

Macrophage Migration Inhibitory Factor Homologs of *Anisakis simplex* Suppress Th2 Response in Allergic Airway Inflammation Model via CD4⁺CD25⁺Foxp3⁺ T Cell Recruitment¹

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We have cloned the macrophage migration inhibitory factor (MIF)-like protein (*Anisakis simplex* (As)-MIF) from larvae of the whale worm (*Anisakis simplex* third-stage larvae). Asthma was induced in the mice using OVA/alum, with or without various concentrations of rAs-MIF treatment before OVA/alum challenge. Treatment with rAs-MIF coupled with OVA/alum during the challenge period induced a complete inhibition of eosinophilia and goblet cell hyperplasia within the lung and profoundly ameliorated the development of lung hyperreactivity. Also, rAs-MIF was shown to reduce profoundly the quantity of Th2-related cytokines (IL-4, IL-5, and IL-13) in the bronchial alveolar lavage fluid and allergen-specific IgG2a in sera. IL-10 and TGF- β levels in the bronchoalveolar lavage fluid of the rAs-MIF-treated group were significantly higher than in the other groups. Additionally, CD4⁺CD25⁺Foxp3⁺ T cells (regulatory T) were recruited to the spleen and lungs of the rAs-MIF-treated mice, but this recruitment was inhibited by anti-rAs-MIF Ab. *The Journal of Immunology*, 2009, 182: 6907–6914.

Helminth parasites can survive for an extended period within a host's body using strategies established over their long coevolutionary interaction with mammalian host immune systems (1, 2). The long life span of the helminth parasites constitutes sufficient evidence that they are extremely skilled at immune evasion, and it is also apparent that interference and modulation are among the very first events occurring in infection (2). Their strategy appears generally to be adaptive, as they defuse aggressive immune reactions and induce certain forms of immunological tolerance, thereby facilitating their long-term survival (3). Although diseases of parasitic infection are quite common in many developing countries, reports of parasitic infection have been reported only rarely in industrialized Western countries over the past century.

In contrast with parasitic infections, the prevalence of asthma has been increasing at an alarming rate in industrialized countries (4). This observation (the relationship between parasites and allergies) has been attributed to a phenomenon referred to as the "hygiene hypothesis" (5). Nagler-Anderson (6) previously demonstrated that the infection of mice with *Heligmosomoides polygyrus* induced a reduction in allergen-specific IgE levels within the serum. In addition, Wohlleben et al. (7) previously reported that an

infection with the helminth *Nippostrongylus brasiliensis* suppressed allergic reactions in mice.

In our previously work, we demonstrated that immunized mice with excretory-secretory protein and total extracts of *Toxascaris leonina* had a thinning of the bronchial epithelial and muscle layer, a disruption and shedding of epithelial cells, a reduction in the number of goblet cells, and a reduction in mucus production compared with those of the OVA-induced asthma group (8). Also, Pearce et al. (9) suggested that only the soluble egg Ag of *Schistosoma mansoni* could induce Th2 responses in the host, in addition to worm infection. Trujillo-Vargas et al. (10) reported that helminth-derived products inhibit the development of allergic responses in mice. This indicates that the excretory-secretory proteins of parasites are likely to harbor molecules that function as host immune system regulators.

A number of parasite-derived proteins, glycoconjugates, and small lipid moieties have been demonstrated to perform known or hypothesized functions in immune interference. Other researchers have already isolated several other immune down-regulatory molecules from parasites, and these molecules have been identified as mammal cytokine homologs, protease inhibitors, abundant larval transcript Ags, glyconetworks, and venom allergen-like proteins (11–17). The cytokine network is a crucial component of host defense against pathogens. It is not, therefore, surprising to find that one of the immune evasion strategies utilized by infectious organisms is the generation of mammalian cytokine homologs, including TGF- β and the macrophage migration inhibitory factor (MIF)³ (18, 19).

MIF was described initially as one of the earliest cytokines to be derived from activated T cells and was believed to prevent the

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³ Abbreviations used in this paper: MIF, migration inhibitory factor; T_{reg}, regulatory T; rMou-MIF, recombinant mouse MIF; BALF, bronchoalveolar lavage fluid; Bm-MIF, *Brugia malayi* MIF; Penh, enhanced pause.

random migration of macrophages (20). MIF has also been demonstrated to be generated abundantly by monocytes/macrophages and to function in an autocrine/paracrine manner in the up-regulation and sustenance of the activation of diverse cell types (21). The profile of the activities of MIF, both in vivo and in vitro, is reflective of a role for MIF in the pathogenesis of a variety of inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, ankylosing spondylitis, and psoriasis (22). MIF performs a crucial function in airway inflammation and airway hyperresponsiveness in asthma (23).

Recently, several MIF homologs have been isolated from parasitic nematodes. Two types of MIF homologs have thus far been identified in nematodes (12). The type 1 MIF homologs bear a greater amino acid similarity with the mammalian MIFs than do the type 2 MIF homologs (24). The type 1 MIF homologs isolated from *Brugia malayi* (Bm-MIF1) induce eosinophil recruitment and alternatively activated macrophage recruitment in vivo when injected into the peritoneal cavities of mice. Mutation of the conserved proline residue induces the abrogation of this activity (25). This ability of parasite MIF homologs was similar to those of mammalian MIF. However, recently Cho and colleagues (26, 27) reported that hookworm MIF (structurally type 2 MIF) functions differently from mammalian MIF.

In this study, we have isolated another type 2 MIF homolog (As-MIF) from *Anisakis simplex* (whale worm) third-stage larva, which causes anisakidosis in humans (28). To confirm its potential as a host immune down-regulation factor, constructed rAs-MIF and applied rAs-MIF were i.p. administered along with OVA/alum to mice, after which we attempted to determine whether OVA-specific Th2 responses were generated in the presence of rAs-MIF. We also assessed the mechanism underlying the down-regulation of OVA-specific Th2 responses by rAs-MIF.

Materials and Methods

Preparation of recombinant macrophage MIF-like protein isolated from parasite

The human MIF-like protein coding gene (*As-MIF*) was isolated by chance from an *A. simplex* third-stage larva cDNA library during the expressed sequence tags analysis of the parasite (28). After the sequence of the PCR product was confirmed, *As-MIF* was extracted for ligation into the PET 28a expression vector (Novagen). After gene ligation, the constructed plasmids were transformed into the *Escherichia coli* BL21 strain. The expression and purification of rAs-MIF protein was conducted in accordance with the manufacturer's instructions (Novagen). LPS was depleted (endotoxin levels <0.01 µg/ml) from rAs-MIF using Detoxi-Gel Affinity Pak prepacked columns (Pierce) in accordance with the manufacturer's instructions. Recombinant mouse MIF (rMou-MIF) was purchased from R&D Systems. Polyclonal anti-rAs-MIF Ab was produced in mice that were boosted twice every 2 wk with 500 µg of rAs-MIF and CFA and IFA (Sigma-Aldrich).

Evaluation of the effects of rAs-MIF in a mouse asthma model

Female BALB/c mice, 6 wk of age and free of murine-specific pathogens, were acquired from Hyochang-Science. The mice were housed throughout the experiments in a laminar flow cabinet and maintained in individual ventilation cages (Three's Shine). All animal studies were approved by the Pusan National University School of Medicine Animal Care and Use Committee.

The experiments were conducted in two stages. The rAs-MIF allergic airway response inhibitory effects, in accordance with concentration, were evaluated in the first stage, and the functional relationship between rAs-MIF and rMou-MIF on allergic airway responses was evaluated in the second stage. All techniques used in the two stages were identical.

These mice were grouped into six groups (each group consisting of 10 mice) and were sensitized on days 1 and 7 via i.p. injections of 75 µg of OVA (Sigma-Aldrich) emulsified in 2 mg of aluminum hydroxide (Sigma-Aldrich) in a total volume of 200 µl. The negative control group was sensitized with PBS on the same day. On days 13, 14, 19, and 20, the mice were treated with PBS (positive control), 200 µl of 1, 10, and 100 µg/ml rAs-MIF (stage 1), PBS, rAs-MIF (10 µg/ml), rMou-MIF (100 ng/ml), and

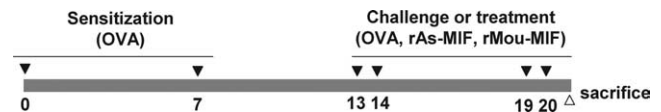


FIGURE 1. Experimental protocol of airway inflammation-inducing model.

a mixture of rAs-MIF (5 µg/ml) and rMou-MIF (50 ng/ml) (stage 2) via i.p. injection administered before 30 min of OVA challenge. They were then challenged for 30 min with an aerosol of 3% (w/v) OVA in PBS (or with PBS as a control) with an ultrasonic nebulizer (Omron, Korea). The negative group was challenged by PBS on the same day as were the other groups (Fig. 1).

Analysis of bronchoalveolar lavage fluid (BALF)

BALF was conducted 24 h after final challenge. At the time of lavage, the mice were sacrificed with 200 µl of ketamine:lumpun:PBS (2:3:5). The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter was secured with ligatures. Pre-warmed PBS was slowly inflated into the lungs and withdrawn. The pooled sera were then centrifuged and the supernatants were maintained at -70°C until use for ELISA. The cell pellets were resuspended and washed twice in PBS. The total cell numbers were counted using a hemocytometer after RBC lysis using ACK lysis buffer. The BALF cell smears were prepared with a cytospin apparatus. The smears were then stained with Diff-Quik solution (Dade Diagnostics) to determine the cell differentials in accordance with conventional morphological criteria. The results were calculated after three consecutive experiments.

Lung histopathology

Histological analyses were conducted as described previously (29). In brief, the lung tissues were fixed with formaldehyde and embedded in paraffin. The thin sections of the embedding tissues were then stained with H&E and periodic acid-Schiff stain. After staining, we microscopically examined the stained sections.

ELISA

OVA-specific Igs (IgG1, IgG2a, and IgE) in the serum and cytokine (IL-4, IL-5, IL-10, IL-13, IFN- γ , and TGF- β) levels in the BALF were determined via ELISA, conducted in accordance with the manufacturer's instructions (eBioscience and R&D Systems). The absorbance of the final reactant was determined at 450 nm using an ELISA plate reader.

Airway hyperresponsiveness measurements

The enhanced pause (Penh), a measure of bronchoconstriction, was evaluated at baseline and after treatment with increasing doses of aerosolized methacholine (2.5–50 mg/ml) via whole-body plethysmography for animals (Allmedicus). In the plethysmography procedure, the mice were allowed to acclimate for 3 min, exposed to nebulized saline for 4 min, then subsequently treated with increasing concentrations (0, 2.5, 5, 10, and 50 mg/ml) of nebulized methacholine (Sigma-Aldrich) in saline using an ultrasonic nebulizer. After each nebulization, recordings were obtained for 3 min and the Penh values measured during each 3-min period were averaged. Graphs of the Penh values were constructed in response to the increasing methacholine concentrations for each dose-matched group of mice.

Flow cytometry

To evaluate the recruitment of CD4⁺CD25⁺Foxp3⁺ T cells (regulatory T (T_{reg}) cell) induced by rAs-MIF, live lymphocytes were gated from the spleen, lungs, and lung lymph nodes of OVA-induced airway inflammatory mice with rAs-MIF, anti rAs-MIF treatment, or no treatment. The cell surfaces were stained with CD4 (FITC) and CD25 (Cy5) Ab (eBioscience) in accordance with the manufacturer's recommended protocols. After permeabilization buffer treatment, the cells were stained with anti-mouse Foxp3 (PE) Ab. The three-color samples were acquired using a FACSCalibur cytometer (BD Biosciences) equipped with CellQuest software (BD Biosciences).

Statistical analysis

Normally distributed data were evaluated via the Student *t* test. ANOVA and data that were not normally distributed were evaluated using nonparametric tests as appropriate.

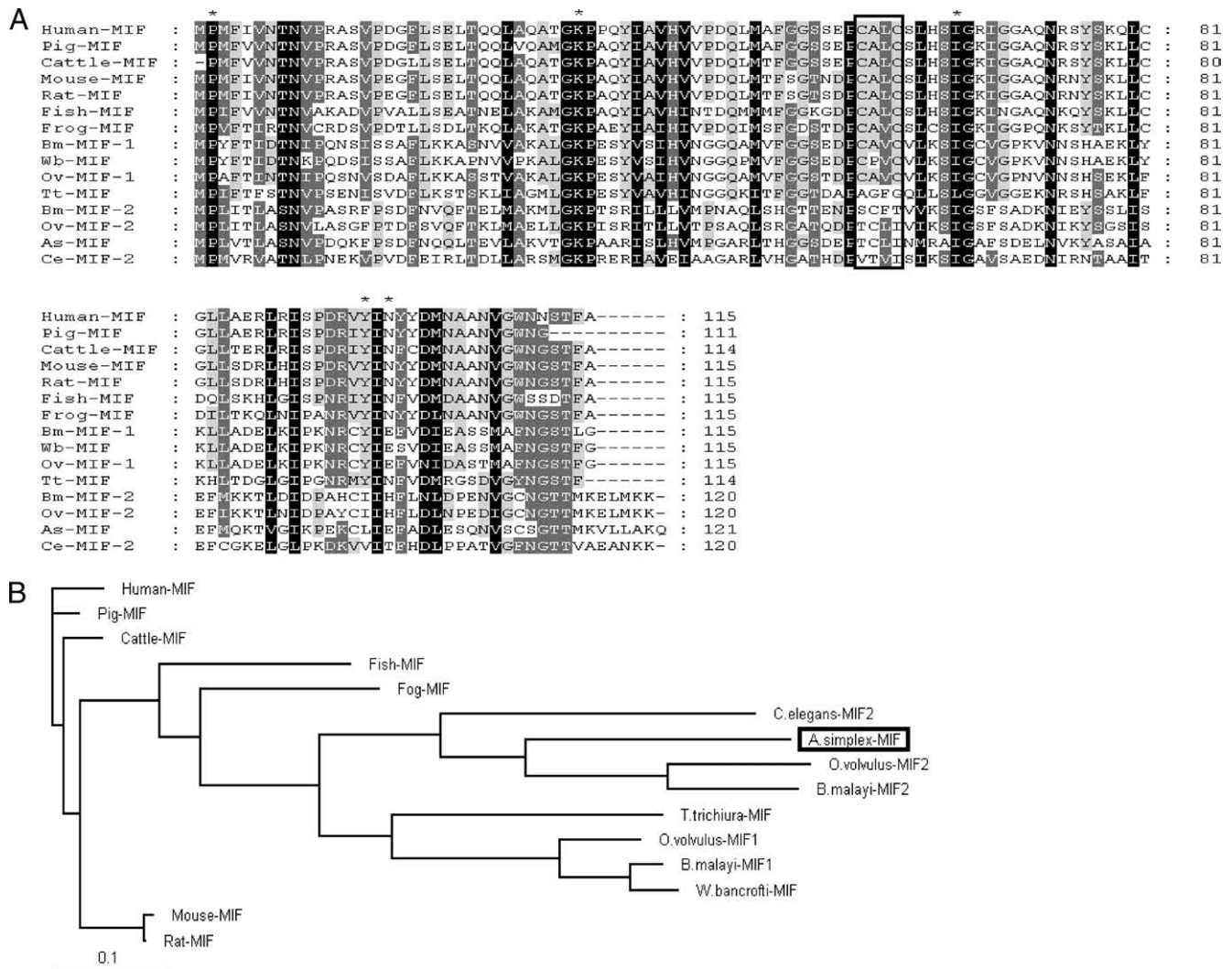


FIGURE 2. Alignment amino acid sequences of MIF homologs from As-MIF with other previously reported MIFs (A) and a neighbor-joining phylogenetic tree derived via the alignment of the amino acid sequences of As-MIF with those of other MIFs in the database (B). Enzyme activity residues are marked and the same residues are highlighted in black. The box indicates each from the 57th to 60th amino acid sequences; sequences of type 1 MIFs were conserved with CXXC residue but those of type 2 MIFs were various (A). Phylogenetic reconstruction was used to generate a gene tree via the maximum-parsimony neighbor-joining method, as executed by the ClustalW program (version 1.8) (B). Human (human-MIF (18699569)), bovine (cattle-MIF (693791)), pig (pig-MIF (116175251)), rat (rat-MIF (1359198)), mouse (Mou-MIF (6754696)), frog (frog-MIF (67677987)), fish (fish-MIF (74096408)), Bm-MIF1 (2829751), Bm-MIF2 (9717249)), *O. volvulus* (Ov-MIF1 (14532265) and Ov-MIF2 (14532268)), *Wuchereria bancrofti* (Wb-MIF (2789663)), *Trichuris trichiura* (Tt-MIF (5327285)), *Caenorhabditis elegans* (Ce-MIF2 (17562710)), and As-MIF (EF165019).

Results

As-MIF belongs to the type 2 MIF and excretory-secretory proteins

The cDNA (As-MIF) obtained from *A. simplex* third-stage larva expressed sequence tags was 366 bp long and was found to code for 121 aa, whose amino acid sequence was quite similar to various MIFs. The active site for tautomerase enzyme activity of human MIF appears to be located around Pro² as a catalytic residue. The substrate also interacts with the Lys³³, Ile⁶⁵, Leu⁹⁶, and Glu⁹⁸ residues, the latter two of which are contributed by the neighboring monomer and the Pro², Asn⁹, Pro³⁴, Pro⁵⁶, Ser⁶⁴, and Leu⁸⁸ residues, which are invariant across the whole MIF family (30). In the deduced As-MIF amino sequence, Ser⁶⁴ was altered to Ala⁶⁴ (Fig. 2A). Interestingly, the Cys⁵⁷ and Cys⁶⁰ of As-MIF and other type 2 MIF were replaced with other amino acid sequences. This site is known as a critical site for the oxidoreductase activity of MIF, which has been demonstrated to be necessary for an important immunological activity of MIF (Fig. 2A). In the initial BLAST

search, As-MIF was matched closely with representative members of the MIF family from nematode, human, pig, cattle, rat, mouse, fish, and frog. The deduced amino acid sequence of As-MIF evidenced 54% identity (77% similarity) with *B. MIF*-2, 49% identity (75% similarity) with the MIF-2 of *Onchocerca volvulus*, and 31% identity (53% similarity) with human and mouse MIF. After clustering with other known MIF sequences, As-MIF was grouped with the type 2 MIFs of nematodes (Fig. 2B). Additionally, As-MIF was detected in the excretory-secretory proteins of *A. simplex* third-stage larvae; thus, they may bind to and stimulate or regulate host molecules of the host (supplemental Fig. 1⁴).

rAs-MIF inhibits OVA-induced airway inflammation in a dose-dependent manner

The administration of 200 μ l of rAs-MIF (1, 10, and 100 μ g/ml in PBS) four times with OVA resulted in a significant reduction

⁴ The online version of this article contains supplemental material.

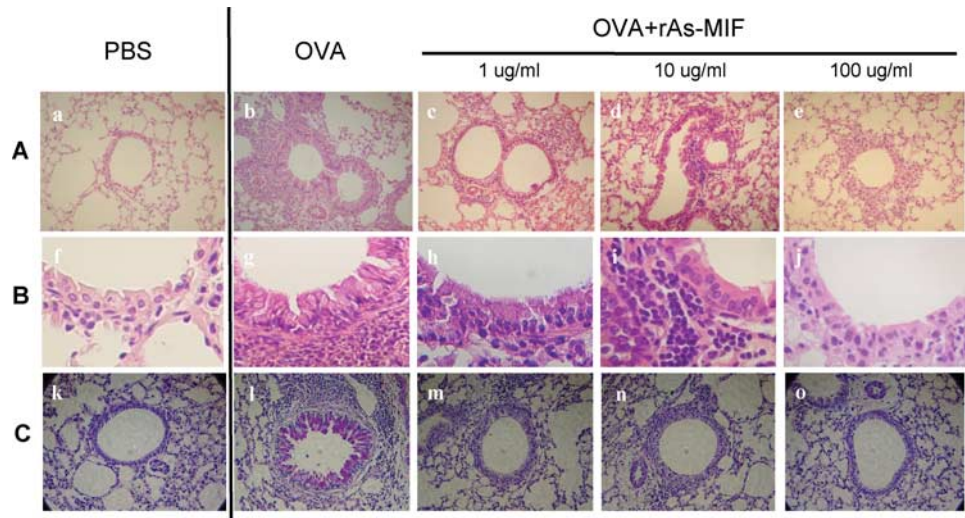


FIGURE 3. Histological appearance of lungs after challenge with PBS, OVA, and rAs-MIF by concentration (H&E stain). Original magnifications: A, $\times 100$; B, $\times 600$; and C, periodic acid-Schiff stain. C, a, f, and k, PBS treated; b–e, g–j, and l–o, OVA plus alum treated (induced asthma); c, h, and m, challenged with 1 $\mu\text{g/ml}$ rAs-MIF; d, i, and n, challenged with 10 $\mu\text{g/ml}$ rAs-MIF; e, j, and o, challenged with 100 $\mu\text{g/ml}$ rAs-MIF. In asthma-induced mice, a massive peribronchial infiltration with immune-related cells and hyperplasia of bronchial epithelial cells were observed. Upon challenge with 1 $\mu\text{g/ml}$ rAs-MIF treatment (c, h, and m), asthma-induced mice evidenced thinner bronchial epithelial cells than were observed in the asthma-induced mice (b, g, and l). Mice challenged with treatment with 10 and 100 $\mu\text{g/ml}$ rAs-MIF evidenced thinner than normal bronchial epithelial cells and decreased numbers of immune-related cells. Goblet cells and immune-related cells in the airway walls of mice exposed to PBS, OVA, and OVA challenge with 1, 10, and 100 $\mu\text{g/ml}$ rAs-MIF. In asthma-induced mice (g), a massive peribronchial infiltration of inflammatory cells and hyperplasia of bronchial epithelial cells were detected. However, goblet cell hyperplasia was reduced in the bronchial epithelial cells of the rAs-MIF-treated mice (h–j).

of the airway infiltration of inflammatory cells in a dose-dependent manner as compared with the OVA challenge group (Fig. 3A). After the induction of asthma, we noted influxes of inflammatory cells in the pulmonary parenchyma. These influxes of inflammatory cells induced severe inflammation and pulmonary edema of the alveolar wall. In the lungs of mice treated with 100 $\mu\text{g/ml}$ rAs-MIF, pulmonary parenchymal inflammation occurred as a mild, diffuse cellular infiltration. In the other groups

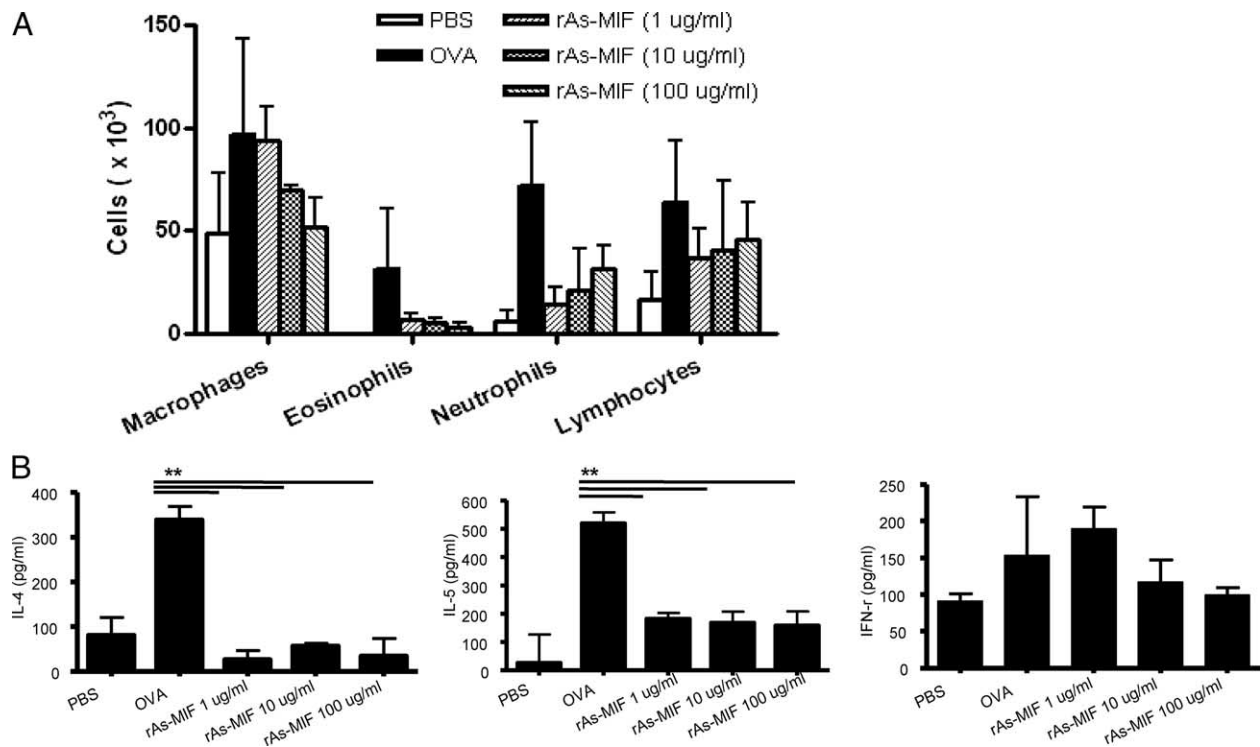


FIGURE 4. OVA-induced Th2 response was reduced as the result of rAs-MIF treatment. The number of inflammatory cells, particularly the numbers of eosinophils and macrophages, were significantly lower in mice challenged with OVA plus 1, 10, and 100 $\mu\text{g/ml}$ rAs-MIF than in the OVA-challenged mice (A). IL-4 and IL-5 cytokine levels in the BALFs of rAs-MIF-treated mice were lower than those of asthma-induced mice on a whole (B). Level of IFN- γ cytokine in BALF was similar to that of IFN- γ cytokine from asthma-induced mice. The data are expressed as the means $\pm$ SD of individual mice (**, $p < 0.01$, $n = 4$ –6 mice/group, three independent experiments).

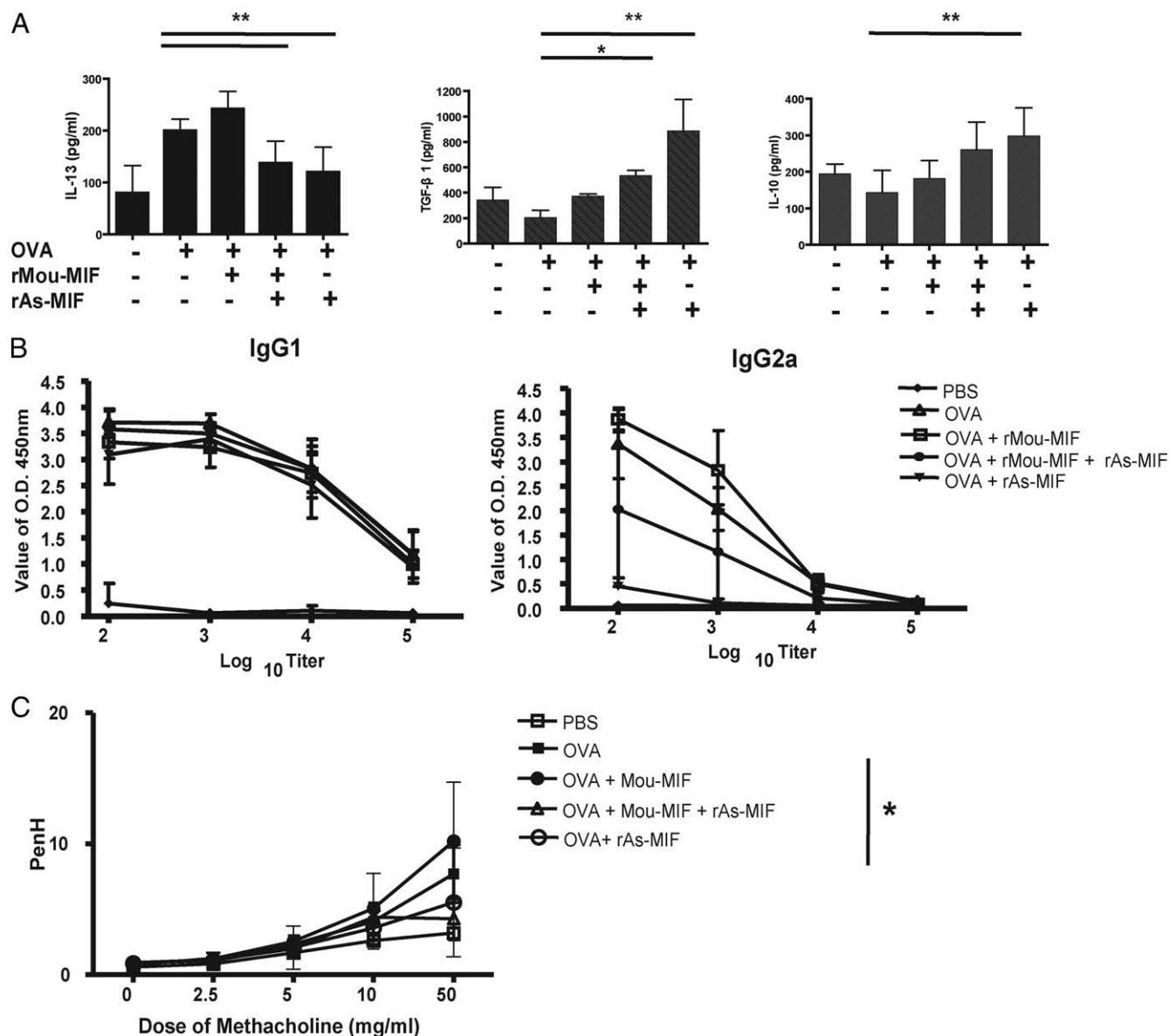


FIGURE 5. rAs-MIF can inhibit host MIF function. The levels of all cytokines of the rAs-MIF-treated mice were contrasted with those of the rMou-MIF-treated mice (A). IgG1 levels were reduced in rAs-MIF-treated mice, but IgG2a levels in BALF were similar to those of all groups, with the exception of the PBS-treated group (B). rAs-MIF reduces airway hypersensitivity (AHR) after asthma induction in mice (C). *, $p < 0.05$ and **, $p < 0.01$, $n = 4-6$ mice/group, three independent experiments.

(1 and 10 $\mu\text{g/ml}$ rAs-MIF-treated mice), we noted slight cellular infiltrations around the bronchial tree, with a small quantity of inflammatory cells. The rAs-MIF-treated mice groups also evidenced a thinning of the bronchial epithelium and a reduction in the number of goblet cells, as well as a reduction in mucus production, compared with what was observed in the OVA-challenged mice, which was more evident at higher rAs-MIF concentrations (Fig. 3, B and C). No goblet cell hyperplasia was observed in the airways of the PBS and all rAs-MIF-treated groups, but this effect was not as marked as that observed in the OVA-challenged group. Bronchial epithelial cell hyperplasia was detected only in the 1- $\mu\text{g/ml}$ rAs-MIF-treated (Fig. 3, B and C) and OVA-challenged groups.

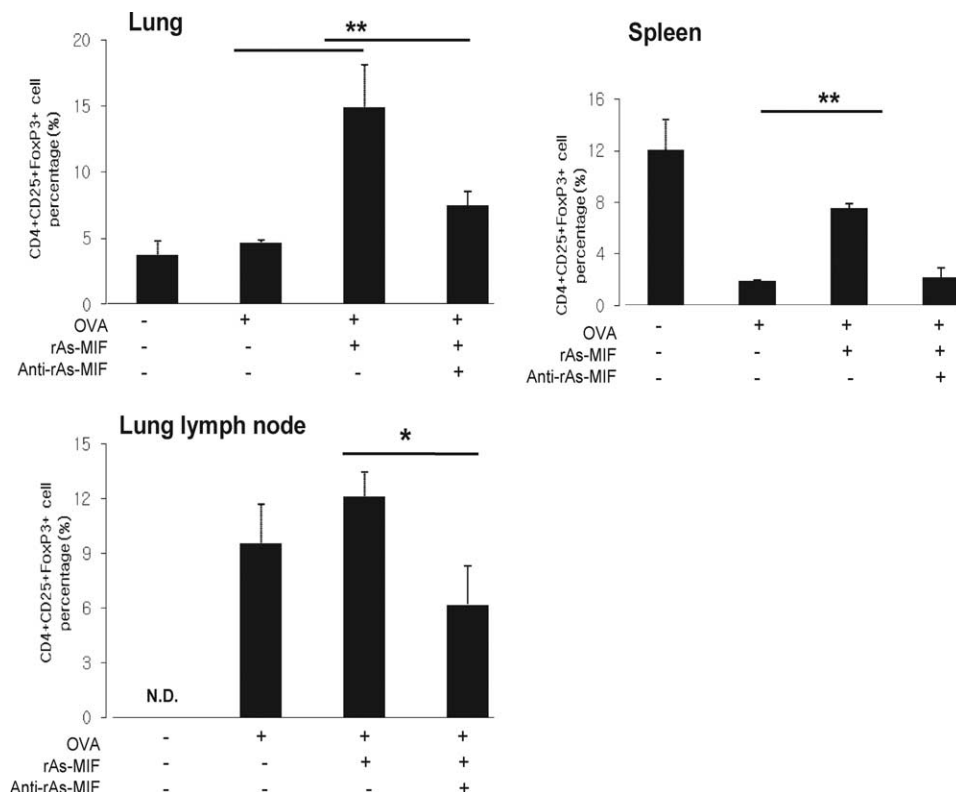
Fig. 4A shows immune-associated cells in the BALFs of asthma-induced and rAs-MIF-treated mice. In the OVA-challenged group after treatment with 100 $\mu\text{g/ml}$ rAs-MIF, the number of macrophages and eosinophils in the BALF was reduced by 74 and 97%, respectively, compared with those of the OVA-challenged group.

The administration of 1 and 10 $\mu\text{g/ml}$ rAs-MIF also inhibited the recruitment of inflammatory cells into the airway to a similar degree. In an effort to determine the manner in which rAs-MIF influences cytokine secretion in the BALF, ELISA was conducted for Th1 cytokines (IFN- γ) and Th2 cytokines (IL-4 and IL-5) (Fig. 4B). The rAs-MIF administration group evidenced a significant reduction of IL-4 and IL-5 concentrations compared with what was observed in the OVA-challenged group ($p < 0.01$). Although the IFN- γ level of 1 $\mu\text{g/ml}$ in the rAs-MIF-treated group was similar to that of the OVA-challenged group, the IFN- γ levels of 10 and 100 $\mu\text{g/ml}$ observed in the rAs-MIF-treated groups were reduced compared with what was observed in the OVA-challenged group.

rAs-MIF antagonized the effect of host MIF in airway inflammation model

To determine the manner in which rAs-MIF and rMou-MIF could influence cytokine secretion in the BALFs, ELISA was conducted for IL-4, IL-5, TGF- β 1, IL-10, and IL-13. rAs-MIF administration

FIGURE 6. $CD4^+CD25^+Foxp3^+$ T (T_{reg}) cell production could be induced by rAs-MIF treatment. T_{reg} cell populations in the lungs and spleen were significantly increased by rAs-MIF treatment, but this effect was inhibited by rAnti-As-MIF (*, $p < 0.05$ and **, $p < 0.01$, $n = 5$ mice/group, three independent experiments). N.D., Not defined.



resulted in a significant reduction in the concentration of IL-13 (Fig. 5A). In addition, the rAs-MIF-treated group exhibited a significant increase in the levels of TGF- β 1 and IL-10 ($p < 0.05$). By way of contrast, rMou-MIF treatment (100 ng/ml) induced an elevation in the levels of IL-10, IL-13, and TGF- β 1 compared with what was observed in the OVA-inhalation group. Also, the mouse group treated with a combination of rAs-MIF (5 μ g/ml) and rMou-MIF (50 ng/ml) evidenced slightly reduced IL-13 levels compared with the OVA-challenged group, but the levels of TGF- β 1 and IL-10 were higher than those of the OVA-challenged mice. These findings show that rAs-MIF could inhibit allergic airway reactions via the inhibition of Th2 cytokine production by increasing the production of TGF- β 1 and IL-10 cytokines, rather than by enhancing the Th1 response. Additionally, rAs-MIF effected a significant reduction in OVA-specific IgG2a serum levels, although the production of OVA-specific IgG1 remained unaffected (Fig. 5B). By way of contrast with what was observed in the rMou-MIF-treated asthma-induced group, the IgG2a levels were higher than those observed in the asthma-induced group.

To determine whether the lung inflammation induced by rAs-MIF or rMou-MIF was associated with alterations in lung function, variations in intratracheal pressure in response to increasing methacholine doses were evaluated. The values of Penh after methacholine (from 2.5 to 50 mg/ml) administration were significantly increased in the OVA-inhalation mouse group compared with the PBS-challenged mice. In response to increasing doses of methacholine, the rMou-MIF-treated mice exhibited enhanced airway hyperresponsiveness compared with the OVA-treated mice (Fig. 5C). Treatment with rAs-MIF induced a significant suppression of Penh values compared with the OVA-treated mice ($p < 0.05$), and these values were also reduced in comparison to the rMou-MIF-treated mice. In the group treated with a combination of rMou-MIF and rAs-MIF, we noted a slight reduction in Penh values as compared with the OVA-treated mice.

T_{reg} cells were recruited by rAs-MIF treatment and this effect was inhibited by anti-As-MIF Ab

Although rAs-MIF reduced airway inflammation as the result of IL-10 and TGF- β production, it was unclear as to whether or not regulatory T cells ($CD4^+CD25^+Foxp3^+$ T cell: T_{reg}) were involved. Thus, we evaluated the T_{reg} cell subset in the lung, spleen, and lung draining lymph nodes following OVA/alum treatment with As-MIF or without treatment. The subset of T_{reg} cells in the asthma-induced lung was significantly lower than those of the rAs-MIF treatment group (Fig. 6 and supplemental Fig. 2). However, the T_{reg} cell subset was reduced significantly after rAs-MIF and anti-rAs-MIF Ab cotreatment. In the spleen, the subset of T_{reg} cells was also substantially reduced after asthma induction, but their subset was increased significantly as the result of rAs-MIF treatment.

Discussion

In our current study, we attempted to determine whether a cytokine-like protein (As-MIF) isolated from intestinal parasites exerts any detectable antiallergic effects. We isolated mammalian macrophage MIF homologs from mammalian intestinal parasites and evaluated their anti-inflammatory effect using an allergic inflammatory airway mouse model. MIF is increased in asthmatic patients and has been identified as a critical factor in the pathogenesis of asthma (23, 31, 32). Mice lacking MIF failed to develop airway hyperresponsiveness, tissue eosinophilia, and mucus metaplasia. Additionally, analysis of the bronchoalveolar fluids revealed a substantial reduction of IL-13, eotaxin, and cysteinyl leukotrienes (32). However, rAs-MIF proved highly effective with regard to the inhibition of goblet cell hyperplasia and inflammatory responses in the airways of OVA-induced asthma model mice. Increasing concentrations of rAs-MIF induced an increase in the anti-inflammatory effects on asthma model mice. Additionally, the

function of rAs-MIF was antagonized compared with the function of host MIF (rMou-MIF).

How does As-MIF suppress allergy responses in mice? There have been many reports demonstrating that Th2-type effector responses may be regulated by T_{reg} cells (33–35). Additionally, nematode infections can induce and expand naturally occurring T_{reg} cells in both humans and mice (36, 37), thereby suggesting a role for these T_{reg} cells in the helminth-induced modulation of inflammatory diseases (38, 39). In particular, the clinical symptoms of allergic airway inflammation in the mouse model was clearly modulated by T_{reg} cell-mediated immune suppression, which was itself activated by helminth infection or Ag treatment (40, 41). In this study, we could determine the increase in T_{reg} cells as the result of rAs-MIF treatment in OVA/alum asthma-induced mice.

IL-10 and TGF- β were produced primarily by T_{reg} cells and they are known to suppress immune response effects. The IL-10 and TGF- β levels measured in BALFs from rAs-MIF-treated asthma-induced mice were higher than those of the asthma-induced mice; the IL-10 and TGF- β levels occurred in accordance with their treated concentrations (Fig. 5). The helminthic parasites stimulate the production of immunoregulatory mediators, which likely perform a function in the maintenance of the chronicity of infection, without any marked induction of pathology. In particular, elevated IL-10 levels have been associated with responses against allergic diseases in helminth-infected individuals (37). Also, Nagler-Anderson (6) showed that in mice sensitized with peanut plus cholera toxin, anti-IL-10 treatment abrogated the ability of helminths to protect against allergic symptoms and to down-regulate allergen-specific IgE. IL-10, which is referred to as the cytokine synthesis inhibitory factor, is an anti-inflammatory cytokine, which is capable of inhibiting the synthesis of proinflammatory cytokines (42).

The IL-10 requirement is critical to several important human diseases, including schistosomiasis, wherein marked increases in host morbidity and mortality are observed when IL-10 levels are low or absent (43). In cases of murine *S. mansoni* infection, IL-10 attenuates the hepatocyte damage induced by the eggs of the parasite. IL-10 is also essential for the maintenance of nonlethal chronic infections, in addition to the inhibition of inappropriate immune responses in experimental models (44). In our study, the IL-10 levels in BALFs from rAs-MIF-treated asthma-induced mice were also shown to be higher than those observed in asthma-induced mice; the level of IL-10 was found to be dependent on the concentrations administered (Fig. 5). TGF- β 1 is also a strong candidate for immune suppression by T_{reg} cells from helminth-infected mice and has already been recognized to alleviate experimental airway allergy symptoms (45) and to instruct peripheral T cells to develop their regulatory capacities (46). Thus, the inhibition of asthma response by rAs-MIF may be associated with the principal regulatory T cell-associated down-regulatory cytokines, including TGF- β 1 and IL-10.

The explanation of the hygiene hypothesis through the Th1/Th2 paradigm initially was thought to imply that increases in IFN- γ should be associated with a reduction in allergic disease. However, later studies demonstrated the following: high levels of IFN- γ can be found in the BALFs of asthmatic subjects (47); IFN- γ -generating T cells can exacerbate airway inflammation in animal models (48); and protective measures to suppress airway inflammation, such as *Mycobacterium vaccae* administration, exert their effects via mechanisms that are not mediated by IFN- γ (49). We have also observed reductions in the levels of Th1 cytokines associated with inflammation, including IFN- γ , in the BALFs from rAs-MIF-treated asthma-induced mice (Fig. 4). These results demonstrate

that the mechanism underlying the down-regulatory effects of rAs-MIF on OVA-induced asthma were associated with the reduction of OVA-specific Th1 responses, as well as OVA-specific Th2 responses.

Recently, Cho and colleagues (26, 27) reported that the structure of nematode parasite MIF (rAceMIF, type 2 MIF) was similar to that of human MIF as type 1 MIF and they can bind equal receptor, CD74, although binding activity of rAceMIF is 4.5-fold lower than that of human MIF (26, 27). However, parasite type 2 MIF could not be inhibited by the mammalian MIF inhibitor ISO-1. They hypothesized that the parasite MIF (AceMIF) exerts its biological effect through a direct interaction with the MIF receptor CD74 a competitor of host MIF. This study and a previous report showed that As-MIF also was classified to the type 2 MIF group (27); therefore, As-MIF also might have a similar function to that of AceMIF. Actually rMou-MIF can elevate the clinical signs of airway inflammation by OVA challenge; also, we found that these effects were quite inhibited by rAs-MIF treatment (Fig. 5). These results suggested that although we could not determine whether rAs-MIF can bind to the same receptor of host MIF (Mou-MIF), it might function as an antagonist of host MIF.

Mammalian MIF evidences the widely conserved Cys-Xaa-Xaa-Cys motif (50) Cys⁵⁷-Ala-Leu-Cys⁶⁰. Interestingly, vertebrate MIFs and type 1 nematode MIFs harbored conserved amino acid sequences in this study, but type 2 nematode MIFs, including As-MIF, harbored very diverse amino acid sequences (Fig. 2A). This region has been determined to form an intramolecular disulfide bond and oxidoreductase activity was determined to be dependent on the presence of the cysteines (51–53). It is worthy of note that the oxidoreductase activity of MIF has been shown to be necessary for the crucial immunological activity of MIF, as the substitution of Cys⁵⁷ or Cys⁶⁰ by serine resulted in the partial or complete elimination of the MIF-mediated killing of *Leishmania* parasites by macrophages, respectively (52). However, we do not currently understand how this region of As-MIF is associated with T_{reg} cell recruitment. To assess the mechanisms of the As-MIF immunosuppressive effect in detail, their crystallized structure should be determined and further molecular research should be conducted. The N-terminal region of MIF was crucial in the binding of its ligand, and six histidine residues were attached to the N-terminal region of rAs-MIF. This structure was required for elution and was thought to be relevant to the binding ability of rAs-MIF. However, we repeated the experiment without any fusion system structure and obtained identical results.

In conclusion, we detected As-MIF in the excretory-secretory proteins of *A. simplex* third-stage larva. This is relevant in that the protein functions as a host immune modulator. Parasites regulate or suppress their host immune response, maintaining their parasitism for a prolonged period, using unknown molecules. As-MIF may be one of the molecules that affects host immune regulation; the further characterization of As-MIF may ultimately result in the design of novel therapeutic intervention strategies for the treatment of asthma.

Disclosures

The authors have no financial conflict of interest.

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