

Immunomodulatory effect of the antiasthma Chinese herbal formula MSSM-002 on T_H2 cells

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Background: T_H2 cytokines play a central role in the pathogenesis of allergic asthma. We previously showed that the “antiasthma” Chinese herbal formula MSSM-002 exhibited therapeutic effects on established allergic airway responses in a murine model of allergic asthma. However, the mechanisms underlying these effects are largely unknown.

Objective: The objective of this study was to determine whether and how MSSM-002 modulates an established T_H2 response and whether the actions of MSSM-002 on T_H2 cell differs from corticosteroids.

Methods: T_H2 polarized splenocytes (T_H2-SPCs) from mice with antigen-induced airway hyperresponsiveness and T_H2 cloned cells, D10 G4.1 (D10), were cultured in the presence or absence of antigen with or without MSSM-002 and dexamethasone, and the proliferative responses and cytokine profiles were determined. Apoptosis and T_H2 transcription factor GATA-3 expression and binding to IL-4 gene promoter and V_A enhancer in MSSM-002-treated D10 cells were also determined.

Results: MSSM-002 significantly decreased antigen-induced proliferation and IL-4 and IL-5 production but increased IFN- γ production by T_H2-SPCs, whereas dexamethasone suppressed IFN- γ as well as IL-4 and IL-5. Anti-IL-12 antibody, although abrogating MSSM-002 induction of IFN- γ , had no significant effect on MSSM-002 suppression of IL-4 and IL-5 secretion. MSSM-002 also suppressed T_H2 cytokine secretion by D10 cells, and in contrast to dexamethasone, MSSM-002 did not induce apoptosis of D10 cells. MSSM-002 markedly suppressed GATA-3 mRNA and protein expression and the binding to IL-4 gene promoter and V_A enhancer in D10 cells. **Conclusion:** MSSM-002, in contrast to the overall suppression of T cells by dexamethasone, exhibits immunomodulatory actions on T_H2 cells caused, at least partially, by downregulation of GATA-3. (*J Allergy Clin Immunol* 2004;113:268-76.)

Key words: Asthma, Chinese herbal medicine, T_H1-T_H2 responses, immunomodulation

Abbreviations used

BALF: Bronchoalveolar lavage fluid(s)

CA: Conalbumin

D10 cells: D10 G4.1 cells

GAPDH: Reduced glyceraldehyde-phosphate dehydrogenase

OD: Optical density

T_H2-SPCs: T_H2 polarized splenocytes

TUNEL: Transferase-mediated deoxyuridine triphosphate nick end labeling

Asthma has emerged as a major public health problem worldwide, particularly in Westernized societies.¹ Allergic asthma is characterized by airway hyperresponsiveness associated with elevated serum IgE and bronchial inflammation. Although corticosteroids are effective nonspecific anti-inflammatory agents, the side effects of corticosteroid treatment are a significant concern, particularly for children, in whom the morbidity and mortality of asthma have been increasing.² The need for safe and effective treatments of this disease is greater than ever.

Complementary and alternative medicine approaches are increasingly being considered by patients, and the use of complementary and alternative medicine has grown substantially during the last 10 years in Western countries.^{3,4} A recent study reported that up to 50% of asthmatic patients were using some form of complementary and alternative medicine, and that a growing number of asthma patients wish to use complementary and alternative medicine.⁵ However, a role for complementary and alternative medicine in asthma therapy is uncertain because there are few well-controlled scientific studies demonstrating efficacy, and any beneficial actions of these materials on allergic processes are largely unknown.⁵

Traditional Chinese medicine has played a central role in treating diseases including asthma for centuries and is still widely used in modern medical practice in China and other Asian countries. Although basic research into herbal interventions for allergy and asthma is limited, recent review articles of the English language literature by Bielory and Lupoli⁶ and Ziment and Tashkin⁷ provide some scientific evidence supporting the use of herbal formulas and herbal derivatives for the treatment of asthma. These findings also suggest that there is a great potential in traditional Chinese medicine

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for development of herbal interventions to treat asthma and allergy.⁵ We previously developed an herbal formula, MSSM-002, on the basis of an empirical traditional Chinese medicine prescription for treating childhood asthma and bronchitis, and we tested the therapeutic effect of this formula on allergen-induced airway hyperreactivity in a well-characterized murine model of allergic asthma. We found that MSSM-002 significantly reduced airway hypersensitivity, eosinophilic inflammation, serum IgE and bronchoalveolar lavage fluid (BALF) T_H2 cytokine levels,⁸ and that these effects were comparable with that of dexamethasone treatment. In contrast to dexamethasone, MSSM-002 did not suppress IgG2a or IFN- γ production. These results showed that MSSM-002 and dexamethasone suppression of pulmonary allergic reactions might be attributable to different mechanisms. However, the precise mechanisms of the therapeutic effects of MSSM-002 on allergic airway responses are largely unknown, and whether the mechanisms of actions of MSSM-002 and corticosteroids are indeed different is an important and frequently asked question.^{9,10}

T_H2 -type cytokines such as IL-4, IL-13, and IL-5 produced by activated $CD4^+$ T cells play a central role in the pathogenesis of allergic asthma.¹¹ IL-4 and IL-13 promote B-cell switching to IgE production and mucus hypersecretion, whereas IL-5 is the primary determinant of eosinophil recruitment, activation, and survival.¹¹ IL-13 and IL-4 also have been implicated in smooth muscle contraction.¹² Although the precise mechanisms responsible for T_H1/T_H2 cell differentiation are not fully understood, it has been shown that T_H2 cytokine gene expression is regulated at the level of transcription,¹³ and that GATA-3, the key transcription factor inducing T_H2 cell lineage, is a critical factor in T_H2 cell-mediated allergic asthma¹⁴ and is pivotal for T_H2 cytokine memory by inducing T_H2 cytokine expression.¹⁵

Because MSSM-002 exhibited a therapeutic effect on established allergic reactions and this effect was accompanied by reduction of T_H2 cytokines, we hypothesized that MSSM-002 might modulate the established T_H2 phenotype. In the present study we sought to examine whether and how MSSM-002 affects T_H2 responses by using antigen-specific T_H2 polarized splenocytes (T_H2 -SPCs) and the T_H2 clone, D10 G4.1 (D10) cells. We found that unlike dexamethasone, which, as expected, suppressed IFN- γ as well as IL-4 and IL-5 production, MSSM-002 increased IFN- γ secretion while suppressing IL-4 and IL-5 secretion by T_H2 -SPCs. We further found that MSSM-002 suppression of T_H2 cytokines was not IL-12 dependent, because anti-IL-12 antibody, although abrogating MSSM-002 induction of IFN- γ , had no significant effect on MSSM-002 suppression of IL-4 and IL-5. Additional experiments also demonstrated direct MSSM-002 suppression of T_H2 cytokine secretion by the T_H2 clone, D10 cells. In contrast to dexamethasone, which induces lymphocyte apoptosis, MSSM-002 suppression of T_H2 cells was not associated with apoptosis or toxicity, but it was associated with reduced expression

of the T_H2 transcription factor GATA-3. These results demonstrate that MSSM-002 modulation of the T_H2 phenotype differs from both corticosteroids and IL-12 inducing immunologic adjuvants.

METHODS

Reagents

Conalbumin (CA) was purchased from Sigma (St Louis, Mo). D10 cells were purchased from American Type Culture Collection (Manassas, Va). Antibodies for measuring IL-4, IL-5, and IFN- γ by ELISA and anti-IL-12 antibodies were purchased from PharMingen (San Diego, Calif). Standard lyophilized MSSM-002 was prepared as previously described.^{8,16}

Antigen sensitization, challenge, and SPC preparation and culture

Male AKR/J mice (6- to 8-week-old), purchased from the Jackson Laboratory (Bar Harbor, Me), were maintained in the animal facility at Mount Sinai School of Medicine. To generate T_H2 polarized cell line, we followed the same antigen sensitization and challenge protocol used to generate a murine model of allergic asthma.⁸ Briefly, mice were sensitized by 2 intraperitoneal injections of 200 μ g of CA and 2 mg of alum in 0.3 mL of PBS (1 week apart). Seven days after the last sensitization, mice were anesthetized and challenged intratracheally with 100 μ g of CA in 0.05 mL of PBS followed by 2 more intratracheal challenges on days 20 and 30. Three days after the last challenge, mice were killed, and spleens were collected from at least 10 mice of each experiment and pooled.

Splenocytes were prepared as previously described¹⁷ and were then suspended in complete cell culture medium (RPMI 1640 containing 10% FCS, 1% penicillin/streptomycin). For proliferation assay, splenocytes (1×10^6 cells per well in 0.2 mL) were cultured with CA (50 μ g/mL) in triplicate in microwell plates in the presence or absence of 1, 5, 50, or 500 μ g/mL MSSM-002 or dexamethasone (2.5 μ mol/L). Unstimulated cells and CA-stimulated cells were used as negative and positive controls, respectively. In addition, splenocytes cultured as above were treated with or without anti-IL-12 antibody (10 μ g/mL). Three days later, the cultures received an 18-hour pulse of 1 μ Ci of ^{3} H-thymidine per well. The cells were then harvested, and the incorporated radioactivity was counted in a β -scintillation counter. The results are expressed as cpm.

In parallel, cell suspensions were cultured in duplicate in 24-well plates (4×10^6 /mL/well) as above. Supernatants were collected after 72-hour culture and stored at -80°C until analyzed.

D10 cell culture

D10 cells are a well-characterized CA-specific T_H2 clone generated from AKR mice.¹⁸ Many studies used these cells to characterize the role of T_H2 cells in the pathogenesis of allergic reactions and to test various approaches to modulation of the T_H2 phenotype.¹⁹⁻²¹ Although T_H2 -SPCs within a mixed cell population mimic physiological conditions, the long-term committed T_H2 cell line provides a tool to determine any direct effect of MSSM-002 on T_H2 cells. In this study we followed the previously described method for culture and expansion of the D10 cells.¹⁹ Briefly, resting D10 cells were stimulated with CA (50 μ g/mL) in the presence of irradiated AKR spleen cells as feeder cells in complete cell culture medium. CA-stimulated cells were treated with or without MSSM-002 (50 μ g/mL). Proliferative responses were determined. Supernatants were collected 72 hours after culture for cytokine measurement. Cells were cultured in triplicate for proliferation and in duplicate for collection of supernatants.

Quantification of secreted cytokines

Cytokines IL-4 and IL-5 and IFN- γ levels in culture supernatants in the supernatants of T_H2-SPCs and D10 cells were measured by ELISA according to the manufacturer's instructions (PharMingen) and as previously described.¹⁹

Trypan blue exclusion and transferase-mediated deoxyuridine triphosphate nick end labeling assays

In parallel with cell cultures for proliferation assays and cytokine measurements, additional CA-stimulated T_H2-SPCs and D10 cells were cultured with MSSM-002 for 72 hours as above, and viability was determined by trypan blue assay.²² Dead cells (trypan blue positive cells) and viable cells (trypan blue negative cells) were counted by using a light microscope, and the percent viable cells was calculated.

Apoptotic cells were identified by transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay. Cytospins (2×10^4 cells/slide) of aliquots of the same D10 cells were prepared and stained by using a TUNEL assay kit according to the manufacturer's instructions (Fluorescein Apoptosis Detection System KIT; Promega, Madison, Wis). Slides were then cover-slipped and observed by using a fluorescent microscope (Olympus BX60, Melville, NY) equipped with a triple band-pass filter at 200 $\times$ magnification. Apoptotic cells, which incorporate FITC-nucleotide, appeared green or yellow, whereas viable cells with nonfragmented DNA appeared red. The percentage of apoptotic cells was evaluated by counting 500 cells per slide.

Semiquantitative RT-PCR

Semiquantitative RT-PCR was performed as previously described.^{23,24} Briefly, cultured D10 cells (5×10^6 cells) were harvested immediately after collection of the supernatants and subjected to total mRNA isolation by using Trizol reagent (Gibco BRL, Rockville, Md), as described by the manufacturer. The total RNA concentration was measured and adjusted to 0.5 μ g/ μ L by triplicate optical density (OD) readings (Bio-RAD SmartSpect 3000; Bio-Rad, Hercules, Calif). RT was performed by using the Superscript First-Strand Synthesis System for RT-PCR including Oligo(dT)₁₂₋₁₈ primer (Invitrogen Life Technologies, Carlsbad, Calif), as described by the manufacturer and a previous publication.²⁴

PCR was performed as described previously^{23,24} by using AmpliTaq DNA polymerase according to manufacturer's protocol (Clontech Laboratories, Palo Alto, Calif). PCRs were carried out beginning with 95°C for 5 minutes followed by 25 cycles for reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) and 35 cycles for GATA-3 by using the following temperature profile: denaturation at 94°C for 45 seconds, primer annealing at 60°C for 45 seconds, and primer extension at 72°C for 90 seconds. The final extension was at 72°C for 10 minutes. Our preliminary study showed that these cycles sufficiently amplified the GAPDH and GATA-3 expression and avoided amplification saturation. Once the PCR reactions were complete, 10 μ L of the reaction mixture was separated by electrophoresis through a 1.2% agarose gel and visualized by ethidium bromide staining and UV irradiation. Gel images were captured by using a Gel Doc Image Analysis (BioRad), and PCR product quantitation was performed by densitometry with Quantity One Software (BioRad) and normalized against GAPDH from the same mRNA preparation. Results were expressed as an OD ratio (cytokine/ β -actin). Before analysis, the PCR product band intensities were checked to ensure that they had not reached saturation. The primers GATA-3 (sense: 5'-TCTGGAGGAGGAAACGC TAATGG-3'; antisense: 5'-GAAGTCTTCGCACACTTGGG GACT-3', 408 bp) and GAPDH (5'-GGTGAAGGTCGGTGT GAACGGATT; antisense: 5'-GGTGAAGGTCGGTGTGAACG

GATT-3', 501 bp) used in the PCR were synthesized by Life Technologies (Grant Island, NY).

Western blot analysis

Western blot analysis for determination of GATA-3 protein synthesis was performed by using monoclonal anti-GATA-3 antibody, HG3-31 (Santa Cruz Biotechnology, Santa Cruz, Calif) as previously described.²⁵ Briefly, D10 cells (1×10^7) were lysed, and 40 μ g of total proteins was separated by SDS-PAGE and blotted onto a nitrocellulose membrane. After blocking, the membranes were incubated with anti-GATA-3 HG3-31 antibody (1:1000 dilution) and β -actin (1:3000 dilution), respectively. The membrane was then incubated with a secondary antibody containing peroxidase-conjugated anti-mouse IgG (1:2500). Immunoreactive bands were visualized by using the ECL Western detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was performed by using a ChIP Assay Kit according to the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY) and as previously described.²⁶ In brief, 1×10^6 D10 cells were fixed with 1% paraformaldehyde at 37°C for 10 minutes. Cells were sedimented, washed, and lysed with SDS lysis buffer. The lysates were sonicated to reduce DNA lengths to between 200 and 1000 bp (20 μ L was kept to quantitate the amount of DNA present in different samples at the PCR protocol as input chromatin). The soluble fraction was diluted and precleared with salmon sperm DNA/protein A-agarose. Chromatin was incubated with 4 μ g of anti-mouse GATA-3 antibody (Santa Cruz Biotechnology) or no antibody as negative control overnight at 4°C. The immune complexes were then precipitated, washed, and eluted. The eluted material was incubated at 65°C for 4 hours to reverse the formaldehyde cross-links, and DNA was extracted with phenol and chloroform. Ethanol-precipitated DNA was solubilized in water (20 μ L). Semiquantitative PCR was performed with 4 μ L of DNA as described above.²³ PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide as described above. The following primers were used: IL-4 P: 5'-TTGGTCTGATTTCACAGG-3', 5'-AACAAATGCAAT GCTGGC-3' (182-bp product); IL-4 E (VA): 5'-AGGGCACT TAAACATTGC-3', 5'-ACGCCTAAGCACAATTCC-3' (239-bp product); GAPDH: 5'-GGTGAAGGTCGGTGTCAAC-3; 5'-ATGCCAAAGTTGTCATGG-3' (500-bp product).

Statistical analysis

Data were analyzed by using SigmaStat 2.03 statistical software package (SPSS Inc, Chicago, Ill). Differences between groups were analyzed by one-way ANOVA followed by Bonferroni *t* test for all pairwise comparisons, if the data passed the normality test. Differences between groups were analyzed by Kruskal-Wallis one-way ANOVA on ranks followed by all pairwise comparison procedure (Dunn), if the data were not normally distributed, ie, failed the normality test. *P* values less than .05 were considered statistically significant.

RESULTS

MSSM-002 suppressed antigen-induced proliferation and altered the antigen-induced T_H2 phenotype of SPCs

We previously found that SPCs from allergic mice with airway hyperreactivity induced by CA sensitization and challenge exhibited a T_H2 phenotype characterized by secretion of IL-4, IL-5, and IL-13 but little or no IFN-

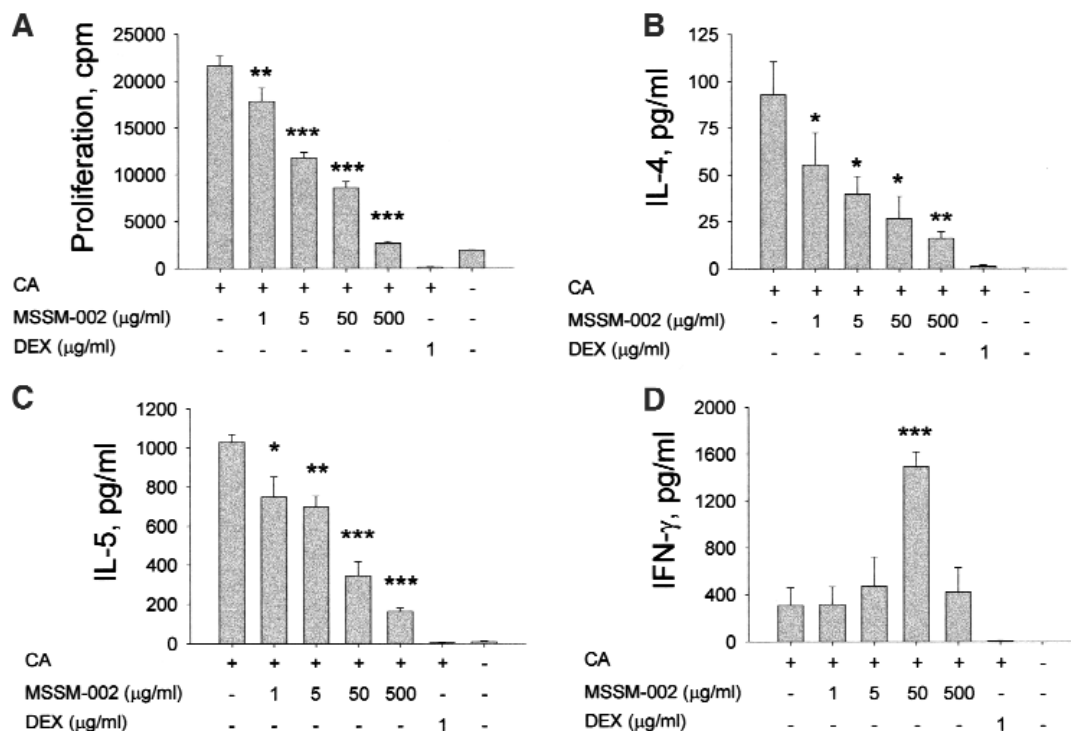


FIG 1. Effect of MSSM-002 on T_H2 -SPCs. Pooled splenocytes isolated from mice ($n = 10$) with pulmonary allergic reactions induced by intraperitoneal sensitization and intrathecal challenge with CA were cultured with or without CA and varying doses of MSSM-002 or dexamethasone. Proliferation assay data represent mean $\pm$ SEM of 3 sets of data from 3 separate experiments. Data for cytokines are mean $\pm$ SEM of 3 sets of data from 3 separate experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$ vs CA.

γ .^{8,27} To determine whether MSSM-002 can directly alter the polarized T_H2 phenotype, we isolated SPCs from mice with allergic airway hyperreactivity, stimulated them with CA as described previously,⁸ and treated the cells with or without MSSM-002 or dexamethasone. As expected, dexamethasone abolished antigen-induced proliferation, IL-4 and IL-5 production, and IFN- γ production (Fig 1). MSSM-002 also significantly decreased antigen-induced proliferation and IL-4 and IL-5 secretion. However, MSSM-002 did not suppress IFN- γ production at any dose and significantly increased IFN- γ production at a concentration of 50 μ g/mL. These results showed that unlike the overall immunosuppressive effect of dexamethasone, MSSM-002 shifted the T_H2 phenotype to a T_H1/T_H0 -like phenotype. MSSM-002 reduction of T_H2 cytokines occurred in a linear dose-dependent manner, whereas increased IFN- γ secretion followed a bell-shaped dose response, ie, slightly increased IFN- γ at the lowest and highest doses (1, 5, and 500 μ g/mL) and significantly increased IFN- γ at the medium dose (50 μ g/mL) (Fig 1) as compared with MSSM-002-untreated SPCs. We tested additional doses between 50 and 500 μ g/mL and found that IFN- γ levels began to decline at 100 μ g/mL (data not shown). The reason for lack of a linear increase in IFN- γ is unknown, but it is not due to toxicity because the numbers of viable cells in all MSSM-002-treated cultures were not significantly different from

untreated control cultures (data not shown). This might be due to induction of T-regulatory/suppressor cytokines such as IL-10 or TGF- β , which downregulate both T_H1 -mediated autoimmune responses and T_H2 -mediated allergic responses.²⁸⁻³² Because 50 μ g/mL MSSM-002 produced marked reduction of T_H2 cytokines and the greatest increase in IFN- γ , this concentration was used in subsequent experiments.

MSSM-002 alteration of antigen-induced T_H2 phenotype was not solely dependent on IL-12

We next determined whether MSSM-002 alteration of the T_H2 phenotype was mediated by IL-12. In a separate experiment, T_H2 -SPCs also prepared from CA-allergic mice with airway hyperreactivity were cultured in the presence of antigen and MSSM-002 with and without the addition of anti-IL-12 antibodies. As shown in Fig 1, we found that MSSM-002 treatment resulted in a significant reduction of IL-4 and IL-5 and increased IFN- γ compared with CA alone cultures. As noted, the absolute cytokine levels differed from those shown in Fig 1 because of variations in responses between mice in different experiments. Interestingly, neutralizing IL-12 antibodies abrogated MSSM-002 induction of IFN- γ (Fig 2, C), but they did not significantly affect MSSM-002 reduction of IL-4 and IL-5 synthesis (Fig 2, A and B). These results show that although MSSM-002 induction

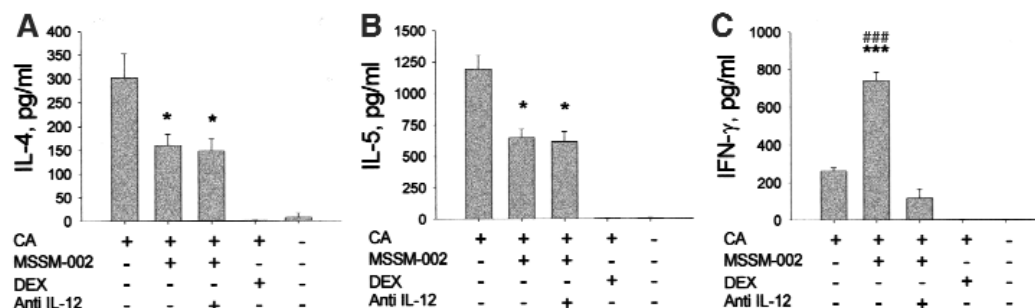


FIG 2. Effect of IL-12 on MSSM-002 alteration of the T_H2 phenotype. Pooled splenocytes were isolated from mice ($n = 10$) with pulmonary allergic reactions induced by CA intraperitoneal sensitization and challenge. T_H2 -SPCs were cultured in the presence or absence of antigen and MSSM-002, with or without anti-IL-12 antibodies. Data for cytokines are mean $\pm$ SEM of 3 sets of data from 3 separate experiments. * $P < .05$, *** $P < .001$ vs CA, and ### $P < .001$ vs CA + MSSM-002 + anti-IL-12.

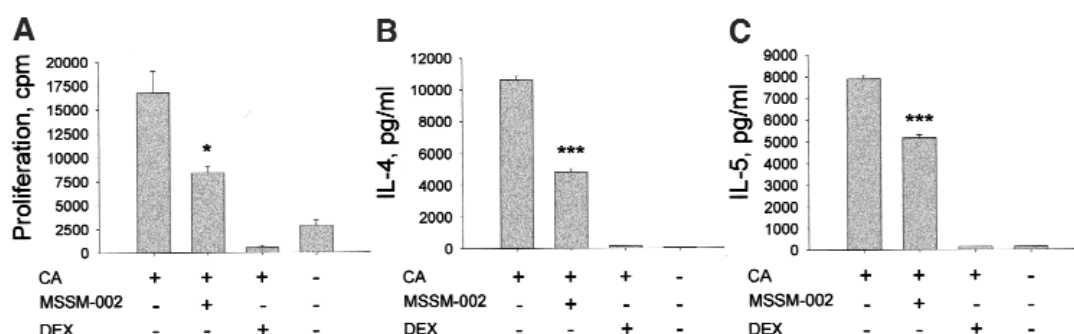


FIG 3. Effect of MSSM-002 on the long-term committed T_H2 clone (D10 cells). D10 cells were cultured in the presence or absence of CA with or without MSSM-002 treatment. Proliferation data are mean $\pm$ SEM of triplicate, and cytokines are the mean of 4 cultures. These results were repeated in at least 3 separate experiments. * $P < .05$, *** $P < .001$ vs CA.

of IFN- γ by T_H2 -SPCs is IL-12 dependent, reduction of T_H2 cytokines involves other mechanisms. To explore the IL-12 independent effect further, we also cultured T_H2 -SPCs with anti-CD3 and CD28 in the presence and absence of MSSM-002 (50 μ g/mL) and found that MSSM-002 also suppressed T_H2 cytokine production by anti-CD3/CD28 stimulated T_H2 -SPCs (data not shown). These results suggest that MSSM-002 directly modulates T_H2 cells.

MSSM-002 suppressed T_H2 cytokine synthesis by the long-term committed T_H2 clone, D10 cells

To determine whether MSSM-002 can directly suppress T_H2 cell activities we used T_H2 clone, D10 cells that produce high levels of T_H2 cytokines but virtually no IFN- γ and do not respond to IL-12 because of lack of IL-12 β 2 receptors.³³ As we found with SPCs, MSSM-002 significantly suppressed proliferation and IL-4 and IL-5 production by D10 cells (Fig 3, $P < .01$). These results show that MSSM-002 suppression of T_H2 cytokine secretion is IL-12 and IFN- γ independent. As expected, dexamethasone abolished IL-4 and IL-5 production by D10 cells.

MSSM-002 downregulation of T_H2 responses is not due to direct cytotoxicity or induction of apoptosis

To analyze the mechanisms by which MSSM-002 suppresses T_H2 cell cytokine secretion, we first determined whether MSSM-002 exposure resulted in T_H2 cell death. In parallel with D10 cell cultures for proliferation and cytokine analyses, we cultured antigen-primed D10 cells in the presence or absence of MSSM-002 and dexamethasone for 72 hours and then assessed cell viability and apoptosis by trypan blue exclusion and TUNEL assays. Dexamethasone-treated cultures contained few viable cells, whereas the percent of viable cells in MSSM-002-treated cultures was essentially the same as untreated cultures (data not shown). In another experiment, D10 cells were cocultured with MSSM-002 for 7 days. The percent viable cells in this culture were not different from CA alone cultured cells (data not shown). These results show that MSSM-002 is not toxic at this concentration, at which T_H2 cytokines were significantly reduced. Corticosteroid induction of lymphocyte apoptosis is well-known,³⁴ and as expected, dexamethasone also induced apoptosis of D10 cells (84% TUNEL-positive cells; Fig 4, C). MSSM-002, however, did not induce

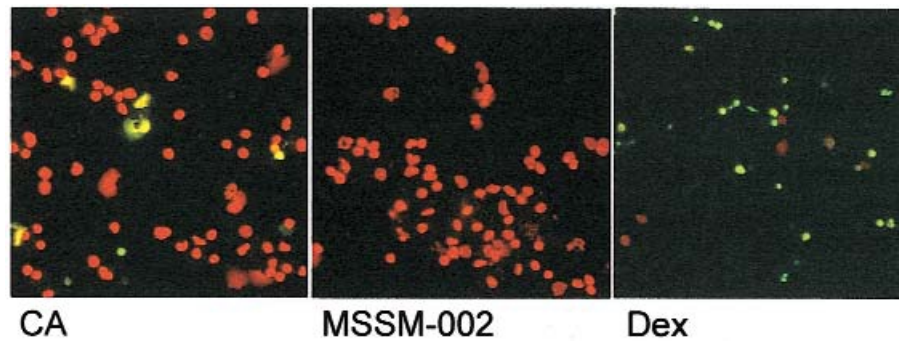


FIG 4. MSSM-002 did not induce T_H2 cell apoptosis. CA-stimulated D10 cells were cocultured with or without MSSM-002 or dexamethasone, and apoptosis was assessed by TUNEL assay. Green fluorescence shows DNA fragmentation. Red fluorescence indicates normal nuclei.

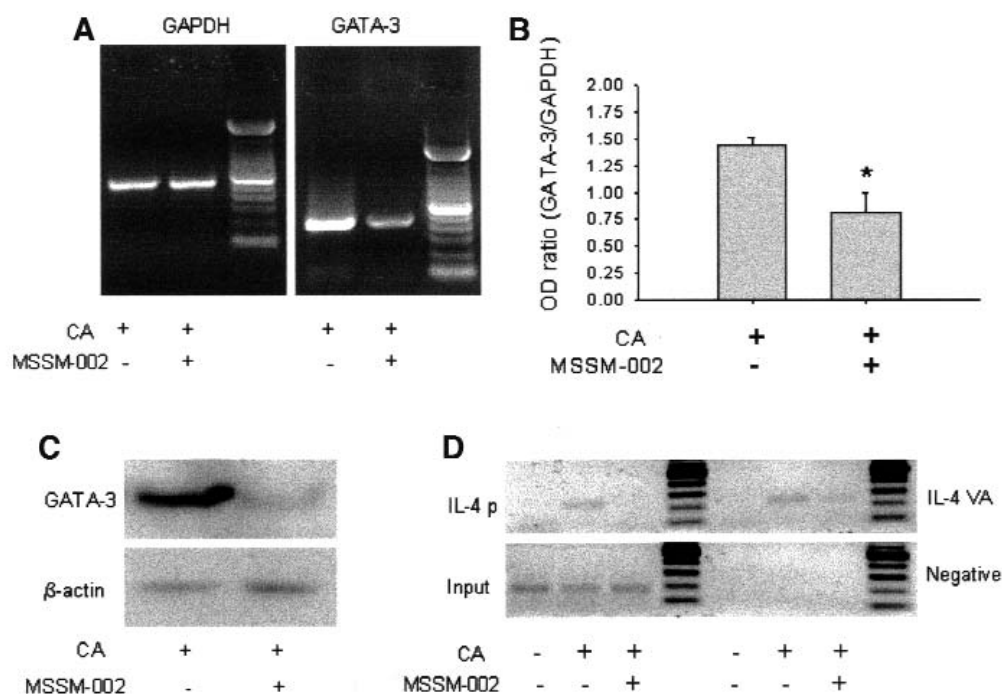


FIG 5. Effect of MSSM-002 on D10 cell GATA-3 expression. **A** and **B**, GATA-3 mRNA expression. Total RNA was extracted from CA-stimulated D10 cells in the presence or absence of MSSM-002. Semiquantitative RT-PCR was performed. **A**, Gel illustrating GATA-3 mRNA expression by D10 cells under different culture conditions as indicated. **B**, OD ratios of GATA-3 vs GAPDH. The results are expressed as mean $\pm$ SEM of 3 PCR reactions. * $P < .05$ vs CA. **C**, Gel showing Western blotting results of GATA-3 protein expression from the CA-stimulated D10 cells in the presence or absence of MSSM-002. **D**, Illustration of chromatin immunoprecipitation assay indicates GATA-3 binding to IL-4 promoter and enhancer in the D10 cells with or without CA stimulation in the presence or absence of MSSM-002-treated cells.

apoptosis (2% TUNEL positive cells; Fig 4, B) as compared with CA alone cultured cells (10% TUNEL positive cells; Fig 4, A). Therefore, MSSM-002 suppression of T_H2 cytokine secretion is not due to direct cytotoxicity or induction of apoptosis. It appeared that MSSM-002-treated cultures had fewer TUNEL-positive cells than CA alone cultured cells. The reason for this is unknown, but one of the herbs in MSSM-002 (Ling Zhi) has been reported to inhibit radiation or chemotherapy induced DNA fragmentation.^{35,36}

MSSM-002 suppresses T_H2 -specific transcription factor GATA-3 expression and activation

Because MSSM-002 directly suppressed T_H2 cytokine synthesis in a nontoxic manner, we hypothesized that MSSM-002 might directly suppress T_H2 transcription factors. Because GATA-3 determines T_H2 cell commitment,¹⁴ we first used semiquantitative RT-PCR to assess the possible effect of MSSM-002 on GATA-3 expression by CA-stimulated D10 cells. As shown in Fig 5, MSSM-

002 markedly suppressed GATA-3 mRNA expression in D10 cells as compared with CA alone cultured D10 cells (Fig 5, A). The difference in OD ratios (GATA-3 vs GAPDH) between these 2 groups was statistically significant (Fig 5, B; $P < .05$). Consistent with this finding, GATA-3 protein synthesis in MSSM-002-treated, CA-stimulated D10 cells was also suppressed as assessed by Western blotting (Fig 5, C). These results showed that MSSM-002 suppression of GATA-3 occurred at both transcriptional and post-transcriptional levels.

IL-4 is a key T_H2 cytokine,³⁷ and previous studies showed that GATA-3-directed IL-4 gene activity involves regulation of both IL-4 promoter³⁸ and enhancer regions.³⁹ Therefore, to further understand the molecular mechanism underlying MSSM-002 modulation of GATA-3-associated T_H2 cytokine expression, we compared GATA-3 binding with IL-4 promoter and enhancer regions in MSSM-002-treated and -untreated D10 cells. Although binding of GATA-3 to both IL-4 promoter and enhancer regions in CA-stimulated D10 cells (Fig 5, D) was observed, there was virtually no binding of GATA-3 to IL-4 promoter and reduced binding to IL-4 enhancer regions in the MSSM-002-treated, CA-stimulated D10 cells. These findings suggest that MSSM-002 suppresses GATA-3 binding to the regulatory sequences of T_H2 cytokine IL-4 gene, resulting in downregulation of IL-4 expression.

DISCUSSION

Although the use of herbal medicines is increasing in Western countries,^{3,4} there is little basic scientific research into their efficacy or effects on cellular and molecular functions. We previously demonstrated a therapeutic effect of an antiasthma herbal formula MSSM-002 on a well-characterized murine model of allergic asthma. In this study we explored the effects of MSSM-002 on an established T_H2 response in vitro and found that MSSM-002 suppressed antigen-induced proliferative responses, reduced IL-4 and IL-5 secretion, and increased IFN- γ secretion by T_H2 -SPCs, thereby showing that MSSM-002 modulates an established T_H2 phenotype to T_H0/T_H1 -like phenotype. To our knowledge, this is the first report that an established murine T_H2 phenotype of antigen-primed SPCs can be modulated. A previous study showed that IL-12 can reverse the human T_H2 phenotype,⁴⁰ but it is believed that polarized murine T cells are more difficult to modulate.⁴¹ MSSM-002 modulation of established T_H2 phenotype might be a fundamental mechanism underlying the previously reported therapeutic effect on an allergic asthma model.

IL-12, produced by antigen-presenting cells, promotes differentiation of T_H1 cells, IFN- γ production, and inhibits differentiation of T_H0 cells into IL-4-secreting T_H2 cells.⁴²⁻⁴⁵ IL-12 is also important in mediating the immunotherapeutic effects of T_H1 response stimulating bacteria and bacterial DNA adjuvants on allergic diseases model.⁴⁶ Because MSSM-002 induced IFN- γ and reduced IL-4 and IL-5 production by splenocyte cultures, we asked whether this effect was dependent on IL-12. We

found that anti-IL-12 blocked MSSM-002 induction of IFN- γ , but it had no significant effect on MSSM-002 suppression of T_H2 cytokines. We also found that MSSM-002 suppressed T_H2 cytokine secretion by anti-CD3/CD28 stimulated T_H2 -SPCs. These results indicate that the mechanisms by which MSSM-002 modulation of T_H2 cells differ from those of T_H1 cytokine-inducing adjuvants, and they suggest that MSSM-002 directly suppresses T_H2 cytokines. To this end, we used the T_H2 clone D10 cells, which do not produce IFN- γ or express IL-12 β 2 receptors,^{18,33} and we found that MSSM-002 significantly reduced antigen-specific proliferation and IL-4 and IL-5 production by these cells. These results, together with the discrepancy between dose responses of MSSM-002 in reducing IL-4/IL-5 and inducing IFN- γ by T_H2 -SPCs, suggest that MSSM-002 modulation of T_H2 -SPCs does not require T_H1 cytokines.

Corticosteroids are the most powerful nonspecific anti-inflammatory drugs routinely used to treat asthma. However, potential side effects, such as adrenal suppression, decreased bone metabolism, and decreased growth, are of particular concern in children, in whom the morbidity and mortality of asthma have been increasing.² It also is well-known that corticosteroids produce overall immune suppression, which results in increased susceptibility to infections. Corticosteroid immunosuppression is due to induction of T-lymphocyte apoptosis.^{34,47} In this study we also found that dexamethasone induction of T_H2 cell apoptosis was responsible for suppression of T_H2 cell activation and cytokine production. In contrast, MSSM-002 was not toxic and did not induce apoptosis. These findings, together with the findings that MSSM-002 suppression of T_H2 cytokines was accompanied by induction of IFN- γ secretion by T_H2 -SPCs, clearly demonstrate that MSSM-002 actions on T cells differ from corticosteroids and suggest that MSSM-002 might be of more benefit to asthma patients, particularly children.

T_H2 cytokine gene expression is regulated primarily at the level of transcription.¹³ GATA-3 appears to exert overall control over T_H2 lineage,¹⁴ as well as subsequent development of allergic airway hyperreactivity. For example, increased GATA-3 gene expression has been found in human asthmatic airways,⁴⁸ and recent animal studies have shown that both antisense-induced local blockade of GATA-3 expression and expression of a dominant-negative mutant of GATA-3 inhibits allergic inflammation in murine model of asthma accompanied with marked reduction of IL-4.^{22,51} Recently, several GATA-3 regulatory mechanisms have been identified at the transcriptional and post-translational levels.¹⁵ In this study we demonstrated that MSSM-002 significantly suppressed GATA-3 mRNA and protein expression by CA-stimulated D10 cells. MSSM-002 also suppressed GATA-3 binding to the gene regulatory regions of T_H2 cytokine IL-4. Because GATA-3 is also pivotal for T_H2 cytokine memory by inducing T_H2 cytokine expression,¹⁵ downregulation of GATA-3 expression and transactivation on T_H2 cytokine gene might, at least in part, account for MSSM-002 suppression of T_H2 cytokine

production by established T_H2 cells. However, the precise mechanisms underlying MSSM-002 modulation of GATA-3 transcription activation and perhaps other T-cell transcription factors require future investigation.

In conclusion, we demonstrated that MSSM-002 can modulate the T_H2 phenotypes of established T_H2 cells, perhaps via downregulation of GATA-3. This activity might be a fundamental mechanism underlying its anti-asthma and antiallergy actions. The finding that MSSM-002 inhibits secretion of T_H2 cytokines without inducing the characteristic overall immunosuppression of corticosteroids suggests that this herbal formula might prove to be of benefit to patients with asthma.

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