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Macrophage Migration Inhibitory Factor Is an Essential Immunoregulatory Cytokine in Atopic Dermatitis

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Macrophage migration inhibitory factor (MIF) is one of the immunoregulatory cytokines involved in T-cell activation and delayed-type hypersensitivity. To elucidate involvement of this cytokine in the pathogenesis of atopic dermatitis (AD), we examined serum MIF concentrations of patients with AD and non-atopic normal healthy individuals. The mean serum MIF concentration of the AD patients (n = 36) was 36.4 ± 3.7 ng/ml (mean ± SEM), whereas that of the non-atopic dermatitis patients (n = 17) or healthy individuals (n = 61) were 13.1 ± 1.8 or 6.5 ± 0.45 ng/ml, respectively. Accordingly, immunohistochemistry of the inflammatory skin lesion of an AD patient demonstrated that MIF protein was diffusely expressed throughout the whole epidermal layer. After 4-week steroid ointment treatment, the MIF concentration decreased as clinical symptoms improved. The serum level of TNF-α was also decreased in parallel with that of MIF. Considering the T-cell dysfunction and disordered cytokine-network reported in AD, it was strongly suggested that MIF was a critical protein for immunoregulation in the pathophysiological mechanism of AD. In this context, MIF may become a useful laboratory parameter to comprehend the clinical course of the disease.

Macrophage migration inhibitory factor (MIF) was the first lymphokine reported to prevent random migration of macrophages and to recruit them at inflammatory loci (1). MIF was long considered to be expressed exclusively in activated T cells; however, it has been revealed that a variety of cells have the potential to produce the immunoregulatory protein. Accordingly, various novel immunological and hormonal functions have been reported. MIF is the major secretory protein released by anterior pituitary cells, potentiating lethality of endotoxin shock (2), and the protein was found to be a glucocorticoid-induced immunomodulator for other proinflammatory cytokines (3). We previously reported the presence of MIF in human epidermal keratinocytes, indicating that MIF might regulate epidermal immunity, inflammation, and cellular differentiation.

Atopic dermatitis (AD) is a chronic pruritic inflammatory skin disorder, and numerous reports have documented its pathogenesis in relation to genetic and immunological abnormalities as well as environmental factors (4, 5). In particular, dysregulation of cytokine production of peripheral mononuclear cells has been considered to be one of major causes of AD (6–8). However, the precise mechanism of the initiation and persistence of skin inflammation remains to be elucidated. In recent years, a number of reports have focused on abnormal populations of Th1 and Th2 subsets of helper T cells (Th1/Th2 imbalance) as a cause for the pathogenesis (9-11). Accordingly, cytokine production by T-helper cells has been extensively studied. Moreover, depressed delayed-type hypersensitivity (DTH) in AD is considered to involve more than Th1/Th2 imbalance (12). Hence, Th1/Th2 imbalance and depressed DTH could be serious underlying immunoregulatory abnormalities. The physiological function of MIF in association with T cell function has not been well understood, but it is of importance that MIF is essential for T cell activation and possibly contributes to maintaining Th1/Th2 imbalance (13).

During the past few years, we have cloned rat MIF cDNA, revealed physicochemical properties of MIF protein, e. g., the tertiary structure, and demonstrated MIF expression in various tissues (14-17). On the skin, we identified the expression of MIF in human keratinocytes, in particular that of the basal layer of the epidermis (18). In the present study, we present for the first time serological and immunohistochemical evidence, suggesting that MIF might function as a critical immunoregulatory protein in the pathophysiology of AD. Ac-

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cordingly, we address its usefulness as a laboratory parameter to comprehend the clinical course of the disease.

MATERIALS AND METHODS

Materials. The following materials were obtained from commercial sources. Nylon membrane filters were purchased from Schleicher & Schuell (Keene, NH, USA); Isogen RNA extraction kit from Nippon Gene (Tokyo, Japan); M-MLV reverse transcriptase from GIBCO (Grand Island, NY, USA); Taq DNA polymerase from Perkin-Elmer (Norwalk, CO, USA); Cosmedium-001 from Cosmo Bio Co. (Tokyo, Japan); Histofine SAB-PO kit from Nichirei (Tokyo, Japan); Konica HRP-1000 immunostaining kit from Konica (Tokyo, Japan); horseradish peroxidase-conjugated goat anti-rabbit antibody and Micro BCA protein assay reagent kit from Pierce (Rockford, IL, USA); o-phenylenediamine from Wako (Osaka, Japan), and random primer fluorescein labeling kit and nucleic acid chemiluminescence reagent from DuPont NEN (Boston, MA, USA). All other chemicals used were of analytical grade. Polyclonal anti-human MIF serum was generated by immunizing New Zealand white rabbits with purified recombinant human MIF as previously described (16, 17).

Patients. Thirty-six patients (25 males and 11 females; mean age of 22.5 years ranging, from 16 to 33 years) with moderate and severe AD were subjects of this study. All the patients visited the Department of Dermatology of the Hokkaido University School of Medicine. AD was diagnosed according to the criteria of Hannifin & Rajka (19). Most of the patients suffered from an exacerbated form of AD involving the face, limbs and trunk. None of the patients was receiving systemic or topical steroids at the time when the study was begun. Skin scores of five patients were obtained before and after 4-week treatment by topical steroid ointment. Severity of the dermatitis was assessed using the Atopic Dermatitis Area and Severity Index (ADASI) score (20). This scoring system yields values ranging from 1 without any apparent symptoms to 18 with the most severe symptoms, e.g., 100% skin involvement of atopic eczema.

On the other hand, 17 patients (10 males and 7 females; mean age of 43.5 years ranging from 21 to 69 years) with chronic eczema were examined as the non-atopic disease control. The patients did not fulfill the criteria of Hannifin & Rajka, e.g. low levels of total IgE (<200 kU/l) and neither IgE antibodies nor immediate-type skin reactions to a routine panel of relevant aero- and food allergens.

Enzyme-linked immunosorbent assay (ELISA). The anti-human MIF antibody dissolved in 50 μl of PBS was added to each well of a 96-well microtiter plate. After incubation for 1 hr at room temperature, the plate was washed three times with PBS. All wells were filled with PBS containing bovine serum albumin (1%) for blocking and left for 1 hr at room temperature. After removal of the blocking solution, the plate was washed three times with PBS containing 0.05% Tween 20 (washing buffer), and samples were added to individual wells and incubated for 1 hr at room temperature. After the plate was washed three times with the washing buffer, 50 μl of biotin-conjugated anti-human MIF antibody was added. Following incubation for 1 hr at room temperature, the plate was again washed three times with the washing buffer. Then avidin-conjugated horseradish peroxidase was added to individual wells, which were further incubated for 1 hr at room temperature. The plate was again washed three times with the washing buffer. Next, 50-μl of substrate solution containing 200 μg of o-phenylenediamine and 10 μl of 30% hydrogen peroxide in 10 ml of citrate-phosphate buffer (pH 5.0) were added. After incubation for 20 min at room temperature, the reaction was terminated with 50 μl of 1 N sulfuric acid. The absorbance at 492 nm was measured with an ELISA plate reader (Bio-Rad, Model 3550).

Immunohistochemistry. Immunohistochemistry for a skin of an AD patient was performed using a Histofine SAB-PO kit according to the manufacturer's protocol. The skin sections were mounted on gelatinized glass slides and immersed in methanol containing 0.3% H_2O_2 for 30 min to block endogenous peroxide activity. Following three washes in cold sucrose-PBS (PBS containing 10% sucrose), nonspecific staining was blocked by incubation with 10% normal goat serum in PBS for 10 min. The sample was incubated with the anti-human MIF antibody (1:50 dilution in PBS) for 30 min at room temperature. Following three washes in cold sucrose-PBS, the specimens were incubated with a biotinylated goat anti-rabbit IgG and avidin-biotin-peroxidase complex at room temperature for 30 min. After an additional three washes, the reaction was developed in 3,3′-diaminobenzidine tetrahydrochloride containing 0.01% H_2O_2.

RT-PCR/Southern blot analysis. The total RNAs were extracted from epidermis of a non-atopic individual and an AD patient with an Isogen RNA extraction kit. The reverse transcription of RNAs was carried out with M-MLV reverse transcriptase using oligo-dT primer. Subsequent PCR amplification was performed essentially as described using a thermal cycler (Perkin-Elmer, Model 2400). Human MIF primers used were 5′-CTCTCCGAGCTCACCCAGCAG-3′ (58-78) (forward) and 5′-CGCGTTCATGTCGTAATAGTT-3′ (292-312) (reverse). Human β-actin primers used were 5′-CGTTCTGGCGGCACCACCAT-3′ (936-955) (forward) and 5′GCAACTAAGTCATAGTCCGC-3′ (1170-1189) (reverse). For quantitative analysis of the MIF mRNA expression level, we preliminarily examined the kinetics of MIF mRNA amplification. The amplification of MIF mRNA remained in the exponential phase at least up to 20 cycles, but the amplification product reached a plateau at 25 cycles. Since the amplification products at 20 cycles could not be visualized on an electrophoresed agarose gel stained with ethidium bromide, we carried out Southern blot hybridization to detect and quantify the amplified products. In brief, the PCR products were electrophoresed with a denatured 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 hr. The DNA probe was prepared using the cloned human MIF insert labeled using a random primer fluorescein labeling kit according to the manufacturer's protocol. The hybridization was carried out in a solution containing 5 M NaCl, 2 M Tris-HCl (pH 8.0), 0.5 M EDTA, 10× Denhardt's solution, 1 mg/ml salmon sperm DNA and the fluorescein-labeled DNA probe. The hybridization was carried out at 65°C overnight. The membrane was washed in 0.2× standard saline citrate (SSC), 0.1% SDS at 65°C for 30 min. The bands were visualized with a nucleic acid chemiluminescence reagent. The quantitative analysis was carried out using an MCID image analyzer (Fuji Film, Tokyo, Japan).

Western blot analysis. Western blot analysis was carried out by the method of Towbin et al (21). In brief, epidermis of non-atopic and individual and an AD patient were suspended in 20 μl of Tris-HCl (50 mM, pH 6.8) containing 2-mercaptoethanol (1%), sodium dodecyl sulfate (SDS) (2%), glycerol (20%) and bromophenol blue (BPB) (0.04%), heated at 100°C for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described (22). The electrophoresed proteins were transferred onto a nitrocellulose membrane at 50 mA for 1 hr using a semi-dry blot transfer apparatus (Bio-Rad). Then the membrane was intensively washed with phosphate-buffered saline (PBS), treated with 1% skim milk, and incubated with an anti-human MIF antibody (1:1000 in dilution) for 1 hr at room temperature. Furthermore, the membrane was reacted with peroxidase-conjugated anti-rabbit IgG (1:1000 in dilution) for 1 hr at room temperature. After the reaction, proteins were visualized with Konica immunostaining HRP-1000 as recommended in the manufacturer's protocol. Protein concentration was determined with a Micro BCA protein assay reagent kit.

Statistics. Intergroup comparisons were made using Student's t-test to compare the mean values. Calculations were done using the statistical software package Statview from Abacus Concepts (Berkeley, CA, USA).

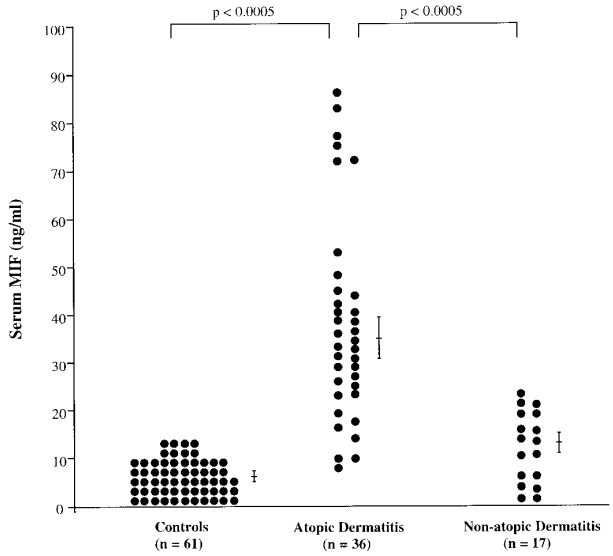

FIG. 1. Serum MIF concentrations of atopic patients in comparison to non-atopic dermatitis patients with chronic eczema and healthy individuals.

RESULTS

Serum MIF content of AD patients. Most of the patients suffered from an exacerbated form of AD involving the face, limbs and trunk. None of the patients was receiving systemic or topical steroids at the time when the study was initiated. First, the serum level of MIF was measured by ELISA as described to elucidate the involvement of MIF in AD (16). As shown in Fig. 1, the serum MIF concentrations were 6.5 ± 0.45 ng/ml and 13.1 ± 1.8 ng/ml (mean ± SEM) in healthy individuals and non-atopic dermatitis patients with chronic eczema, respectively. In contrast, the serum level of the AD patients was 36.4 ± 3.7 ng/ml, significantly higher than that of the non-atopic dermatitis patients or healthy individuals (p0.0005). The elevated serum MIF levels of AD patients decreased as the clinical features improved (Fig. 2). The results showed that the serum MIF concentration correlated with the severity of the clinical symptoms. It has been reported that increased serum TNF-α correlates with the severity of atopic dermatitis (23). As shown in this study, the TNF-α concentration decreased with improvement of dermatitis. On the other hand, no significant correlation of the serum IgE concentration to the clinical features was found as reported (24, 25).

Immunohistochemical analysis of MIF in epidermis. We previously identified MIF protein and expression of MIF mRNA in human epidermis (18). Immunohistochemical analysis showed that MIF protein is present, but mostly limited to the basal layer (Fig. 3a). In contrast, MIF protein was diffusely distributed throughout the whole epidermal layer in a skin lesion of an AD patient (Fig. 3b). On the other hand, MIF protein was largely identified in a few layers near the basal cell layer for a chronic eczema case used as a disease control (Fig. 3c). After a 4-week treatment with a steroid ointment, the histological features appeared to improve as the overall MIF protein expression decreased and the dominant expression was limited to the basal cell layer (data not shown).

MIF mRNA expression of epidermis. To further confirm the high expression of MIF in the epidermis of AD, we performed RT-PCR/Southern blot and Western blot analyses on atopic and non-atopic skin biopsies. The results showed higher (4-fold) expression of MIF mRNA in the AD lesional epidermis than in the normal epidermis (Fig. 4). On Western blot analysis, we found a specific band corresponding to the MIF protein at about 12.5 kDa for AD and the normal samples (Fig. 5). Consistent with the results of RT-PCR/Southern blot analysis, MIF protein was present in a larger (2-fold) amount in the lesional epidermis of AD compared to the normal individual. It was apparent from these results that MIF mRNA and MIF protein were highly expressed in the epidermis of AD patients.

DISCUSSION

In this study, we demonstrated that the serum MIF concentration was significantly elevated in most patients who suffered from AD. Moreover, serum MIF concentrations decreased when clinical features of AD were improved. In accordance with the serological data, over-expression of MIF in keratinocytes of an inflammatory skin lesion was identified by immunohisto-

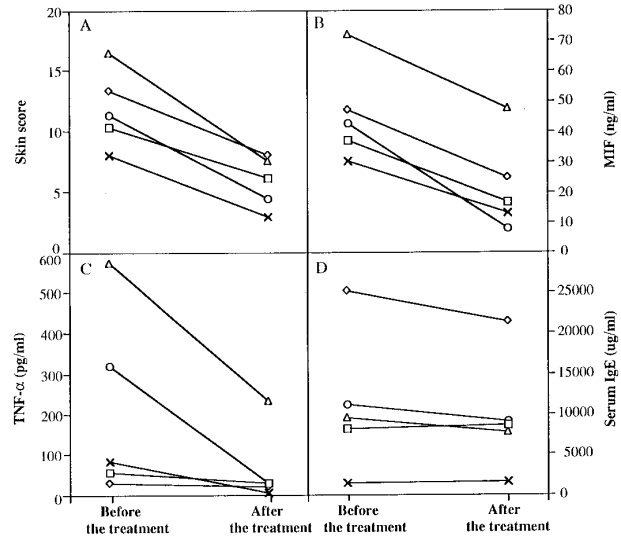

FIG. 2. Changes of skin scores (A), serum concentrations of MIF (B), TNF-α (C), and serum IgE levels (D) before and after 4-week treatment with steroid ointment in patients with atopic dermatitis.

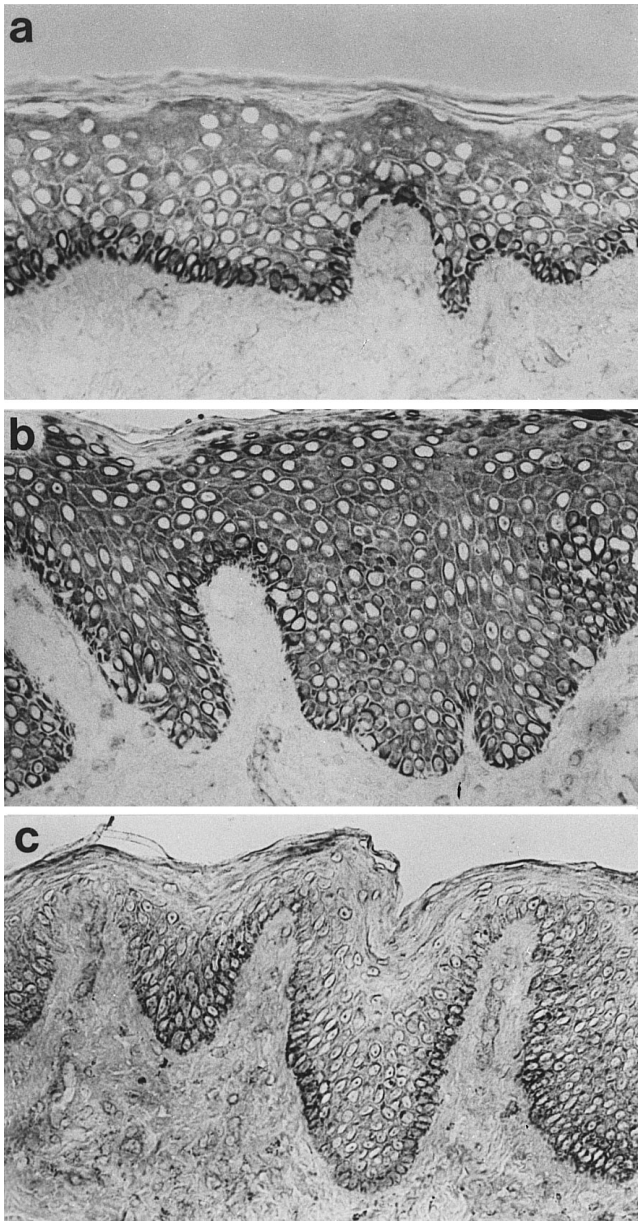

FIG. 3. Immunohistochemistry of MIF of normal and atopic skin. a, normal skin (×80); b, inflammatory skin lesion of AD (×80); c, a skin of non-atopic dermatitis with chronic eczema as a disease control (×80).

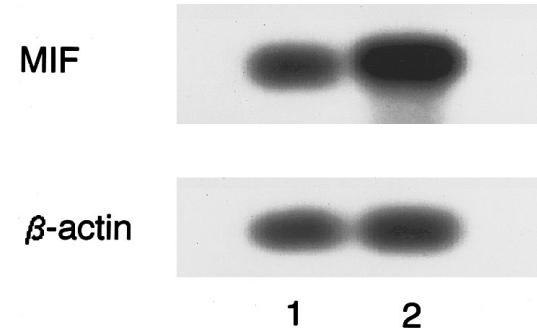

FIG. 4. RT-PCR/Southern blot analysis of MIF mRNA expression of atopic skin epidermis. RT-PCR was carried out as described in Materials and Methods. The PCR products were electrophoresed on 2% agarose gel. Lane 1, normal skin epidermis; lane 2, epidermis of AD skin lesion. The results shown are the typical of experiments. The product of β-actin is shown at the bottom of each lane.

chemical analysis. The source of MIF in the sera of AD patients was not precisely demonstrated; however, keratinocytes of the eczematous skin lesion were considered to the major source, and might induce local inflammatory and immunological reactions. However, it is still possible that activated T cells or monocytes are other sources, because AD is not limited to skin lesions, but is a systemic disease.

There is emerging evidence on the pathogenesis of AD showing deficiencies of the immune system, e. g., T-cell dysfunction and a disordered cytokine-mediated response. However, the pathogenesis of AD appears to be complex and multifactorial. Recent reports have suggested that alteration of cytokine production may play a part in the pathogenesis of AD. For example, for INF-γ, a major pathogenic factor in AD, the mRNA showed a biphasic pattern of 48-hr duration after allergen stimulation, indicating that a switch from a Th2-like to a Th1-like response occurs. Subsets of helper T cells, Th1 and Th2, tend to be self-amplifying and inhibitory to the reciprocal subset. This fact suggests that there exist immunomodulators in the pathogenesis of AD. Interestingly, it was found that INF-γ production is markedly suppressed by an anti-MIF anti-

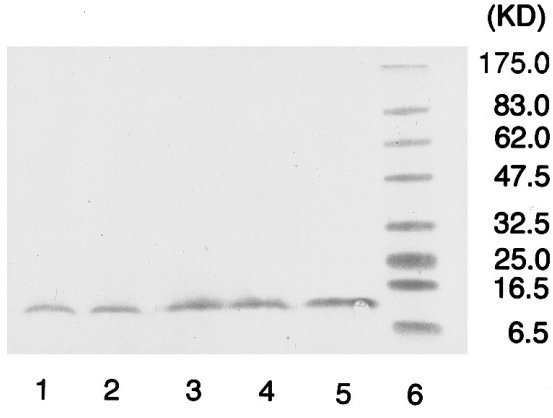

FIG. 5. Western blot analysis of atopic skin epidermis for MIF. The cells were collected, electrophoresed, transferred to a nitrocellulose membrane, and visualized using a Konica immunostaining kit as described in Materials and Methods. The results shown are the typical for 4 samples of normal and AD skin lesion. Lanes 1 and 2, normal human skin epidermis (50 μg protein); lanes 3 and 4, epidermis of AD skin lesion (50 μg protein); lane 5, recombinant human MIF (100 ng); lane 6, prestained molecular marker (New England Biolabs).

body (26). This fact indicates that IFN-γ production is regulated by MIF production. The Th1 subset produces IFN-γ, IL-2 and TNF-α, whereas the Th2 subset produces IL-4, IL-5 and IL-10. It is known that skin lesions of AD predominantly contain the Th2 subset (27, 28), which may facilitate systemic Th1/Th2 imbalance. Th1/Th2 imbalance is also considered to be critical for this pathogenesis. Recently, MIF was found to play an important role in T-cell activation induced by mitogenic and antigenic stimuli, and expression of MIF mRNA was limited to the Th2 subset (13). This finding indicates the possibility that MIF released from the Th2 subset causes the Th1/Th2 imbalance. Thus, it is assumed that MIF is directly or indirectly involved in the pathogenesis of AD.

As for another possible pathogenic mechanism of AD, depressed DTH has been presented. In association with DTH, MIF has been assessed play a critical role (29). In brief, the Th1 subset mediates DTH through recruitment of monocytes and activation of macrophages, in which the role of IL-10 is considered to be critical. IL-10 produced by the Th2 subset is known to depress DTH, and marked expression of the cytokine has been immunohistochemically identified in monocytes infiltrating atopic skin lesions (30). Although there is no evidence for the regulation of IL-10 production by MIF, it is possible that the elevated serum MIF level in keratinocytes of AD may be an immune response for regulation of excessive depression of DTH induced by IL-10. In support of this possible regulatory role of MIF, it was found that MIF has the potential to function as a "counter-regulatory protein" to suppress the excessive anti-inflammatory action of glucocorticoid in inflammatory and immunological responses, thus initiating inflammation (3).

Finally, MIF plays a pivotal role in the inflammatory response in endotoxemia and in the DTH response, but its potential as a regulator of immune-mediated diseases is still poorly understood. Very recently, it was demonstrated that MIF is involved in the inflammatory reaction of immunologically induced glomerulonephritis (31). This fact suggests that MIF is also important in AD, an immune-mediated skin disease. Moreover, it was reported that MIF might function as an immunomodulator, antagonizing the anti-inflammatory action of glucocorticoid in adult respiratory distress syndrome (32), which indicates that MIF may function as a hormone in systemic inflammatory diseases. In conclusion, we here hypothesize that the elevated serum level of MIF in AD patients may enhance atopic eczema via dysregulation of the immune system, e. g., Th1/Th2 imbalance and DTH. Furthermore, we believe that MIF could become a reliable marker to help comprehend the clinical features of AD, and the current serological and histological results could become a major departure point for further elucidation of the pathogenesis of AD.

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