

IMMUNOPATHOGENESIS

Macrophage Migration Inhibitory Factor Isolated from a Parasite Inhibited Th2 Cytokine Production in PBMCs of Atopic Asthma Patients

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Background. In a previous study, we demonstrated that the human macrophage migration inhibitory factor (MIF)-like protein (As-MIF) isolated from helminths could inhibit allergic airway inflammation via the recruitment of CD4⁺CD25⁺Foxp3⁺ T cells. **Objective.** To evaluate the clinical importance of As-MIF as an antiasthma drug, we evaluated immune responses after recombinant As-MIF (rAs-MIF) treatment in peripheral blood mononuclear cell (PBMC) cultures. **Methods.** PBMC was isolated from 10 patients with atopic asthma, 8 patients with nonatopic asthma, and 12 nonatopic healthy subjects, and various concentrations of rAs-MIF were transferred into the PBMC culture medium. After 3 days, we measured the levels of T helper 2 and T helper 1 cytokines via ELISA. **Results.** In atopic asthma, IL-4 and IL-5 production was significantly reduced in the PBMC cultures after rAs-MIF treatment. These inhibitory effects were not observed in the nonatopic asthma group. By way of contrast, IL-10 production in the PMBC cultures was significantly increased after rAs-MIF treatment in all experimental groups. **Conclusion.** The results of this study are similar to those previously reported in a mouse study, suggesting that As-MIF might be a candidate for the specific treatment of asthma.

Keywords asthma, macrophage migration inhibitory factor (MIF), parasite, Th2 cytokines

INTRODUCTION

In several recent reports, helminthic infections could reduce the reactivity of skin to aero-allergens, and treatment with antihelminthic drugs resulted in increased frequency of positive skin-prick test results and increased production of allergen-specific IgE (1–5). Immune responses share many important features in helminthic infections and allergies, most notably the T helper 2 (Th2)-dominant cytokine milieu, which is associated with upregulation of IL-4- and IL-5-mediated IgE production and eosinophilia. Although, like allergens, the parasites elicit Th2 responses, they may downregulate the host immune response via immune modulatory molecular activity (6, 7).

Thus, after infestation, the helminths generate modulatory molecules to downregulate host response and to improve the chances of their own survival. Some of these molecules may inhibit host immune responses (8). The parasites generate bioactive substances, including human cytokine homologues, such as TGF- β and macrophage migration inhibitory factor (MIF), which can proactively modify the immunological environment,

in order to promote parasite survival (9, 10). Recently, filarial infection has been shown to induce Foxp3 expression in activated T cells, thus performing a key function in parasite immune avoidance (9, 11). In our previous study using a mouse asthma model, we demonstrated that the MIF homologues (As-MIF) of *Anisakis simplex* third-stage larvae suppressed airway inflammation and Th2-related cytokine production in bronchoalveolar lavage fluid, and elicited the recruitment of CD4⁺CD25⁺Foxp3⁺ T (T_{reg}) cells to the spleen and lung (12). Additionally, recombinant As-MIF (rAs-MIF) ameliorated experimental intestinal colitis and increased IL-10 production in both dendritic cells (DCs) and intestinal epithelial cells, via the toll-like receptor 2 (TLR2) (13).

In an effort to investigate the potential of As-MIF as an asthma treatment, we evaluated immune responses after rAs-MIF treatment on the peripheral blood mononuclear cell (PBMC) of asthma patients, by measuring the levels of both Th2 and Th1 cytokines and calculating the IL-4/IFN- γ ratio.

MATERIALS AND METHODS

Study Subjects

This study included 12 healthy nonatopic adult volunteers, 10 atopic asthmatics, and 8 nonatopic asthmatics. Asthma was diagnosed by history and positive

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airway hyper responsiveness to methacholine challenge. Airway hyperresponsiveness was expressed as the drug concentration required to cause a fall of 20% in the FEV1 (PC20) in noncumulative units by methacholine challenge testing, and was regarded as positive if the PC20 was less than 8 mg/mL. Atopy in response to one or more common aero-allergens was documented by positive skin-prick tests. Allergy skin-prick tests were conducted using 55 commercially available inhalant allergens (Allergopharma Co., Hamburg, Germany), and positive (1 mg/mL histamine) and negative (solvent) controls. After 15 min, the mean diameters of the wheals formed by the allergens (A) were compared with those formed by histamine (H). If the former was equal to in size or larger than the latter (i.e., A/H ratio ≥ 1.0), the reaction was considered positive. All subjects were off medication at the time of the study, and none of the patients had received either systemic or inhaled corticosteroids within the 3 months prior to the study. Informed consent was obtained in all cases and the experimental protocols were approved by the ethics committee of our institution.

Preparation of the Recombinant MIF-Like Protein Extracted from the Parasite

In our previous study, rAs-MIF was cloned into the pET 28a expression vector (Novagen, Darmstadt, Germany). Then the rAs-MIF was expressed and purified, as described in a previously published report (12).

PBMC Isolation and Culture Procedures

PBMCs were isolated from heparinized venous blood via centrifugation on a Ficoll-Paque system. PBMCs were washed in RPMI 1640 medium (Gibco, Eggenstein, Germany), supplemented with 2 mM L-glutamine and 50 μ M gentamicin, then re-suspended in the same medium complemented with 5% heat-inactivated human AB serum (Sigma, Munich, Germany). PBMCs (1×10^6 cells/mL) were cultured in a total volume of 200 μ L/mL RPMI/5% human AB serum with or without phytohemagglutinin (PHA) (final concentration 15 μ g/mL) in 96-well round-bottom microtiter plates. Cells were left either untreated or treated with different concentrations of rAs-MIF (0.1, 1, and 10 μ g/mL). After 3 days of culture, 100 μ L supernatant was removed from the culture wells for cytokine detection.

Measurement of IL-4, IL-5, IFN- γ , IL-10, and TGF- β Cytokine Levels

The levels of IL-4, IL-5, IFN- γ , IL-10, and TGF- β in the supernatants were measured via ELISA, according to the manufacturer's recommended protocols. The lower limits of detection of IL-4, IL-5, IFN- γ , IL-10, and TGF- β were 2, 4, 4, 2, and 60 pg/mL, respectively.

Statistical Analysis

The data were expressed as means $\pm$ standard error of mean (SEM). The mean values of the measured variables were compared between the two groups via the

nonparametric Kruskal–Wallis test. A p -value of $<.05$ was considered statistically significant.

RESULTS

Table 1 shows the characteristics of the patients included in the study. All subjects were nonsmokers. The control subjects had negative allergy skin tests and negative results on methacholine challenge, that is, no respiratory symptoms. In terms of cytokine production in the PBMCs, the levels of IL-4 and IL-5 were higher in the atopic asthmatics than in the normal controls (IL-4: 2.36 ± 0.55 μ g/mL vs. 0.79 ± 0.09 μ g/mL; $p = .014$; IL-5: 12.93 ± 7.96 pg/mL vs. 1.23 ± 0.31 pg/mL; $p = .04$). However, the levels of IL-10 and IFN- γ did not differ significantly between the atopic asthmatics and normal controls (Table 2). The level of TGF- β 1 was higher in the atopic asthmatics than in the normal controls (350.34 ± 181.96 pg/mL vs. 93.25 ± 18.37 pg/mL; $p = .015$).

Treatment with 0.1, 1, and 10 μ g/mL of rAs-MIF significantly suppressed IL-4 production in the PBMC cultures from atopic asthmatics, in a concentration-dependent manner ($p = .037$, $p = .007$, and $p = .007$, respectively, Figure 1). Similarly, treatment with 10 μ g/mL of rAs-MIF significantly suppressed IL-5 production in the PBMC cultures from atopic asthmatics ($p = .037$). In addition, the production of IL-13 in the PBMC cultures from atopic asthmatics was significantly reduced by 0.1, 1, and 10 μ g/mL rAs-MIF treatment ($p = .023$, $p = .022$, $p = .022$, respectively). However, these effects were not observed in the nonatopic asthmatics. Treatment with rAs-MIF significantly and dose-dependently increased the concentration of IL-10 in the supernatants from the PBMC culture in all of the experimental groups (Figure 1). TGF- β concentrations

TABLE 1.—Demographic characteristics of subjects.

	Atopic asthma ($n = 10$)	Nonatopic asthma ($n = 8$)	Normal controls ($n = 12$)
Age (years)	31.7 ± 7.5	39.3 ± 8.2	24.4 ± 1.0
Sex (M/F)	5/5	4/4	7/5
FEV1 (%)	92.3 ± 7.6	98.5 ± 6.4	98.1 ± 3.8
FEV1/FVC	79.2 ± 3.7	82.0 ± 1.8	82.6 ± 1.8
PC20 (mg/mL)	2.27 ± 1.20	4.24 ± 1.42	>25
Skin-prick test	Positive	Negative	Negative

Note: Data are presented as mean with standard error of mean (SEM).

TABLE 2.—Serum cytokine profiles of the subjects.

	Atopic asthma	Nonatopic asthma	Normal controls
IL-4 (ng/mL)	$2.36 \pm 0.55^*$	1.28 ± 0.98	0.79 ± 0.25
IL-5 (pg/mL)	12.93 ± 7.96	3.72 ± 1.71	1.23 ± 0.31
IFN- γ (pg/mL)	58.69 ± 33.37	25.31 ± 11.46	12.26 ± 0.51
IL-10 (pg/mL)	$21.15 \pm 12.70^{**}$	$11.8 \pm 2.04^{**}$	2.41 ± 0.51
TGF- β 1 (pg/mL)	$350.34 \pm 181.96^*$	323.25 ± 95.26	93.25 ± 18.37

Notes: Data are presented as mean with standard error of mean.

* $p < .05$ compared with normal controls, ** $p < 0.01$ compared with normal controls.

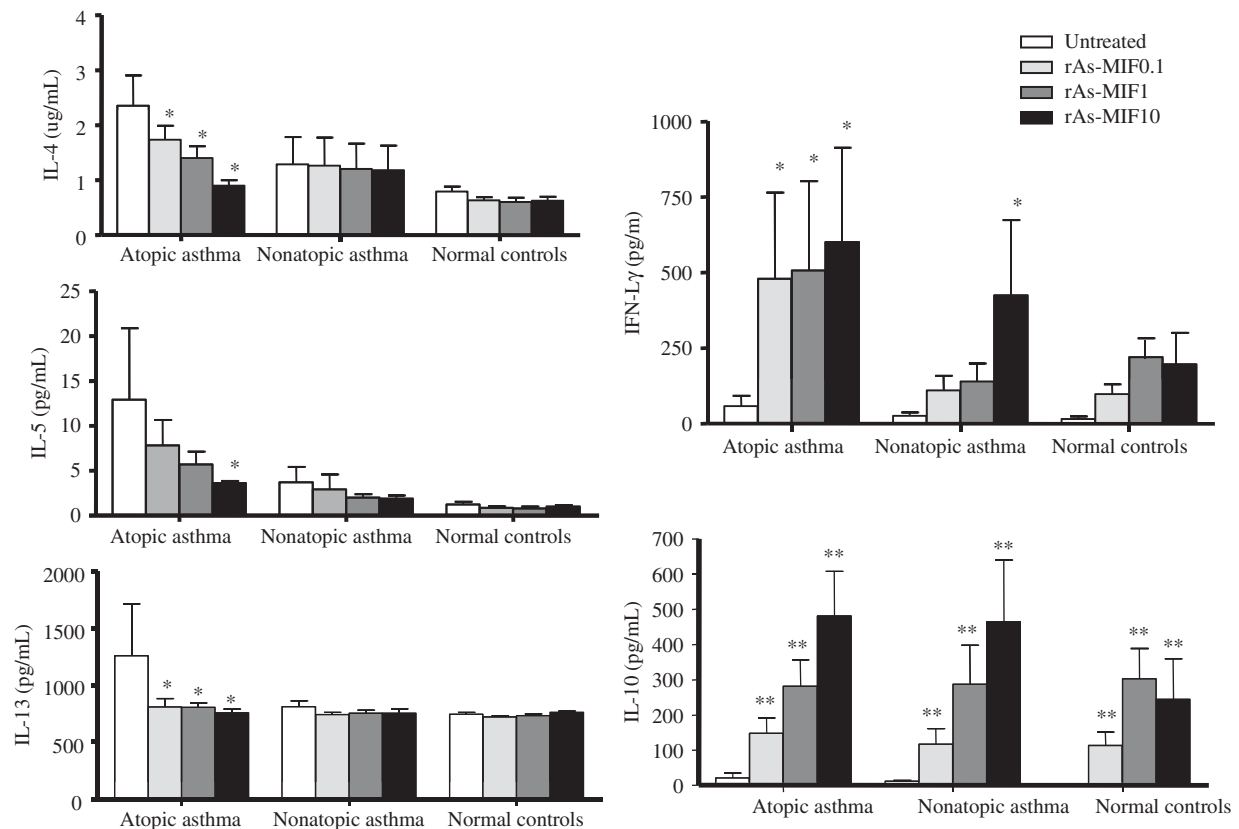


FIGURE 1.—Effects of the rAs-MIF treatment on IL-4, IL-5, IL-10, and IFN- γ production. PBMCs were isolated from subjects with atopic asthma ($n = 10$), nonatopic asthma ($n = 8$), and normal controls ($n = 12$), and incubated for 3 days with culture medium and different concentrations of rAs-MIF (0.1, 1, and 10 $\mu\text{g/mL}$, respectively). Data are expressed as the means with standard error of the mean. * $p < .05$ compared with normal controls, ** $p < .01$ compared with normal controls.

were not significantly altered in the three groups after rAs-MIF treatment (data not shown). Furthermore, the concentration of IFN- γ in the PBMC culture supernatants was increased significantly by rAs-MIF treatment in both the atopic and nonatopic asthmatics (Figure 1).

To find more specific response, we evaluated cytokine production of PBMC after PHA (nonspecific stimuli) treatment. After PHA stimulation, most of cytokine production was increased. The production of Th2 cytokines (IL-4, IL-5, and IL-13) in PBMC cultures from atopic asthmatics was reduced, whereas those of IL-10 and IFN- γ were significantly increased after rAs-MIF (1 μg) treatment (Figure 2). These results were very similar to those of only rAs-MIF treated (without PHA treatment) PBMC from atopic asthmatics (Figure 1). The IL-4/IFN- γ ratio was reduced significantly after rAs-MIF treatment in the atopic asthmatics (Figure 3).

DISCUSSION

The results of recent studies have suggested that helminthic infection may exert protective effects against allergic diseases (4, 5). Many hypotheses have been advanced thus far to explain how helminth infection might protect against allergy. One of these hypotheses is that helminth parasites stimulate the production of immunoregulatory mediators, which likely perform

a function in the maintenance of the chronicity of infection, with no marked induction of pathology. In particular, elevated IL-10 levels have been previously associated with protection against allergic diseases in helminth-infected African children (14). It has been demonstrated that helminth infections can induce T_{reg} cells in hosts, and that these cells secrete IL-10 and suppress the proliferation of other $CD4^+$ T cells (15–17).

In our previous study with mice, we isolated a new immune regulator (rAs-MIF) from an intestinal parasite. rAs-MIF was shown to significantly reduce the levels of both Th2-related cytokines (i.e., IL-4, IL-5, and IL-13) in the bronchial alveolar lavage fluid and allergen-specific IgG2a in the sera. IL-10 and TGF- β levels in the bronchial alveolar lavage fluid of the rAs-MIF-treated group were significantly increased compared with other groups. In addition, T_{reg} cells were recruited to the spleens and lungs of rAs-MIF-treated mice (12). In this study, we found similar immune responses to rAs-MIF treatment in human PBMC. According to the present results, rAs-MIF treatment inhibited the production of Th2 cytokines (IL-4, IL-5, and IL-13) in PBMC cultures from atopic asthma patients. In addition, the IL-10 production of the PBMCs was increased significantly by rAs-MIF treatment in all experimental groups. IL-10 levels might be increased by antigen presenting cells. It has been previously suggested that TLR2 agonists are specialized in inducing

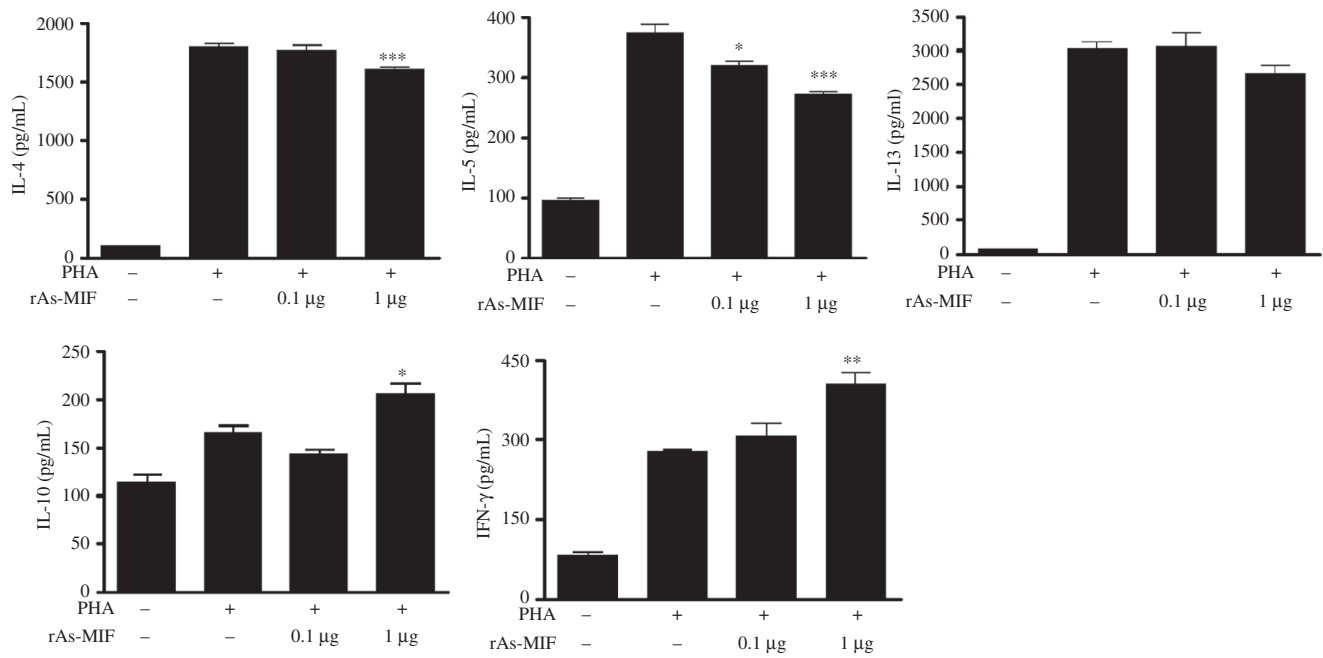


FIGURE 2.—Variation of cytokines production in nonspecific stimulated PBMC cultures from atopic asthmatics after the rAs-MIF treatment. PBMC of atopic asthmatics was incubated with/without PHA and different concentrations of rAs-MIF (0.1 and 1 $\mu\text{g/mL}$, respectively). Data are expressed as means with standard error of mean. * $p < .05$ compared with only PHA treatment, ** $p < .01$ compared with only PHA treatment, *** $p < .001$ compared with only PHA treatment.

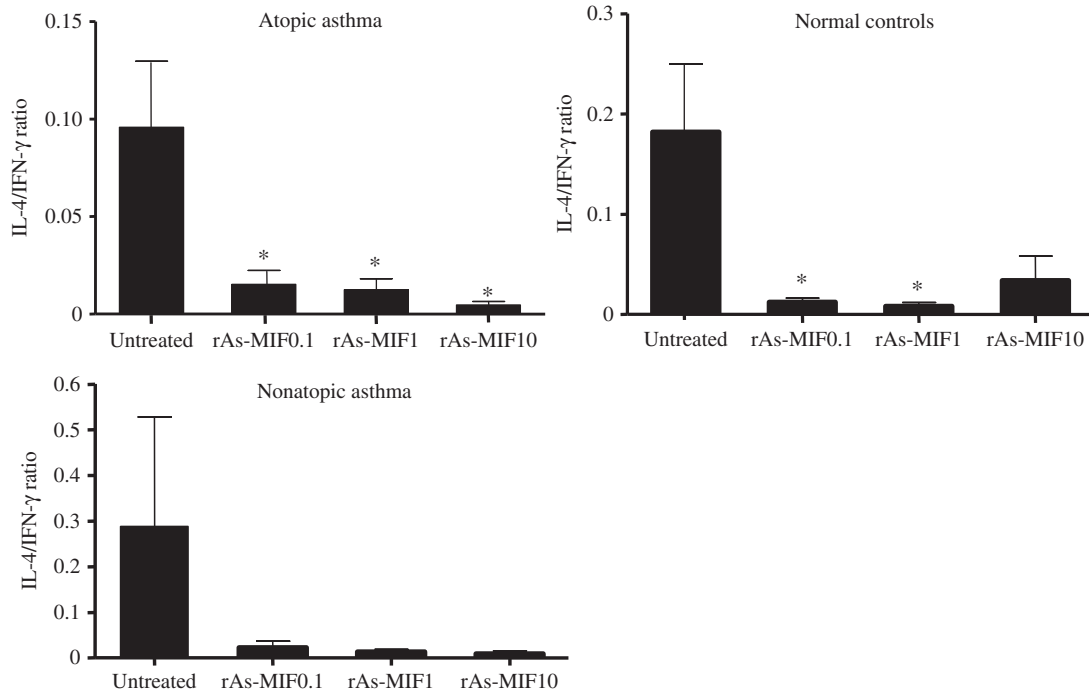


FIGURE 3.—IL-4/IFN- γ ratio after rAs-MIF treatment. Treatment with different concentrations of rAs-MIF (0.1, 1, and 10 $\mu\text{g/mL}$) significantly reduced the IL-4/IFN- γ ratio in atopic asthmatics. Data are expressed as means with standard error of mean. * $p < .05$ compared with untreated control.

IL-10 expression by antigen presenting cells (18,19). For example, TLR2 signaling is crucially important for the induction of IL-10 production either by macrophages or by DCs stimulated with *Mycobacterium tuberculosis* or with lipopeptides and the LcrV antigen of *Yersinia pestis* (19). In our previous study, rAs-MIF has also been shown to induce IL-10 production in DCs via TLR2 (13).

Although no direct evidence has been collected to suggest this, it is likely that the observed enhancement of IL-10 production in human PBMC after rAs-MIF treatment was initiated via TLR2, as has been shown in previous mouse experiments. Additional studies are warranted to determine whether IL-10 production of human PBMC was dependent on TLR2.

In this study, we determined that the concentration of IL-4 in the supernatants of PBMC culture was reduced as a consequence of rAs-MIF treatment, but the concentrations of IFN- γ were increased (Figures 1 and 2). Interestingly, those effects were more distinct in the atopic asthma patient group than in the other groups. In particular, the IL-4/IFN- γ ratio was reduced significantly by rAs-MIF treatment in the PBMC culture supernatants from patients with atopic asthma (Figure 3). Asthmatic lungs contain both Th1 cytokines (IL-1 β , IFN- γ , and TNF- α) and Th2 cytokines (IL-4, IL-5, IL-10, and IL-13). The underlying process that drives and maintains the asthmatic inflammatory process appears to be an imbalance in the equilibrium between Th1 and Th2 immune response types, in which the latter predominates (20, 21). Th2 cytokines promote various elements of allergic inflammation, including propagation of the Th2 phenotype, isotype-switching from IgG1 to IgE synthesis, eosinophil mobilization, and mast cell maturation and activation (21). The expression of T-box transcription factor, a key Th1 transcription factor, was reduced in the airways of patients with asthma as compared with that seen in the control human subjects (22). Therefore, regulation of the Th1/Th2 balance has been considered as a possible therapeutic strategy for patients with atopic asthma. We were unable to determine why the level of IFN- γ was increased by rAs-MIF treatment in the atopic asthma patients, compared with normal controls; in further studies, the relevant mechanisms will be investigated in further detail.

CONCLUSION

In conclusion, we identified the inhibitory effect of a helminthic parasite-derived product on Th2 cytokine production from the PBMCs of atopic asthma patients. Thus far, ethical considerations have precluded detailed studies of the inhibitory effects of rAs-MIF on Th2 cytokine production. Nevertheless, we consider this parasitic worm-derived product to be a possible candidate for an effective asthma treatment in humans.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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