

Increased production of macrophage migration inhibitory factor by PBMCs of atopic dermatitis

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Background: Atopic dermatitis (AD) is a chronic pruritic inflammatory skin disorder. The underlying cause of AD is multifactorial, and several cytokines are considered to be involved in this severe inflammatory skin disease. Macrophage migration inhibitory factor (MIF) is an immunoregulatory cytokine essential for T-cell activation and delayed-type hypersensitivity. Recently we demonstrated that serum MIF content was significantly elevated in patients with AD. Consistent with this, expression of MIF messenger RNA in keratinocytes of the eczematous skin lesion was up-regulated.

Objective and Method: Although keratinocytes are considered to be a potential source of increased serum MIF content in AD, precise evaluation has not been carried out in other tissues. MIF is ubiquitously expressed in various cells, including T cells and macrophages. In this study we examined MIF production and its messenger RNA level of PBMCs from patients with AD to investigate the contribution of these cells to elevated serum MIF content and to its pathologic characteristics.

Results: Consistent with our previous findings, the serum MIF content of patients with AD was significantly elevated compared with nonatopic healthy control subjects and patients with chronic urticaria without eczema. As for the MIF productivity of unstimulated PBMCs, the MIF content in the culture medium of PBMCs obtained from patients with AD (40.4 ± 8.4 ng/mL) (mean $\pm$ SEM) was significantly increased compared with that from healthy control subjects (6.6 ± 1.1 ng/mL) and patients with chronic urticaria (8.5 ± 1.4 ng/mL) ($P < .0001$). When PBMCs were stimulated by concanavalin A, MIF production by PBMCs of patients with AD was more enhanced than in control subjects or patients with chronic urticaria. The increased ratio of MIF production by PBMCs in response to concanavalin A was significantly correlated with the severity of clinical features of AD. Supporting these results, the level of MIF mRNA in PMBCs of patients with AD was significantly higher than in nonatopic healthy control subjects.

Conclusions: The current results showed that PBMCs should be an important source of increased serum MIF in AD. Because MIF has the potential to induce local and systemic inflammatory and immune responses, it is conceivable that

MIF produced by PBMCs may affect local and systemic pathologic features in AD. (*J Allergy Clin Immunol* 1999;104:659-64.)

Key words: Atopic dermatitis, chronic urticaria, cytokine, concanavalin A, macrophage migration inhibitory factor, PBMCs

Atopic dermatitis (AD) is a chronic pruritic inflammatory skin disorder, and numerous reports on its pathogenesis have documented its genetic and immunologic abnormalities, as well as the involvement of environmental factors.¹⁻³ However, the precise mechanism for the initiation and persistence of skin inflammation remains to be elucidated. In recent years a number of reports have focused on abnormalities on Th1 and Th2 subsets of helper T cells (Th1/Th2 imbalance) in the pathogenesis of AD, and production of various cytokines by T-helper cells has been extensively studied.⁴⁻⁶ In parallel, it is suggested that depressed delayed-type hypersensitivity (DTH) is also involved in this immunologic disorder.⁷

The macrophage migration inhibitory factor (MIF) was the first lymphokine reported to prevent random migration of macrophages and to recruit them at inflammatory loci.⁸ In 1989 human MIF complementary DNA was cloned from activated T cells.⁹ Recent findings demonstrated that MIF functions as an initiator of inflammation by regulation of a number of proinflammatory cytokines, including TNF- α , IL-1, and IL-6.^{10,11} Moreover, MIF is essential for T-cell activation and is expressed dominantly in activated Th2 cells.¹² From these findings, it is conceivable that MIF might contribute to dysregulation of inflammatory and immune responses in AD, particularly in Th1/Th2 imbalance.

In human inflammatory diseases it has been reported that MIF has the potential to exacerbate acute respiratory distress syndrome¹³ and asthma.¹⁴ With regard to the skin, we demonstrated the presence of MIF in the epidermal keratinocytes.¹⁵ Moreover, we found excessive expression of MIF messenger RNA (mRNA) as well as MIF protein in the inflammatory skin lesions in AD, and the serum MIF content was changed in parallel with clinical symptoms.¹⁶ In this context it is considered that the major source of increased serum MIF might be keratinocytes; however, the source of this protein in other tissues has not been fully explored. A number of reports

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Abbreviations used

AD:	Atopic dermatitis
ADASI:	Atopic Dermatitis Area and Severity Index
Con A:	Concanavalin A
DTH:	Delayed-type hypersensitivity
MIF:	Macrophage migration inhibitory factor
mRNA:	Messenger RNA
RT:	Reverse transcription
SSC:	Standard saline citrate

have elucidated that MIF is ubiquitously expressed, not limited to T cells, in the brain, kidney, and macrophages.¹⁷⁻²¹ On the basis of these findings, it is speculated that patients with AD may have multiple cellular sources other than keratinocytes, such as T cells and monocytes, for elevated serum MIF content. We here demonstrate that PBMCs obtained from patients with AD have the potential to produce more MIF than those of healthy control subjects and patients with chronic urticaria, examined as a disease control.

MATERIAL AND METHODS

Material

The following materials were obtained from commercial sources. Nitrocellulose membrane filters were purchased from Millipore (Bedford, Mass); Ficoll Paque Plus from Pharmacia (Uppsala, Sweden); concanavalin A (Con A) and polymyxin B from Sigma (St Louis, Mo); Isogen RNA extraction kit from Nippon Gene (Tokyo, Japan); RPMI 1640, ampicillin, streptomycin, and Moloney murine leukemia virus reverse transcriptase from Gibco (Grand Island, NY); Taq DNA polymerase from Perkin-Elmer (Norwalk, Conn), and the random primer fluorescein-labeling kit and nucleic acid chemiluminescence reagent from DuPont NEN (Boston, Mass). All other chemicals were of reagent grade.

Patients

The study groups comprised 23 patients with AD (aged 13 to 32 years, mean age 23.9 ± 1.3 years; 15 male and 8 female), 14 patients with chronic urticaria without eczema (aged 18 to 56 years, mean age 33.8 ± 4.4 years; 8 male and 6 female), and 15 normal healthy control subjects (aged 16 to 36 years, mean age 24.9 ± 1.6 years; 8 male and 7 female). All the patients visited the Department of Dermatology, Hokkaido University School of Medicine. AD was diagnosed according to the criteria of Hanifin and Rajka.²² Most of the patients had an exacerbated form of AD involving the face, limbs, and trunk. Patients were not treated by oral steroid treatment, and none received topical steroids for at least 2 weeks before study. Severity of the dermatitis was assessed with the Atopic Dermatitis Area and Severity Index (ADASI) score.²³ This scoring system yields values ranging from 1 without any apparent symptoms to 18 with the most severe symptoms (eg, 100% skin involvement of atopic eczema). Five of these patients were further examined before and after topical steroid ointment treatment with regard to MIF productivity by PBMCs, serum MIF levels, and skin scores indicating the severity of AD. Patients with chronic urticaria were chosen as a nonatopic-disease control. The duration of urticaria ranged from 8 weeks to 27 years, and all patients had daily or almost-daily wheals at the time of this study. No patient had taken steroids for at least 2 weeks before the study. Patients with urticarial vasculitis, systemic lupus erythematosus, or hepatitis B or C were excluded.

Activation of PBMCs

Human PBMCs were prepared from heparinized blood by Ficoll-Paque Plus density gradient centrifugation. In brief, we collected a cell layer at the density of 1.077 ± 0.001 g/mL, in which little eosinophil contamination was observed by microscopic examination. The PBMC layer was washed 3 times with sterile PBS. PBMCs (1×10^6 /mL) were cultured in RPMI 1640 containing ampicillin (100 IU/mL), streptomycin (50 μ g/mL), and polymyxin B (30 μ g/mL) with use of 24-well plates at 37°C in a humidified atmosphere with 5% carbon dioxide. Cells were stimulated with 10 μ g/mL of Con A or left unstimulated. After 48-hour culture, supernatants were collected and frozen until the assay for MIF.

ELISA

The supernatants of cultured PBMCs and serum MIF were subjected to ELISA for measurement of MIF as previously described.¹⁶ For this assay we used recombinant human MIF to obtain the standard curve, in which good linearity was demonstrated between MIF contents (1 to 200 ng/mL) and absorbency.

Reverse transcription-PCR and Southern blot analysis

Total RNA was extracted from PBMCs with an Isogen RNA extraction kit. The reverse transcription of RNA was carried out with use of Moloney murine leukemia virus reverse transcriptase with oligodeoxythymidine primers and subsequent amplification with Taq DNA polymerase. The PCR was carried out for 23 cycles of denaturation at 94°C for 1 minute, annealing at 53°C for 2 minutes, and extension at 72°C for 1 minute with a thermal cycler (Perkin-Elmer, model 2400). Human MIF primers used were 5'-CTCTCCGAGCTCACCCAGCAG-3' (58-78) (forward) and 5'-CGCGTTCATGTCTCGTAATAGTT-3' (292-312) (reverse) and those for β -actin were 5'-CGTCTGCGGCACCACCAT-3' (936 to ~955) (forward) and 5'-GCAACTAAGTCATAGTCCGC-3' (1170 to ~1189) (reverse). The PCR products were separated on 2% agarose gel and transferred onto a nylon membrane by capillary action. The DNA was immobilized on the membrane by baking at 80°C for 2 hours. The DNA probes for MIF and β -actin were prepared with use of the full-length rat MIF and β -actin complementary DNA insert labeled with a fluorescein-labeling kit according to the manufacturer's protocol. Hybridization was carried out in a solution containing 2 $\times$ standard saline citrate (SSC), 0.5% (wt/vol) dextran sulfate, 1 mg/mL salmon sperm DNA, 0.1% SDS, and the fluorescein-labeled DNA probe at 65°C overnight. The membrane was washed in 2 $\times$ SSC containing 1% SDS at 65°C for 15 minutes and 0.2 $\times$ SSC containing 0.1% SDS at 65°C for 15 minutes. The bands were visualized by a nucleic acid chemiluminescence reagent. Quantitative analysis was carried out with an MCID image analyzer (Fuji Film, Tokyo, Japan).

Statistics

Statistical analysis of the MIF levels in the culture media of the PBMCs was performed with the Student *t* test. Correlation coefficients and statistical significance were determined with use of Pearson's linear regression analysis.

RESULTS

Production of MIF in PBMCs from patients with AD

MIF levels in the sera were significantly high in patients with AD (22.5 ± 2.1 ng/mL) (mean $\pm$ SEM) compared with nonatopic healthy control subjects ($8.6 \pm$

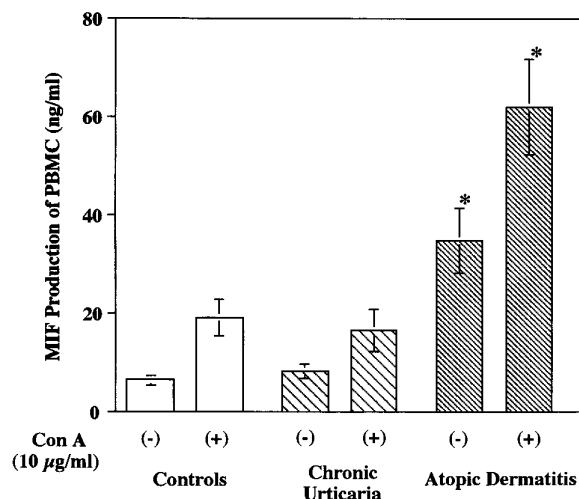


FIG 1. Effect of Con A on MIF production of PBMCs. Con A (10 μ g/mL) was added to culture medium of PBMCs (1×10^6 cells/mL) obtained from nonatopic healthy control subjects ($n = 15$), patients with chronic urticaria without eczema ($n = 14$), and patients with AD ($n = 23$). After 48-hour incubation MIF content in culture medium was measured by ELISA. Asterisk, Statistically significant compared with control subjects or with patients with chronic urticaria ($P < .005$).

TABLE I. Serum MIF contents of atopic and nonatopic control groups

Group	No.	Age (y)	Sex (male/female)	Serum MIF (ng/mL)
Control	15	24.9 $\pm$ 1.6	8:7	8.6 $\pm$ 0.8
Non-AD	14	33.8 $\pm$ 4.4	8:6	10.8 $\pm$ 1.0
AD	23	23.9 $\pm$ 1.3	15:8	22.5 $\pm$ 2.1*

Control subjects were nonatopic healthy individuals; non-AD patients had chronic urticaria without eczema.

*Significantly higher than control ($P < .0001$).

0.8 ng/mL) ($P < .0001$) (Table I), consistent with our previous study.¹⁶ However, we could not find any significant increase of MIF production in the patients with chronic urticaria examined as a disease control. MIF production by PBMCs obtained from 5 patients with AD decreased as clinical symptoms improved with steroid ointment treatment (Table II). On the basis of this result, we further examined MIF production by PBMCs. As for PBMCs of healthy control subjects, the MIF content was 7.1 ± 0.8 ng/mL. The MIF content in the culture medium was increased to 13.8 ± 1.3 , 17.2 ± 1.8 , and 29.4 ± 2.1 ng/mL in response to 5, 10, and 20 μ g/mL Con A after 48 hours, respectively ($n = 3$). The cell viability of PBMCs was significantly reduced by 20 μ g/mL Con A in 72 hours. Therefore we used 10 μ g/mL Con A and a 48-hour incubation for the following experiments. The MIF content of healthy control subjects was significantly increased from 6.6 ± 1.1 ng/mL to 19.2 ± 3.6 ng/mL by the 48-hour incubation with Con A ($n = 15$) ($P < .001$) (Fig 1). Patients with chronic urticaria did not show any significant difference in MIF content from healthy control subjects, showing 8.5 ± 1.4 ng/mL without Con A

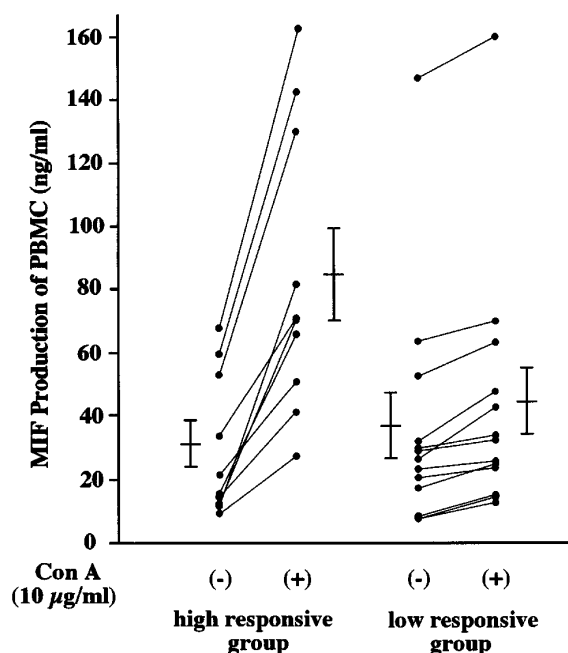


FIG 2. Ratio of increase of MIF production by PBMCs in response to Con A. AD patients, as described in Fig 1, were divided into 2 groups, depending on increased ratio of MIF production by Con A stimulation. Increased ratio was determined as MIF contents in culture medium with Con A stimulation divided by those without stimulation for each individual. *Left*, High-responsive group (ratio ≥ 2) ($n = 10$), ranging from 31.9 ± 6.9 ng/mL to 84.8 ± 14.5 ng/mL; *right*, low-responsive group (ratio < 2) ($n = 13$), ranging from 37.1 ± 10.5 ng/mL to 44.3 ± 11.0 ng/mL.

TABLE II. MIF production by PBMCs before and after steroid treatment with regard to clinical scores

	Age (y)/sex	Steroid treatment	Serum MIF (ng/mL)		ADASI
			Con A-	Con A+	
1	16/M	-	30.1	35.3	15.8
		+	26.6	30.7	5.6
2	22/F	-	26.2	43.5	21.0
		+	14.1	24.2	3.4
3	16/M	-	17.2	40.7	17.1
		+	9.7	29.0	12.5
4	15/F	-	29.0	32.6	29.2
		+	13.1	18.6	8.6
5	23/F	-	21.2	48.9	25.6
		+	14.0	28.7	17.3

M, Male; F, female.

stimulation and 16.6 ± 4.2 ng/mL with the stimulation ($n = 14$). On the other hand, the MIF content in the culture medium of PBMCs of patients with AD was much more increased by Con A stimulation, from 34.9 ± 6.6 ng/mL to 61.9 ± 9.6 ng/mL ($n = 23$). Spontaneous production of MIF by PBMCs was not always correlated with the severity of the clinical features of AD. When the increased ratio of MIF production in response to Con A stimulation was evaluated, there were 2 distinct groups, high- (ratio ≥ 2) and low- (ratio < 2) responsive popula-

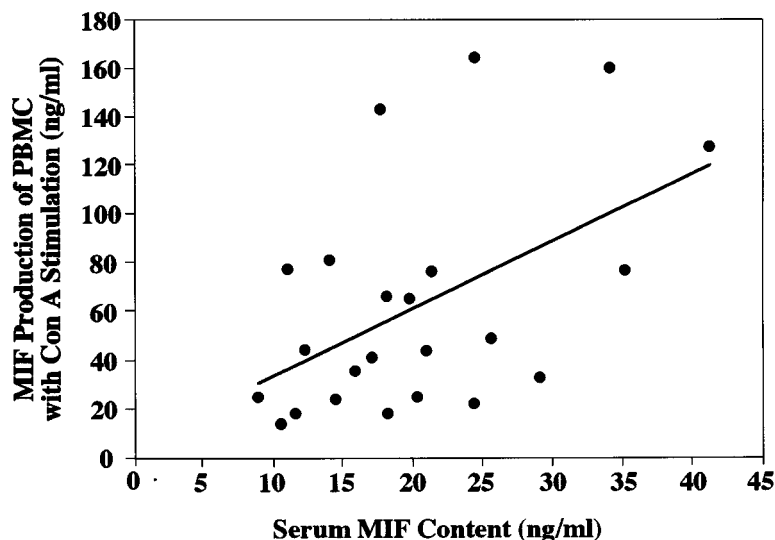


FIG 3. Correlation between serum MIF content and MIF production by PBMCs with Con A stimulation in patients with AD. Significant correlation was observed between serum MIF content and content in culture medium of PBMCs after stimulation with Con A.

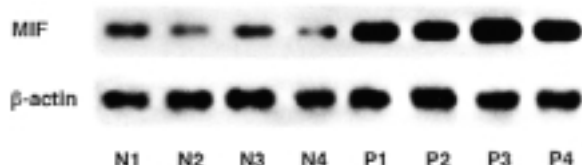


FIG 4. MIF mRNA expression of PBMCs, in patients with AD and in nonatopic healthy individuals. Total RNA was isolated from PBMCs in patients with AD ($n = 4$) and in healthy individuals ($n = 4$). RT-PCR-Southern blot analysis was performed as described in Material and methods. **A**, Comparison of expression of MIF mRNA in PBMCs from patients with AD and control subjects. **B**, Lanes N1 to N4, PBMCs from control subjects; lanes P1 to P4, PBMCs from patients with AD.

tions (Fig 2). The increased ratio was defined by dividing MIF contents in culture supernatants with Con A stimulation by those without the stimulation. The results revealed that the high and low responders showed high and low ADASI scores, respectively. These facts indicated that responsiveness of PBMCs to Con A may reflect the clinical features of atopic dermatitis (Table III). We next analyzed the correlation between serum MIF content and MIF production of PBMCs in patients with AD. The results showed a significant correlation between serum MIF content and MIF production by PBMCs with Con A stimulation ($r = 0.51$, $P < .01$) (Fig 3).

Expression of MIF in PBMCs from patients with AD

Cytokine production by PBMCs of atopic and nonatopic subjects has been extensively studied. Reverse transcription (RT)-PCR has often been used to examine mRNA expression in human materials, although it is semiquantitative in nature, because this method needs a smaller amount of material than other methods such as

TABLE III. Comparison of high and low responders to Con A with regard to MIF production by PBMCs and relationship with clinical scores

Group	Ratio*	No.	Mean ratio	ADASI
High	≥ 2	10	3.0 ± 0.43	$9.5 \pm 0.56^\dagger$
Low	< 2	13	1.3 ± 0.06	7.0 ± 0.63

*Ratio was determined through dividing MIF contents in culture supernatant of PBMCs with Con A stimulation by that without stimulation.
 $^\dagger P < .01$.

Northern blot analysis. For example, up-regulation of IL-13 mRNA expression of PBMCs from patients with AD was revealed by this method.²⁴ We combined RT-PCR with Southern blot analysis to obtain more accurate data and determined the MIF mRNA level in AD. MIF mRNA expression from freshly isolated PBMCs was measured in patients with AD ($n = 4$) and in healthy control subjects ($n = 4$). The results showed a higher level of MIF mRNA expression by PBMCs in patients with AD (about a 1.6-fold increase) than in healthy control subjects (Fig 4).

DISCUSSION

In this study we demonstrated for the first time that spontaneous MIF production by PBMCs in 78% of patients with AD was significantly elevated in comparison with that of nonatopic healthy control subjects and patients with chronic urticaria. In addition, Con A stimulated more MIF production by PBMCs of patients with AD than by those of healthy control subjects and patients with chronic urticaria. We found 2 groups, one more responsive to Con A than the other with regard to MIF production by PBMCs. It should be noted that the responsiveness was significantly correlated with the severity of the clinical symptoms. That is, the high- and

low-response groups had high or low ADASI scores, respectively. This fact indicated that responsiveness of PBMCs might be associated with the pathologic characteristics of atopic dermatitis. Supporting these data, we found that the MIF mRNA level of PBMCs of patients with AD was up-regulated, as demonstrated by RT-PCR–Southern blot analysis.

In our previous report we showed that MIF was highly expressed in keratinocytes in atopic inflammatory skin lesions.¹⁶ On the basis of this result, it was speculated that keratinocytes might be a potential source of serum MIF. However, the current results showed that PBMCs were also important cells inducing elevation of serum MIF in AD. Rossi et al¹⁴ reported that eosinophils are an important cellular source of MIF in asthmatic patients evaluated by examining MIF productivity in response to stimulation with phorbol myristate acetate. Eosinophils have also been implicated as key cells in the pathogenesis of AD.²⁵ Thus it is likely that the high serum MIF content in patients with AD might be the result of increased MIF productivity of multiple cells, including keratinocytes, PBMCs, and eosinophils.

The pathogenesis of AD is complex and multifactorial. There is emerging evidence showing dysregulation of the immune system, in which a disordered cytokine-mediated response is a major pathologic factor.^{2,7,26,27} In atopic skin lesions a large number of lymphocytes, monocyte-macrophages, and granulated mast cells are observed.²⁸ The lymphocytes within AD lesions are CD4⁺ cell dominant,⁵ and T-cell subsets are believed to contribute to the regulation of the immune system. Subsets of helper T cells tend to be self-amplifying and inhibitory to the reciprocal subset. In this context Th1/Th2 imbalance is considered to be critical for this pathogenesis.^{3-5,27} PBMCs of patients with AD show significant reductions of IFN- γ , TNF- α , and IL-2 originating from the Th1 subset.^{3,7} In terms of subsets of T-helper cells, MIF production was induced by mitogenic or antigenic stimuli, and MIF mRNA was dominantly expressed in the activated Th2 subset.¹² Taken together, these facts indicate that it is likely that MIF released from the Th2 subset may contribute to control the balance of cytokine production by Th1 and Th2 subsets in a physiologic state, whereas excessive MIF production may elicit a disorder of Th1/Th2 balance.

Another possible immunologic abnormality of AD, depressed DTH, has been suggested.^{3,7,29} Bernhagen et al¹⁰ reported that administration of neutralizing anti-MIF antibodies to mice inhibited the development of DTH. This finding supports the possibility that MIF plays an important role in immunologic dysfunction of this skin disease. The Th1 subset mediates DTH through recruitment of monocytes and activation of macrophages, whereas cytokines released from the Th2 subset play a reciprocal role to maintain the Th1/Th2 balance. For example, IL-10 produced by the Th2 subset depresses DTH. Indeed, marked expression of IL-10 has been identified in monocytes infiltrating atopic skin lesions.³⁰ Although the mechanism of MIF in DTH of AD remains

to be elucidated, it is conceivable that excessive production of MIF may contribute to the mechanism of DTH features observed in this disease through Th1/Th2 imbalance.

Alternatively, persistent colonization of AD skin with *Staphylococcus aureus* has been recently focused on with regard to the potential role of *S aureus*-derived superantigens.^{3,31} Bacterial superantigens have the potential to trigger chronic T cell-mediated skin inflammation. It should be noted that MIF plays an important role in the innate host response to staphylococcal and streptococcal exotoxins.³² Streptococcal pyrogenic exotoxin A is a very potent inducer of MIF production by immune cells. Thus it is likely that continuous up-regulation of MIF production may cause persistent inflammation of atopic skin lesions.

Finally, MIF functions as a proinflammatory cytokine, pituitary-derived hormone, and glucocorticoid-induced immunomodulator.²⁰ As for glucocorticoid-induced MIF production, we could not find MIF induction by steroid treatment in this experiment. However, this phenomenon might depend on the cell type or culture conditions. Further study is required on the effects of corticosteroids on MIF induction with PBMCs. From the data available to date, including the current results, it is conceivable that enhanced production of MIF by a variety of cells, including PBMCs, may affect local and systemic pathologic features in AD. Recently, we showed that MIF production in the skin is up-regulated by UVB exposure, suggesting its involvement in the cytokine network in the event of skin tissue damage.³³ In this context the current results further encourage us to investigate more precise functions of MIF in terms of cutaneous inflammatory and immune responses.

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