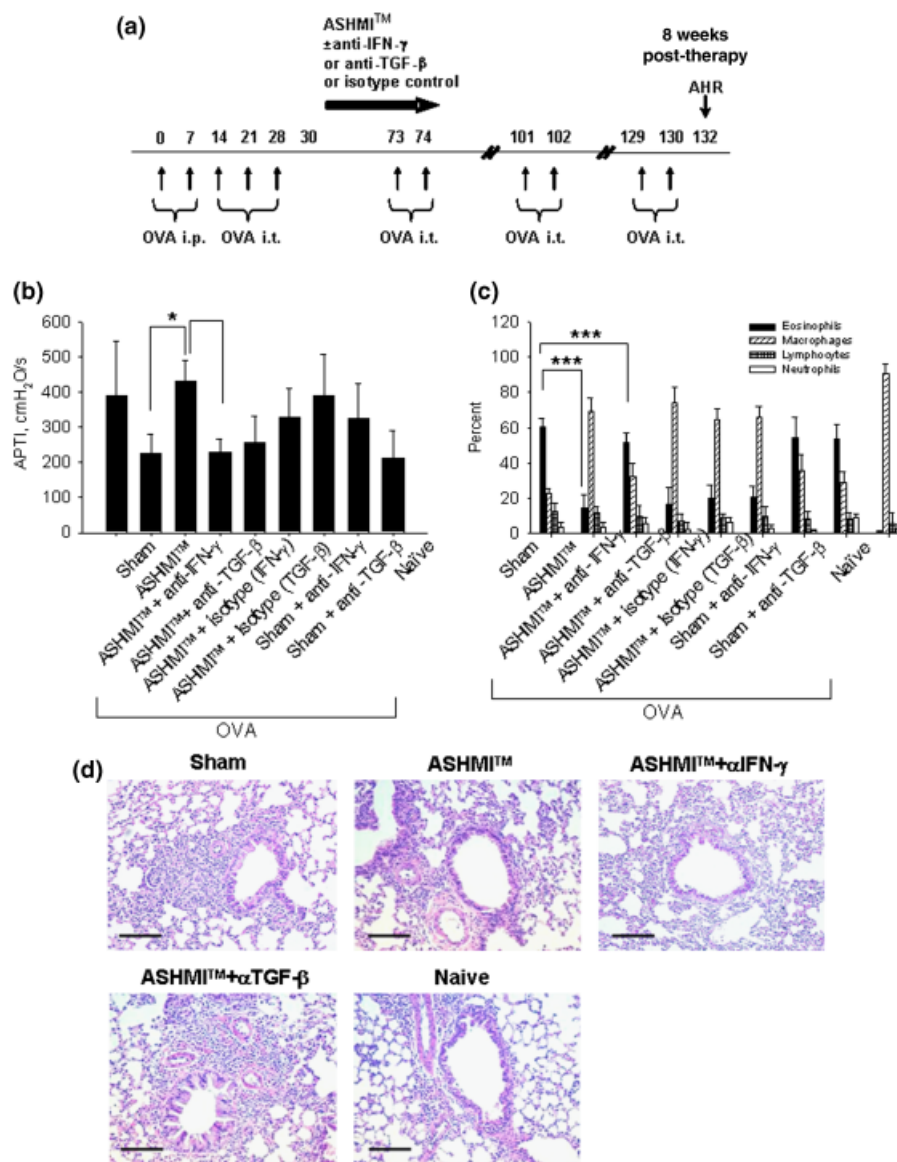


Fig. 3. (a) Mice were sensitized and challenged at times indicated. Six weeks of daily oral ASHMI™ was given with or without neutralizing IFN- γ , TGF- β or isotype control antibodies. Neutralizing antibodies were also given to groups of sham-treated mice. Responses were assessed 8 weeks post-therapy. (b) Airway hyper-reactivity measured 48 h after last ovalbumin challenge. Data shown as mean $\pm$ SD of individual airway pressure time index values ($n = 4$). (c) Percentages of inflammatory cells in bronchoalveolar lavage fluid counted after differential staining. Data shown as mean $\pm$ SD of percentages in individual samples. (d) Haematoxylin and eosin staining of sections of unlabeled lung ($n = 4$). Bars indicate 100 μ m. * $P < 0.05$; *** $P < 0.001$ vs. Sham.

with sham-treated OVA mice ($P < 0.001$ vs. sham, Fig. 6). Neutralization of IFN- γ abrogated ASHMI™-mediated IgE suppression ($P < 0.001$ vs. ASHMI™ alone, Fig. 6). Neutralization of TGF- β or administration of isotype control Abs had no effect. IFN- γ or TGF- β neutralization did not affect IgE levels in OVA/sham mice.

Discussion

Growing concern over the increased incidence and prevalence of asthma in westernized countries has intensified attempts to find new drug therapies. We report here that,

in a murine model of chronic allergic asthma, treatment with the herbal preparation, ASHMI™, eliminated AHR, and reduced pulmonary inflammation and airway remodelling in animals with established asthmatic responses. Therapeutic effects were associated with the suppression of specific IgE and Th2 cytokines well known to play key roles in the pathology of allergic asthma. Importantly, ASHMI™ benefits persisted for at least 8 weeks after treatment was discontinued. Such persistent effects are in marked contrast to steroid withdrawal, which is often accompanied by asthmatic exacerbation and amplified airway inflammation <4–6 weeks after discontinuing

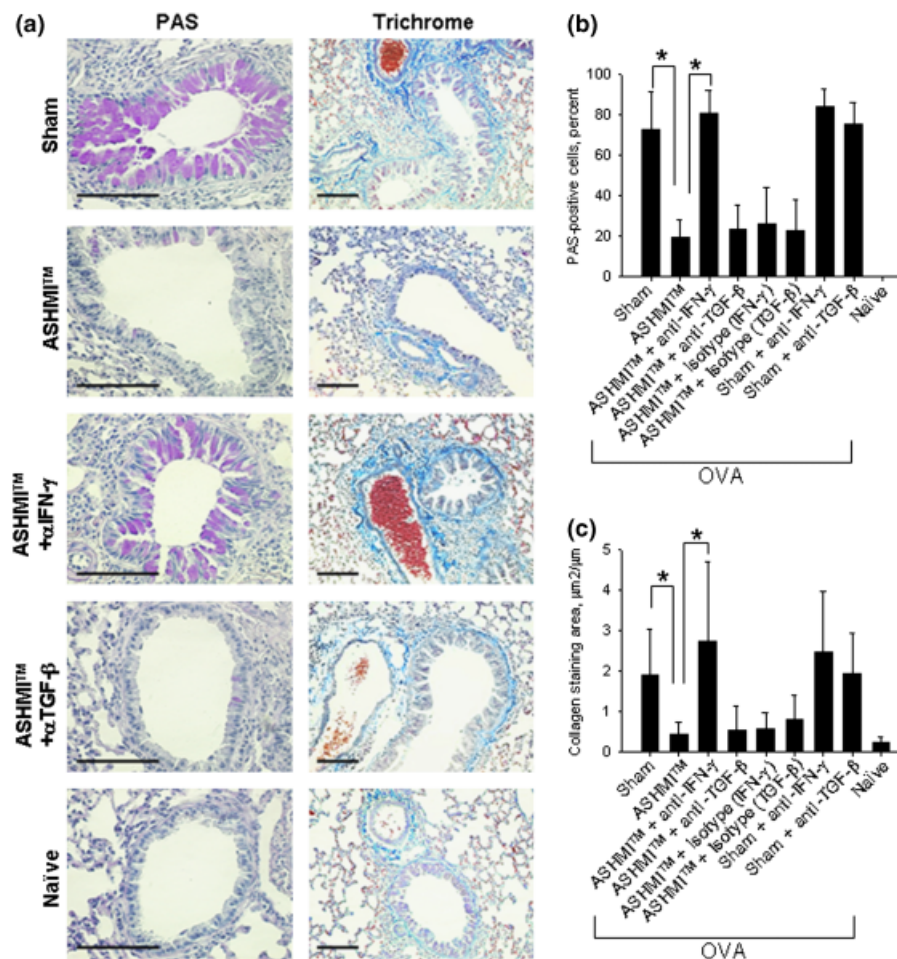


Fig. 4. (a) (Left panel) Periodic acid Schiff (PAS) staining of sections of unlagged lung. Mucin-positive cells stain magenta. (b) Shows percent of PAS-positive cells/airway. (a) (Right panel) Trichrome staining of lung sections. Collagen stains turquoise blue. (c) Shows area of peribronchial collagen staining. All data shown as mean $\pm$ SD of 3–5 airways per animal ($N = 4/\text{group}$). Bars indicate 100 μm . * $P < 0.05$.

treatment [23, 24]. Sustained suppression of IgE levels post-ASHMITM therapy, despite additional OVA challenges suggests that this therapy may be superior to anti-IgE therapy, in which free IgE increased as early as 2 weeks after discontinuation of treatment [8]. It is a common practice in preclinical drug development to treat animals before disease establishment, which is of limited use regarding treatment of human asthma. In this study, allergic airway responses were established before initiating treatment, thereby mimicking human asthma therapy. Furthermore, as in other previously described chronic models of asthma [25, 26], OVA mice received repetitive antigen challenges, and by week 8 post-therapy, mice had received six sets of challenges over ~ 100 days. If airway tolerance is defined by the loss of induction of AHR to antigen exposure, all ASHMITM-treated mice, but no sham-treated mice, developed tolerance, which persisted up to 8 weeks post-therapy. Although, animal models of asthma are not identical to human asthma, this study

suggests that clinical investigation of potential long-term ASHMITM effects in asthma patients is warranted.

The findings of the present study that ASHMITM significantly up-regulated IFN- γ production, slightly reduced IL-10 production and only modestly increased TGF- β production suggest that ASHMITM efficacy is attributable to shifting a Th2-mediated allergic immune response towards a Th1 response, thereby distinguishing it from steroid therapy, where in similar murine models IFN- γ was reduced in dexamethasone-treated mice [27, 28]. To confirm that IFN- γ functionally contributes to ASHMITM effects, we gave ASHMITM concomitantly with IFN- γ neutralizing Abs. This study also used TGF- β neutralizing Abs to rule out a cell-cell contact mechanism of TGF- β function, determinable by measuring TGF- β levels. Anti-IFN- γ Ab administered during treatment, completely abrogated ASHMITM reductions of systemic Th2 cytokines (Fig. S3), AHR, OVA-specific IgE, pulmonary inflammation and airway remodelling. Neutralizing INF- γ ,

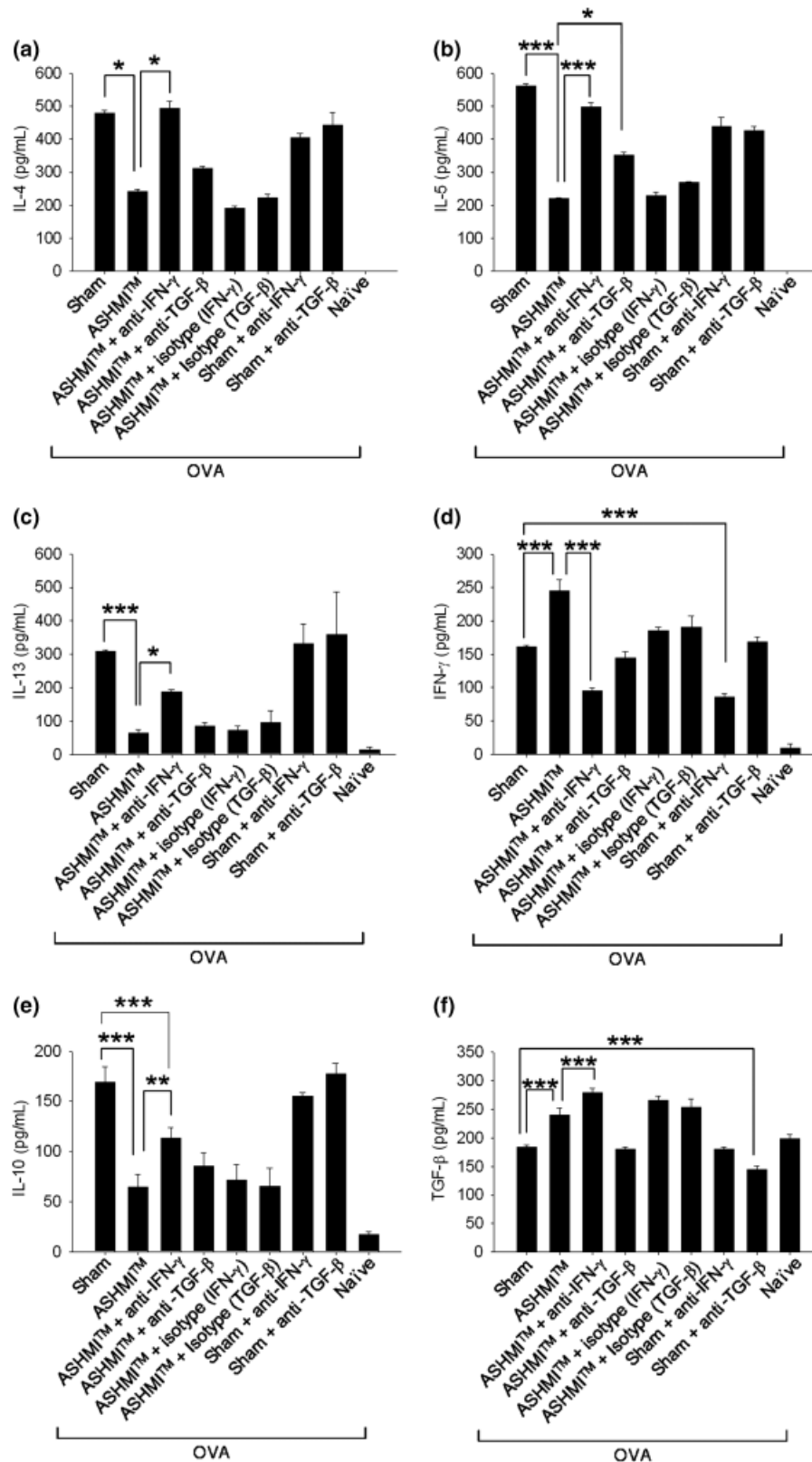


Fig. 5. Bronchoalveolar lavage fluid supernatants were pooled for each group of mice and cytokine levels at 8 weeks post-ASHMITM therapy were determined by ELISA (a) IL-4, (b) IL-5, (c) IL-13, (d) IFN- γ , (e) IL-10 and (f) TGF- β . Results are expressed as means $\pm$ SD of pooled samples measured in triplicate. (N = 4 mice/group). * P < 0.05; ** P < 0.01; *** P < 0.001.

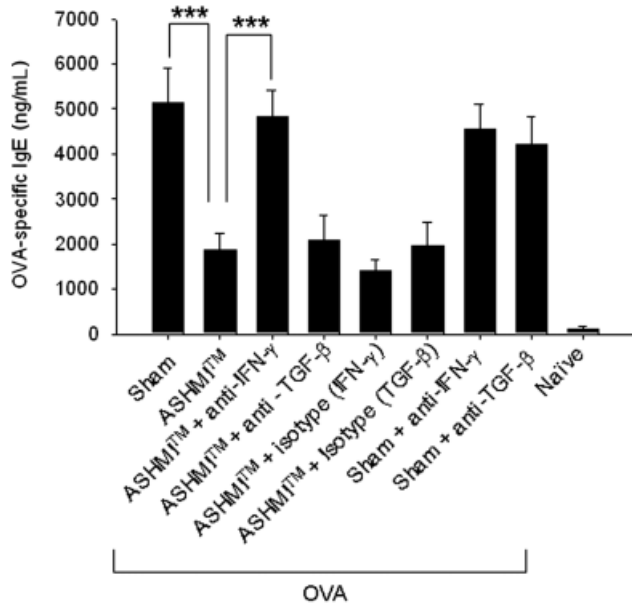


Fig. 6. Serum harvested from mice at the time of airway hyper-reactivity evaluation was assayed for ovalbumin-specific IgE by ELISA. Data are shown as mean $\pm$ SD of individual samples measured in duplicate. $N = 4$ mice/group. *** $P < 0.001$.

dramatically but not completely, reversed ASHMITM-induced reductions in BALF IL-13 and IL-10, implying that yet undetermined IFN- γ -independent mechanisms acting locally may be contributing to ASHMITM effects and warrant further investigation. We have yet to identify the cellular source of the ASHMITM-induced increased IFN- γ secretion in our study; however, CD8⁺T cells and iNKT cells are attractive targets for investigation based on work by us and others [29–32]. We demonstrated previously that ASHMITM and its parent formula MSSM-002 inhibited GATA-3 expression and activity in T cells [13, 22]. This may work to decrease the GATA-3/T-bet ratio in these cells leading to up-regulated IFN- γ production as shown in previous reports [33, 34]. A role for the regulation of T cell/iNKT cell IFN- γ output by macrophages and dendritic cells also warrants investigation because *G. lucidum*, a major component of ASHMITM has been shown to be a potent modulator of macrophage and dendritic cell responses [35–37].

Interestingly, neutralization of TGF- β during treatment although moderately decreasing ASHMITM suppression of IL-5, did not decrease its therapeutic effects. The role of IL-10/TGF- β in chronic asthma remains controversial [38, 39]. A recent study by Goplen et al. [40] in a murine model of asthma induced by combined sensitization of mice dust mite, ragweed and *Aspergillus*, closely mimicking chronic asthma features in humans, showed increased IL-10 and TGF- β in chronic asthmatic mice. Our findings showing the suppression of IL-10 by ASHMITM and a lack of effect of TGF- β neutralization on ASHMITM effects suggest that long-term therapeutic effects of ASHMITM

were not due to T_{reg} activity. That IFN- γ elevation underlies ASHMITM effects is not surprising. Similar immune deviation has been implicated in a previous work in which established asthmatic responses were improved by strategies such as CD137 ligation and allergen vaccination with bacterial adjuvants [41–44]. The protective role of IFN- γ in the allergic setting is well known. Mice with defective IFN- γ production show elevated AHR and specific IgE [31]. In humans, low IFN- γ levels in early life was found to be a predictor of asthma [45, 46]. It must be noted, however, that clinical trials of asthma therapies that increased IFN- γ levels have yielded mixed results. Nebulized IFN- γ was shown not to benefit asthmatics and 1018 ISS, a CpG-ODN therapy despite elevating IFN- γ responses failed to show symptomatic improvement, although concerns regarding effective dose were cited [47, 48]. In contrast, BCG vaccinations, associated with elevated IFN- γ were shown to improve lung function and inhibit Th2 responses in moderate-to-severe asthmatics [49]. While we have demonstrated that ASHMITM therapy in humans is beneficial and elevates IFN- γ , the precise contribution of this elevation in humans is unknown as depletion of the cytokine during human ASHMITM therapy cannot be undertaken. Other mechanisms such as direct suppression of Th2 cells [50], chemokine production [14] and modulation of airway smooth muscle contractility [13] may also contribute to the clinical benefit of ASHMITM and require further investigation.

In conclusion, the herbal formula ASHMITM protected allergic mice against AHR, airway inflammation and remodelling for 8 weeks post-therapy. Because AHR remained in complete remission following OVA challenges for 8 weeks post-therapy, research is underway to determine how long the effect persists to subsequent allergy challenges, and identify the active components in ASHMITM responsible for its benefits on asthma. ASHMITM may be a promising option for human asthma therapy as it provides persistent protection without global immune suppression.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1. Long-term stability analysis of ASHMI™ formula based on the HPLC fingerprint assay. A. Chromatogram acquired on 01/17/2008; B. Chromatogram acquired on 08/15/2007. HPLC fingerprint of A and B are consistent. The peaks identified by using standard samples and/or LC-MS method were labeled for figure B.

Fig. S2. Establishment of asthmatic airway responses. Airway responses were evaluated after the third OVA challenge (day 30). Airway hyperreactivity (AHR) (left panel) and % of BAL eosinophils (right panel) were measured and results indicate establishment of asthmatic responses at this time in the experimental protocol described in Figure 1A. Data shown as Mean ± SD in individual samples (N = 4–5 mice/group). #, $P < 0.05$; ###, $P < 0.001$ vs. Naïve.

Fig. S3. ASHMI suppression of Splenocyte Th2 cytokines is largely IFN-γ dependent. Splenocyte cytokine levels at eight weeks post-ASHMI therapy following recall antigen stimulation *in vitro* were determined by ELISA (A)-IL-4, (B)-IL-5, (C)-IL-13, (D)-IFN-γ, (E)-IL-10, (F)-TGF-β. Results are expressed as means ± SD of individual splenocytes cultures measured in duplicate. (N = 4 mice/group). *, $P < 0.05$.

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SUPPLEMENTARY MATERIALS

**ASHMI™ induced long-lasting tolerance to allergen exposure in an asthma model is interferon
- γ , but not TGF- β dependent**

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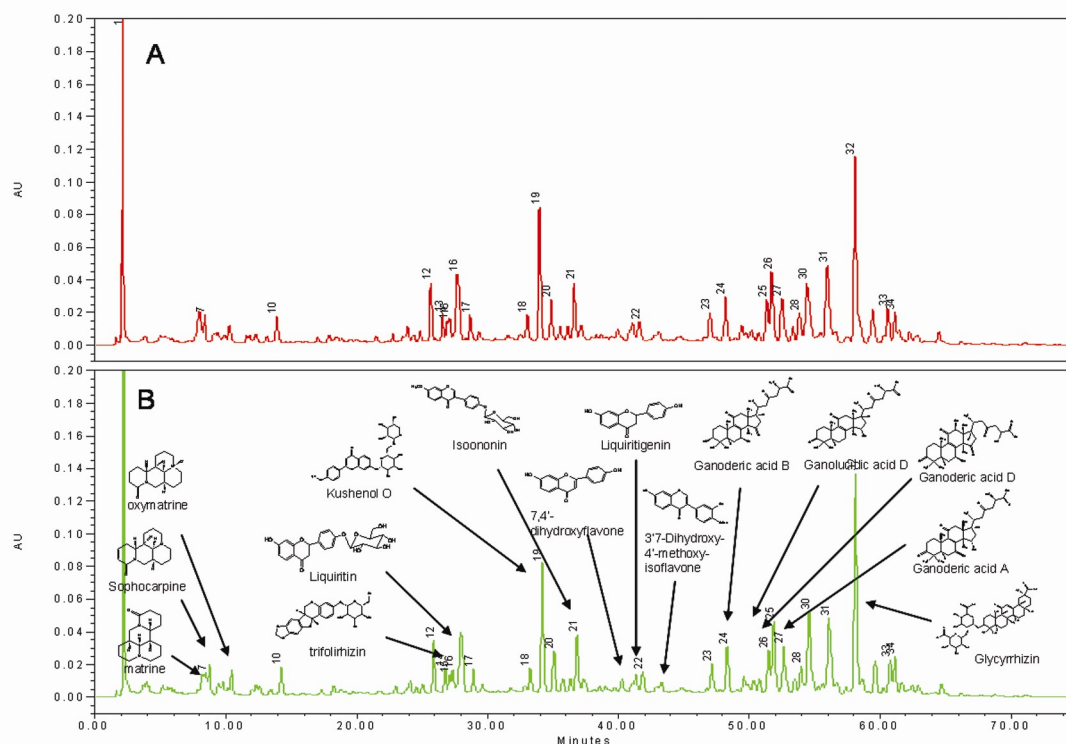
SUPPLEMENTARY METHODS

ASHMITM preparation and quality control

All 3 herbs used for ASHMITM preparation were inspected for identity and quality by Professor WL Duan, a pharmacologist specializing in traditional Chinese medicine at the Department of Materia Medica, Weifang Asthma Hospital, Weifang, China. The botanical information of individual medicinal herbals including geographical location, harvest season, pre-processing, traditional use and known pharmacological actions, heavy metal and pesticides residue has been published previously.^(E1) ASHMITM was prepared by the Sino-Lion Pharmaceutical Company (a GMP certified facility) Weifang, China) as described previously.^(E1) ASHMITM used in this study is the same batch used in our clinical study. Standardization of ASHMITM was established and reported in our previous publication^(E1) and meets US FDA guidance for botanical drug investigation.^(E2) Since the current study involved 5 sets of experiments over 2 years, we monitored the stability of ASHMITM between 2007-2008 by HPLC fingerprinting (Supplementary Figure 1).

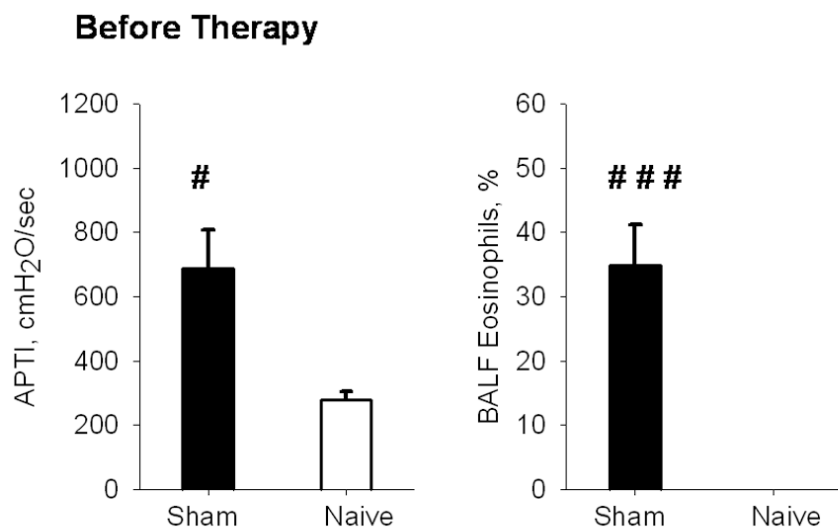
SUPPLEMENTARY FIGURES and LEGENDS

Supplementary Figure 1



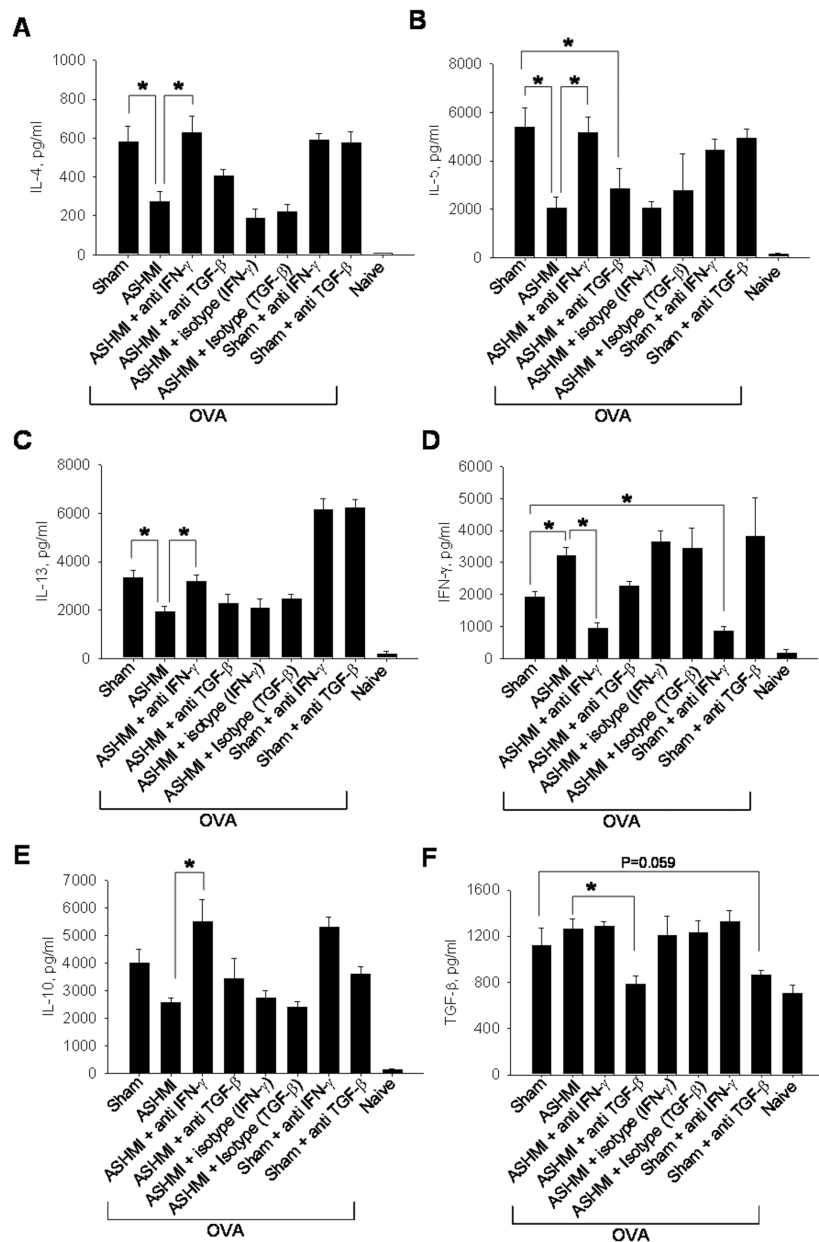
Supplementary Figure 1. Long-term stability analysis of ASHMI™ formula based on the HPLC fingerprint assay. A. Chromatogram acquired on 01/17/2008; B. Chromatogram acquired on 08/15/2007. HPLC fingerprint of A and B are consistent. The peaks identified by using standard samples and/or LC-MS method were labeled for figure B.

Supplementary Figure 2



Supplementary Figure 2. Establishment of asthmatic airway responses. Airway responses were evaluated after the third OVA challenge (day 30). Airway hyperreactivity (AHR) (left panel) and % of BAL eosinophils (right panel) were measured and results indicate establishment of asthmatic responses at this time in the experimental protocol described in Figure 1A. Data shown as Mean $\pm$ SD in individual samples (N=4-5 mice/group). *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$ vs. sham; #, $p < 0.05$; ###, $p < 0.001$ vs naïve.

Supplementary Figure 3



Supplementary Figure 3. ASHMI suppression of Splenocyte Th2 cytokines is largely IFN- γ dependent. Splenocyte cytokine levels at eight weeks post-ASHMI therapy following recall antigen stimulation in vitro were determined by ELISA (A)-IL-4, (B)-IL-5, (C)-IL-13, (D)-IFN- γ , (E)-IL-10,

(F)-TGF- β . Results are expressed as means $\pm$ SD of individual splenocytes cultures measured in duplicate. (N=4 mice/group). *, P<0.05

SUPPLEMENTARY REFERENCES

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