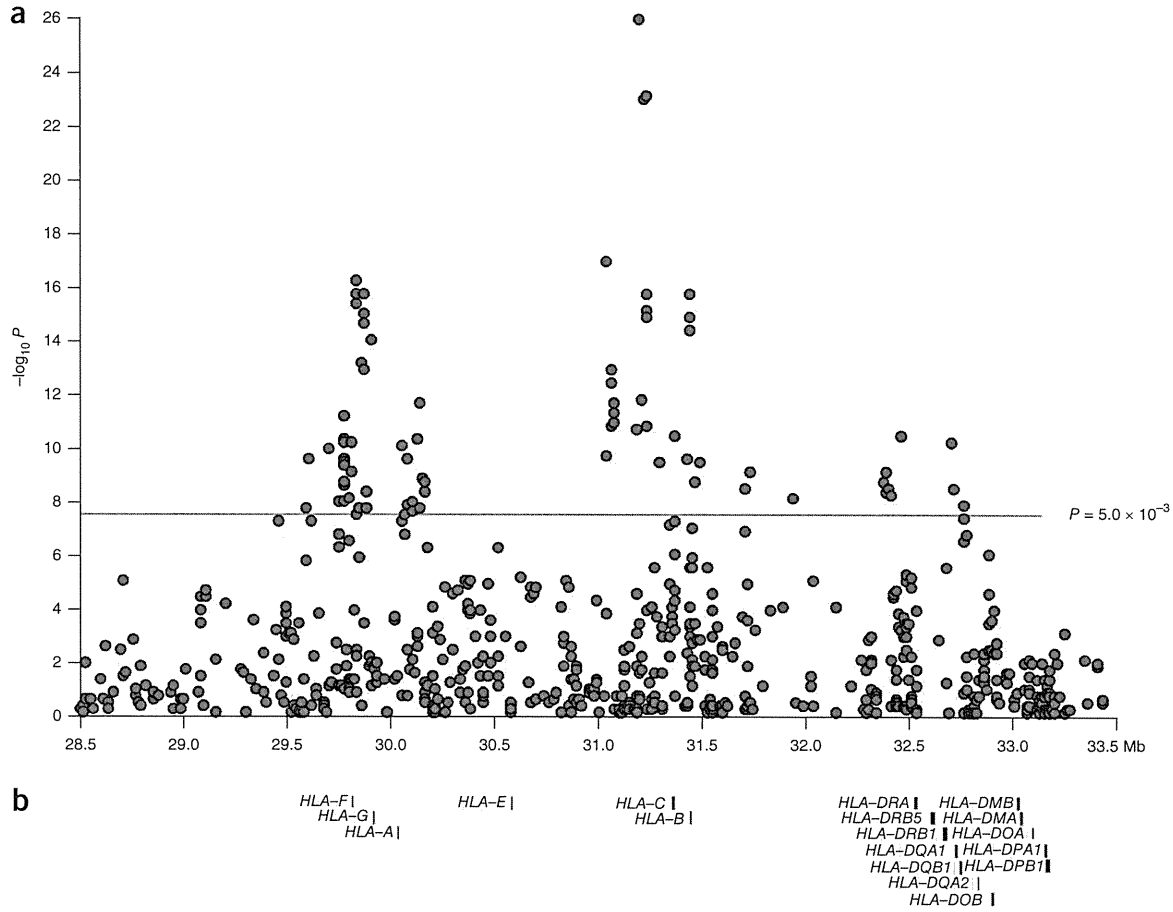

Figure 1 Overview of SNPs across the *HLA* region in the GWAS. (**a**) Association of SNPs located at 28.5–33.5 Mb on chromosome 6. Genotyped SNPs in the GWAS are indicated with a blue circle. (**b**) Location of *HLA* genes from the NCBI RNA reference sequences collection at 28.5–33.5 Mb on chromosome 6.

Outside the *HLA* complex, a total of 54 SNPs at 35 distinct loci showed association with Behçet's disease at $P < 0.0001$ in allele-based tests (**Supplementary Table 3**). Among these SNPs, three of the top four were located on chromosome 1p31.33 and the third was located on chromosome 1q32.1. Genome-wide significant association (using the threshold of $P < 5.0 \times 10^{-8}$) was detected at $P = 2.7 \times 10^{-8}$ for rs12119179, which is located in the 47-kb intergenic region between *IL23R* (encoding interleukin 23 receptor) and *IL12RB2* (encoding interleukin 12 receptor, beta 2). The second strongest region of association was rs1554286, located within the intron 3 of *IL10* (encoding interleukin 10) and also on chromosome 1q32.1 ($P = 8.0 \times 10^{-8}$).

To further validate our GWAS results, we exchanged data with colleagues performing a similar GWAS in a Turkish population[5]. This independent analysis also showed a strong dichotomized association of Behçet's disease with *HLA-B* and *HLA-A* loci in the *HLA* region. In addition, this Turkish GWAS identified the associations at the same two non-*HLA* regions, *IL10* and *IL23R-IL12RB2*, with $P < 0.0001$. This provides cross-validation of these two associations in an independent cohort from a distinct population.

We then fine mapped the association at the *IL23R-IL12RB2* and *IL10* loci using additional SNPs. For the *IL23R-IL12RB2* loci, we genotyped an additional set of 26 SNPs in the 230-kb region covering these genes. Of these SNPs, 23 were genotyped successfully, and 3 were monomorphic. We observed significant associations for several SNPs. The most significant was observed with rs1495965, located in the intergenic region between *IL23R* and *IL12RB2* ($P = 1.2 \times 10^{-8}$) (**Fig. 2a**, **Table 1** and **Supplementary Table 4**). SNPs with strong association in fine mapping or at the GWAS stage (rs11209032, rs11209033, rs12141431, rs12119179 and rs1495965) were in strong LD with each other, and this LD block was located in the intergenic region between *IL23R* and *IL12RB2*. In addition, outside this block, we found a weak association for several SNPs in *IL23R* and for one SNP in *IL12RB2*. To fine-map the *IL10* locus, we genotyped an additional set of 11 SNPs in the 8-kb region covering the gene. All SNPs were genotyped successfully, and we observed strong associations for four SNPs: rs1518111, rs3024490, rs1800872 and rs1800871. The most significant association ($P = 9.5 \times 10^{-9}$) was observed at rs1800872 and rs1800871, located in the promoter region of *IL10* (**Fig. 2b**, **Table 1** and **Supplementary Table 5**). These four SNPs and rs1554286 (found in the initial GWAS) were in strong LD with each other.

Finally, we evaluated the three SNPs showing the most significant *P* values (rs1495965 in *IL23R-IL12RB2* and rs1800872 and rs1800871 in *IL10*) for replication in 2,494 Turkish individuals (obtained from colleagues performing a parallel GWAS in a Turkish population and reported in a coordinated publication in this issue[5]) and 259 Korean individuals (**Table 1**). rs1495965 in *IL23R-IL12RB2* did show evidence of replication in the Turkish cohort ($P = 8.4 \times 10^{-5}$), but this SNP was not significantly associated with Behçet's disease in the Korean cohort ($P = 0.094$). The G allele of rs1495965 showed an increased risk of

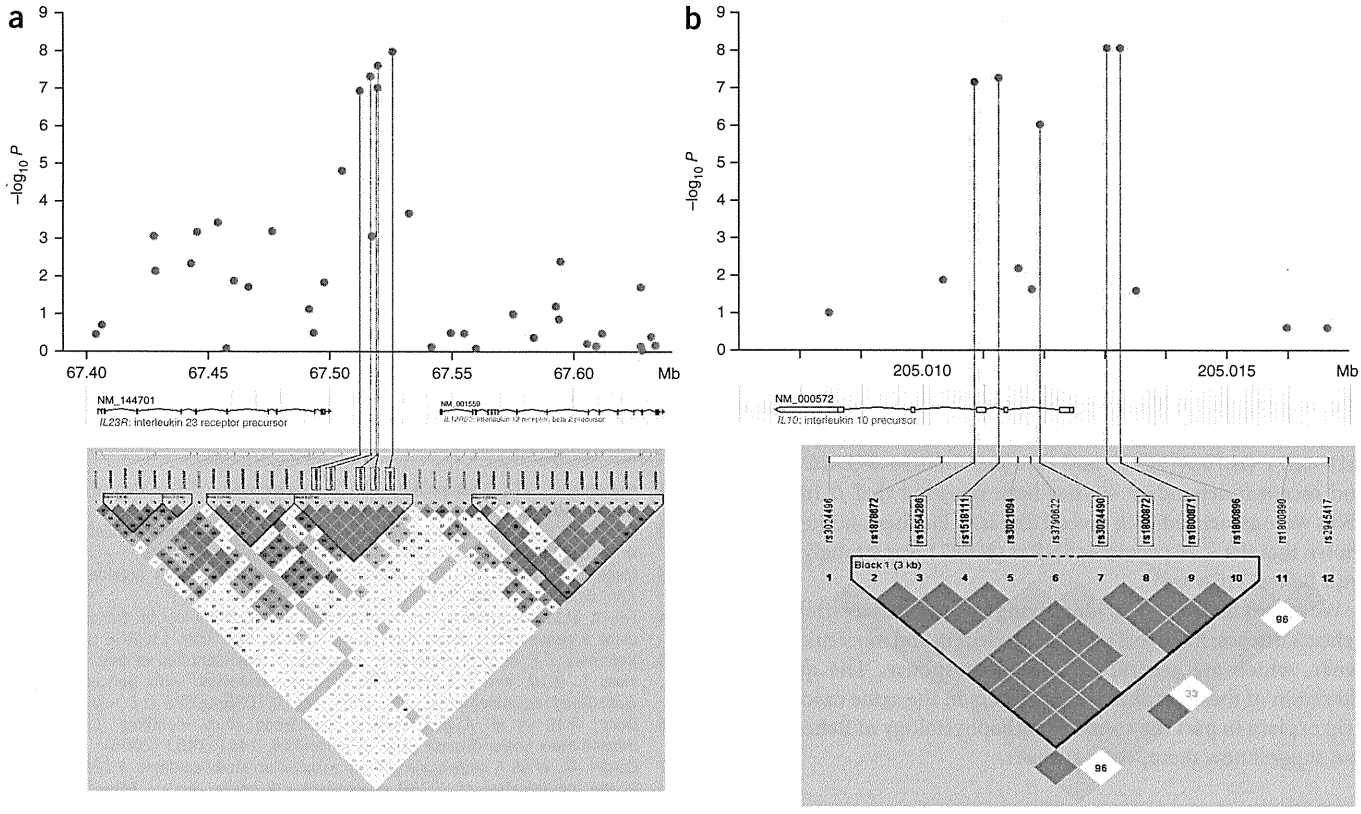

Figure 2 In-depth SNP analysis in target areas. (**a**) Association analysis of SNPs across *IL23R* and *IL12RB2*. (**b**) Association analysis of SNPs across *IL10*. The upper panels show distribution of association results of SNPs in the target gene regions. Genotyped SNPs in the GWAS are indicated with a blue circle, and genotyped SNPs found through fine mapping are indicated with a red circle. The lower panels show LD structure in the target gene regions; higher *D′* is indicated by a brighter red. The yellow and gray segments within exon boxes indicate the coding and untranslated regions, respectively.

Behçet's disease in the three populations ($P = 1.9 \times 10^{-11}$, odds ratio (OR) = 1.35, 95% CI 1.24–1.47). rs1800872 and rs1800871 in *IL10* replicated in both the Turkish and Korean cohorts, and the results from the meta-analysis showed the most significant association (rs1800872, $P = 2.1 \times 10^{-14}$, OR = 1.45, 95% CI 1.32–1.59; rs1800871, $P = 1.0 \times 10^{-14}$, OR = 1.45, 95% CI 1.32–1.60).

Recent genetic surveys including GWAS have identified *IL23R* or *IL12RB2* as susceptibility loci for a number of inflammatory and immune-linked diseases[6–11]. For example, the non-synonymous SNP rs11209026, located within exon 9 of *IL23R*, is primarily associated with inflammatory bowel disease, psoriasis, psoriatic arthritis and ankylosing spondylitis. Although rs11209026 was monomorphic in the Japanese populations, two SNPs in the Behçet's disease–associated LD block, rs11209032 and rs1495965, and other two Behçet's disease–associated SNPs with $P < 0.01$, rs1004819 and rs7517847, have been reported to be associated with inflammatory bowel disease. *IL23R*

Table 1 Allelic association results for SNPs rs1495965, rs1800872 and rs1800871

SNP	Chr. position[a]	Nearest gene	Risk allele	Population	*N*		Risk allele frequency		P^b	OR for risk allele (95% CI)
					Cases	Controls	Cases	Controls		
rs1495965	67,526,096	*IL23R-IL12RB2*	G	Japanese	611	737	0.563	0.451	1.2×10^{-8}	1.56 (1.34–1.83)
				Turkish	1,215	1,279	0.568	0.512	8.4×10^{-5}	1.25 (1.12–1.40)
				Korean	119	140	0.560	0.486	0.094	1.35 (0.95–1.91)
				Overall					1.9×10^{-11}	1.35 (1.24–1.47)
rs1800872	205,013,030	*IL10*	A	Japanese	611	737	0.765	0.664	9.5×10^{-9}	1.64 (1.39–1.95)
				Turkish	1,215	1,279	0.384	0.315	6.1×10^{-7}	1.35 (1.20–1.52)
				Korean	119	140	0.776	0.694	0.038	1.52 (1.02–2.28)
				Overall					2.1×10^{-14}	1.45 (1.32–1.59)
rs1800871	205,013,257	*IL10*	T	Japanese	611	737	0.765	0.664	9.5×10^{-9}	1.64 (1.39–1.95)
				Turkish	1,215	1,279	0.383	0.313	3.1×10^{-7}	1.36 (1.21–1.54)
				Korean	119	140	0.774	0.694	0.044	1.51 (1.01–2.25)
				Overall					1.0×10^{-14}	1.45 (1.32–1.60)

Chr., chromosome.
[a]NCBI build 36; all SNPs were located on chromosome 1. [b]*P* values for each population were calculated using an allele-based test; overall *P* values and ORs in three populations were calculated using the Mantel-Haenzel method.

encodes a subunit of the IL23 receptor. IL23 is a heterodimeric pro-inflammatory cytokine composed of a p19 subunit and a p40 subunit that is shared with IL12. IL23 has been shown to stimulate T helper 17 cell proliferation and increase the production of inflammatory cytokines such as IL1, IL6, IL17 and TNFα (ref. 12). *IL12RB2* encodes an IL12 receptor chain. IL12 plays an important role in T-helper–1 responses, T-cell and NK-cell cytotoxicity, and interferon-γ (IFNγ) production by T cells and NK cells. IL12RB2 has been reported to be essential for high-affinity IL12 binding and IL12 dependent signaling, to be upregulated by interferon-γ (IFNγ) in Th1 cells and to have a crucial role in Th1 cell differentiation[13].

IL10 is a potent suppressor of inflammatory cytokines such as IL1, IL6, IL12, TNFα and IFNγ and inhibits the costimulatory activity of macrophages for T-cell and NK-cell activation. A previous study described three *IL10* promoter SNPs, rs1800896 (IL10-1082A/G), rs1800871 (IL10-819C/T) and rs1800872 (IL10-592A/C) (ref. 14), and recent independent studies have reported that *IL10* promoter SNPs were associated with several diseases. Further, another study has shown that the *IL10* promoter SNP rs1800871 was strongly associated with Behçet's disease[15].

We report here a GWAS identifying two new susceptibility loci for Behçet's disease; these loci include interleukin and interleukin receptor genes, which are central in immune response. The quantitative alteration of these cytokines (and others in the same cascade) could help explain in part the complex pathophysiology of Behçet's disease and suggest new therapeutic avenues.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

N.M. designed and supervised the experiment, provided study samples, performed data analysis and wrote the manuscript. A.M. designed the experiment, performed the SNP selection, supervised genotyping, performed data analysis, wrote the manuscript and prepared the tables and figures. M.O. participated in the experimental design, helped with data analysis and edited the manuscript. S.O. participated in the experimental design, provided study samples and edited the manuscript. T.S., T.K., N.I. and K.Y. performed genotyping. J.K. helped with data analysis. E.O., Y.W.S., E.B.L., N.K., K.N., Y.H., M.T., S.S., M.M. and Y.I. provided study samples. S.B. participated in the experimental design, helped with data analysis and participated in critical revisions of the manuscript. H.I. participated in the experimental design, provided study samples, helped with data analysis and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Kaklamani, V.G., Vaiopoulos, G. & Kaklamanis, P.G. Behcet's disease. *Semin. Arthritis Rheum.* **27**, 197–217 (1998).
2. Ohno, S. *et al.* Close association of HLA-Bw51 with Behcet's disease. *Arch. Ophthalmol.* **100**, 1455–1458 (1982).
3. Meguro, A. *et al.* Genetics of Behcet's disease inside and outside the MHC. *Ann. Rheum. Dis.* **69**, 747–754 (2010).
4. Fei, Y. *et al.* Identification of novel genetic susceptibility loci for Behcet's disease using a genome-wide association study. *Arthritis Res. Ther.* **11**, R66 (2009).
5. Remmers, E.F. *et al.* Genome-wide association study identifies variants in the MHC class I, *IL10*, and *IL23R-IL12RB2* regions associated with Behçet's disease. *Nat. Genet.* advance online publication, doi:10.1038/ng.625 (11 July 2010).
6. Duerr, R.H. *et al.* A genome-wide association study identifies *IL23R* as an inflammatory bowel disease gene. *Science* **314**, 1461–1463 (2006).
7. Cargill, M. *et al.* A large-scale genetic association study confirms *IL12B* and leads to the identification of *IL23R* as psoriasis-risk genes. *Am. J. Hum. Genet.* **80**, 273–290 (2007).
8. Liu, Y. *et al.* A genome-wide association study of psoriasis and psoriatic arthritis identifies new disease loci. *PLoS Genet.* **4**, e1000041 (2008).
9. Hirschfield, G.M. *et al.* Primary biliary cirrhosis associated with *HLA*, *IL12A*, and *IL12RB2* variants. *N. Engl. J. Med.* **360**, 2544–2555 (2009).
10. Rueda, B. *et al.* The *IL23R* Arg381Gln non-synonymous polymorphism confers susceptibility to ankylosing spondylitis. *Ann. Rheum. Dis.* **67**, 1451–1454 (2008).
11. Isomura, M. *et al.* *IL12RB2* and *ABCA1* genes are associated with susceptibility to radiation dermatitis. *Clin. Cancer Res.* **14**, 6683–6689 (2008).
12. Iwakura, Y. & Ishigame, H. The IL-23/IL-17 axis in inflammation. *J. Clin. Invest.* **116**, 1218–1222 (2006).
13. Chang, J.T., Shevach, E.M. & Segal, B.M. Regulation of interleukin (IL)-12 receptor beta2 subunit expression by endogenous IL-12: a critical step in the differentiation of pathogenic autoreactive T cells. *J. Exp. Med.* **189**, 969–978 (1999).
14. Turner, D.M. *et al.* An investigation of polymorphism in the interleukin-10 gene promoter. *Eur. J. Immunogenet.* **24**, 1–8 (1997).
15. Wallace, G.R. *et al.* IL-10 genotype analysis in patients with Behçet's disease. *Hum. Immunol.* **68**, 122–127 (2007).

ONLINE METHODS

Subjects. A total of 612 unrelated individuals with Behçet's disease and 740 unrelated healthy controls, all of Japanese descent, were enrolled in this study (**Supplementary Table 1**). The diagnosis of Behçet's disease was established according to standard criteria[16] proposed by the Japan Behçet's Disease Research Committee at the Yokohama City University, Hokkaido University, Tokyo Medical and Dental University, Kurume University, Yuasa Eye Clinic and Fujioka Eye Hospital. All control participants recruited from the Yokohama City University, Tokai University and Okada Eye Clinic were healthy volunteers unrelated to each other or to cases. Korean participants (119 cases and 140 healthy controls) were recruited at the Department of Internal Medicine, Seoul National University Hospital, South Korea. All cases were diagnosed according to criteria from the International Study Group for Behçet's disease (ISGBD)[17]. All control subjects were healthy Korean volunteers unrelated to each other or to cases. The study methodology complied with the guidelines of the Declaration of Helsinki. The study details were explained to all cases and controls before obtaining their consent for genetic screening. The QIAamp DNA Blood Maxi Kit (QIAGEN) was used to collect peripheral blood lymphocytes and extract genomic DNA from peripheral blood cells. Procedures were carried out under standardized conditions to prevent variation in DNA quality. Following extraction, DNA degradation and RNA contamination were assessed by 0.8% agarose gel electrophoresis.

Genotyping. *Genome-wide association study.* Genotyping with the GeneChip Human Mapping 500K Array Set (Affymetrix) was performed using the standard protocol recommended by Affymetrix. We set the standard for the minimum SNP call rate at 93% for each sample. The SNP call rates for all samples genotyped (612 cases and 740 controls) were ≥93%. To identify genotyping errors and DNA contaminations, we estimated the mean of genome-wide heterozygosity for all samples, and four samples (one case and three controls) that were more than 4 s.d. from the sample mean were excluded. Cryptic relatedness between samples was estimated by the identity-by-descent method using the PLINK software[18], and no samples were excluded by this analysis. This resulted in a dataset with 611 cases and 737 controls.

Quality control was performed on 500,568 SNPs from the GeneChip Human Mapping 500K Array Set, and we excluded 28,702 SNPs with a call rate < 95%, 14,044 SNPs with deviation from Hardy-Weinberg equilibrium in controls ($P < 0.001$) and 137,384 SNPs with a MAF < 5% overall. Therefore, 320,438 SNPs were left for subsequent analyses.

To assess possible population stratification in the GWAS samples, we carried out principal component analysis using EIGENSTRAT[19] in HelixTree, which showed no evidence of population admixture among the samples. To confirm the results of the principal component analysis, we estimated the genomic inflation factor λ based on the median of the χ^2 distribution and obtained an estimated value of $\lambda = 1.05$.

To exclude false-positive results, all SNPs showing significant evidence of association with Behçet's disease (defined as $P < 0.0001$) in the GWAS were assessed by clustering analysis; an association signal ($P < 0.01$) was found for a SNP in strong LD with a lead SNP (which had $P < 0.0001$). This lead SNP was considered to be a true-associated SNP. As a result, of the 83 SNPs with $P < 0.0001$ in non-*HLA* regions, 29 that clustered poorly were excluded from further analysis.

Fine mapping. We selected tagging SNPs covering the regions containing the genes encoding IL23R, IL12RB2 and IL10 from HapMap Japanese data (MAF ≥ 5%, pairwise $r^2 \geq 0.8$ and Hardy-Weinberg equilibrium $P \geq 0.05$) using the Haploview 4.1 software[20]. In addition, known non-synonymous SNPs and/or other disease-associated SNPs in the region containing the genes encoding IL23R, IL12RB2 and IL10 were also selected. Moreover, to cover the entire IL10 region more densely, two SNPs with MAF ~ 1% were selected in the region.

Genotyping of all SNPs was performed by TaqMan 5′ exonuclease assay using primers supplied by Applied Biosystems. The probe fluorescence signal was detected using the TaqMan Assay for Real-Time PCR (7900HT Sequence Detection System, Applied Biosystems) following the manufacturer's instructions.

Replication studies. We tested rs1495965, rs1800872 and rs1800871 using the same TaqMan assays for replication of 259 Korean individuals. We obtained the genotyping results in 2,494 Turkish individuals from colleagues performing a GWAS of Behçet's disease in Turkish individuals[5].

Statistical analyses. All association analyses for our GWAS were carried out using the software HelixTree SVS 7 (Golden Helix, Inc.). The association analyses for the fine-mapping stage were carried out using Haploview 4.1. Haploview 4.1 was also used to infer the LD structure of the targeted regions. The meta-analyses from the various populations were performed using the Mantel-Haenszel method.

16. Mizushima, Y. Recent research into Behçet's disease in Japan. *Int. J. Tissue React.* **10**, 59–65 (1998).
17. Criteria for diagnosis of Behçet's disease. International Study Group for Behçet's Disease. *Lancet* **335**, 1078–1080 (1990).
18. Purcell, S. *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81**, 559–575 (2007).
19. Price, A.L. *et al.* Principal components analysis corrects for stratification in genome-wide association studies. *Nat. Genet.* **38**, 904–909 (2006).
20. Barrett, J.C., Fry, B., Maller, J. & Daly, M.J. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21**, 263–265 (2005).

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LABORATORY INVESTIGATION

Elevation of Surfactant Protein D, a Pulmonary Disease Biomarker, in the Sera of Uveitis Patients with Sarcoidosis

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Abstract

Purpose: Surfactant protein D (SP-D) is found in the epithelial cells of multiple mucosal surfaces. It is commonly used to diagnose and screen for pulmonary diseases. In the present study, serum levels of SP-D were measured in patients with uveitis to ascertain whether SP-D is a clinically useful laboratory parameter to diagnose sarcoidosis.

Methods: Sera were obtained from 81 patients with sarcoidosis, 16 patients with Behçet disease, 40 patients with HLA-B27 associated uveitis, 50 patients with Vogt-Koyanagi-Harada (VKH) disease, and 33 healthy volunteers. Serum SP-D levels were quantified with an SP-D enzyme immunoassay kit.

Results: In the healthy control subjects, the average serum SP-D level was 39.70 ng/ml; in the uveitis patients with sarcoidosis, the mean serum SP-D level was 57.0 ng/ml, and in the uveitis patients with other etiologies the mean levels were 38.63 ng/ml for Behçet disease, 38.18 ng/ml for HLA-B27 associated uveitis, and 31.32 ng/ml for the VKH patients. The average serum SP-D levels of patients with sarcoidosis were significantly higher than those of patients with any other uveitis etiologies or healthy controls ($P < 0.01$).

Conclusions: SP-D may be a less invasive and less expensive laboratory examination for sarcoidosis screening. SP-D should be considered as a new laboratory parameter for the diagnosis of uveitis and sarcoidosis. **Jpn J Ophthalmol** 2010;54:81–84 © Japanese Ophthalmological Society 2010

Keywords: ACE, intraocular inflammation, KL-6, sarcoidosis, SP-D

Introduction

Sarcoidosis is a multisystem chronic inflammatory disorder of unknown etiology[1] in which approximately half of the patients develop ocular inflammation.[2–4] At present, the decision to treat patients is still based mainly on clinical features of worsening respiratory symptoms with assessment by radiography or lung function tests. Even when systemic manifestations are mild, ocular tissues are often severely inflamed.[5] Sarcoidosis is a Th1-mediated disease, and we have demonstrated that the serum levels of a macrophage migration inhibitory factor, secreted by activated T cells, are elevated in uveitis patients with sarcoidosis.[6,7] Recent reports have identified other immunological markers that may be indicators of sarcoidosis, such as upregulated intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 on macrophages, or the presence of inducible NO synthase in bronchoalveolar lavage fluid (BALF).[8] It is well known that angiotensin-converting enzyme (ACE) and lysozyme are helpful serum markers for the diagnosis of sarcoidosis. Although it is not very sensitive, ACE is one of the most useful markers, showing high specificity for sarcoidosis.[9] We have reported that serum KL-6, a MUC-1 mucin-like glycoprotein with a high molecular weight that is strongly expressed on type II alveolar pneumonocytes as

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Jpn J Ophthalmol
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well as on bronchiolar epithelial cells, is a clinically useful complement to ACE measurement as a laboratory parameter for the diagnosis and follow-up of sarcoidosis.[10,11]

Surfactant protein D (SP-D) is found in the epithelial cells of multiple mucosal surfaces, and is additionally present in endothelial cells and in serum, suggesting systemic effects of the protein. It has been demonstrated that either inflammatory diseases or the inflammatory cytokine tumor necrosis factor can induce serum SP-D levels.[12–15]

In the present study, the serum levels of SP-D were measured in patients with uveitis to ascertain whether SP-D might be a clinically useful laboratory parameter for ophthalmologists to diagnose sarcoidosis.

Subjects and Methods

After obtaining informed consent in accordance with the tenets of the Declaration of Helsinki, sera were obtained from 81 patients with sarcoidosis, 16 patients with Behçet disease, 40 patients with HLA-B27 associated uveitis, 50 patients with Vogt-Koyanagi-Harada (VKH) disease, and 33 healthy volunteers. Blood samples were collected during each patient's first medical examination at the Uveitis Survey Clinic of the Hokkaido University Hospital. None of the patients had received systemic treatment with corticosteroids or other immunosuppressive agents. The criteria established by the Diffuse Pulmonary Disease Research Committee of Japan were used for diagnosing patients with sarcoidosis. We chose histological results as the basis for this study. This study was approved by Institutional Review Board of Hokkaido University Hospital for Clinical Research.

Venous blood samples were collected in sterile vacuum tubes and centrifuged for 30 min as described previously.[6,16] Serum SP-D levels were quantified with an SP-D enzyme immunoassay kit from Yamasa Soy Sauce (Tokyo, Japan), which contains all necessary reagents for the assay. The results are presented as mean (standard error), and the statistical analysis of the SP-D levels was performed with the Student *t* test.

Results

The average level of serum SP-D in healthy control subjects was 39.70 (SE, 3.62) ng/ml. In uveitis patients with sarcoidosis, the mean serum SP-D level was 57.0 (4.12) ng/ml, and the uveitis patients with other etiologies had mean levels of 38.63 (5.19) ng/ml in Behçet disease; 38.18 (4.10) ng/ml in HLA-B27-associated uveitis; and 31.32 (2.70) ng/ml in VKH (Fig. 1). The serum levels of SP-D in sarcoidosis patients

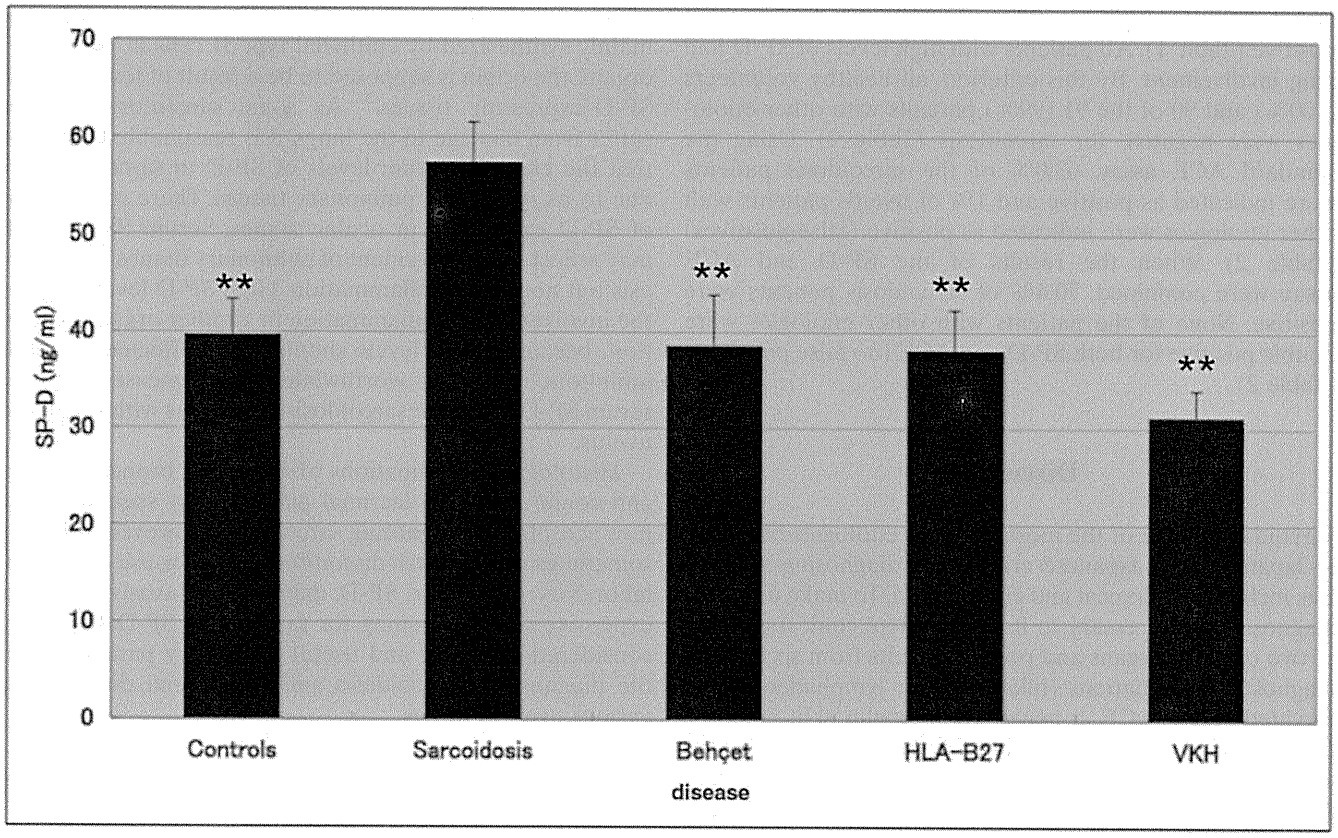

Figure 1. Serum surfactant protein D (*SP–D*) levels. Mean serum SP-D levels were significantly higher in patients with sarcoidosis than in those with Behçet disease, HLA-B27 associated uveitis, Vogt-Koyanagi-Harada (*VKH*) disease, or healthy control subjects. $^{**}P < 0.01$.

Table 1. Serum SP-D and ACE levels in sarcoidosis with uveitis ($n = 81$)

	ACE	
	Positive	Negative
SP-D		
Positive	6	6
Negative	45	24

SP-D, surfactant protein D; ACE, angiotensin-converting enzyme.

Table 2. Serum SP-D and ACE levels in other etiologies of uveitis ($n = 91$)

	ACE	
	Positive	Negative
SP-D		
Positive	0	1
Negative	1	89

were significantly higher than those in healthy controls ($P = 0.0017$), Behçet disease ($P = 0.007$), HLA-B27 associated uveitis ($P = 0.0013$), or VKH disease ($P = 0.0000005$).

Patients were considered to have a positive indication for sarcoidosis if their SP-D serum concentration exceeded 100 ng/ml. We found that, based on their serum SP-D levels, 14.8% (12/81) of uveitis patients with sarcoidosis proved positive (Table 1). All patients with high levels of SP-D had lung involvement. By this criterion, all healthy volunteers (100%) and 90 of the 91 (99%) patients with other etiologies were negative for sarcoidosis (Table 2). Using the standard ACE assay, 63.0% of the sarcoidosis patients were indicated as positive and 1% of uveitis patients with other etiologies were indicated as positive (false positives) (Table 2). When the results of the SP-D and ACE assay were combined, 70.4% of sarcoidosis patients were positive. None of the patients with other etiologies were double positive for both SP-D and ACE (no false positives) (Table 2).

Discussion

Sarcoidosis is one of the most frequent etiologies of uveitis in Japan.[17,18] The Japanese criteria for diagnosing sarcoidosis include histological and clinical data. To make a clinical diagnosis, it is necessary to have complete clinical pictures of two or more organs and positive results from six specific diagnostic examinations (bilateral hilar lymphadenopathy, elevated serum ACE, a negative tuberculin skin reaction, positive Ga scintigram, elevated CD4/CD8 ratio in BALF, and elevated serum/urinary Ca levels). However, serum ACE is not a sensitive indicator of sarcoidosis, making it difficult to clinically diagnose some patients with sarcoidosis, even if their granulomatous uveitis is typical for sarcoidosis. Clinicians already use serum ACE levels as one laboratory marker for sarcoidosis, but ACE measurements identify only half of the uveitis patients with sarcoidosis.[10] As it is thought that granulomatous tissue can release ACE, it is possible that the serum levels of ACE increase in other granulomatous conditions such as tuberculosis, lymphoma, and asbestosis. Serum ACE levels are affected by systemic corticosteroids.[11] In addition, some patients who are suffering from hypertension or heart failure may be treated with ACE inhibitors, which are antihypertensive drugs. In those patients, serum ACE levels often fall below measurable limits.[11]

We previously reported that MUC-1 mucin-like glycoprotein KL-6 levels were also elevated in sarcoidosis patients, and the sensitivity was raised to 87.5%, but there were 10.8% false positives when ACE and KL-6 serum measurements were combined.[10] However, as we later demonstrated, serum KL-6 levels are also elevated in patients with tubulointerstitial nephritis and uveitis (TINU) syndrome as well as sarcoidosis, because KL-6 is expressed in the renal distal tubes in addition to alveolar cells of the lungs.[19] Although it is not known whether the lung surfactant SP-D is expressed in renal tissues, it is unlikely to have a significant impact on serum levels in renal disorders. To examine the serum SP-D level in TINU syndrome may be a subject of future investigation.

Serum SP-D has been used as a prognostic or predictive marker for pulmonary inflammatory diseases (e.g., allergic bronchopulmonary aspergillosis, pneumonia, lung diseases with polymyosis, and acute lung injury).[20] Since SP-D is mainly synthesized by epithelial type II cells in pulmonary organs, the origin is supposed to be a result of leakage from SP-D-expressing tissues.[20] As some sarcoidosis patients suffer from damage to the lungs, it is reasonable to suggest that the elevated serum levels of SP-D in sarcoidosis are due to its release by pulmonary tissues. There is no report of SP-D expression in ocular tissues. Serum SP-D levels may reflect the involvement of pulmonary tissues in sarcoidosis but not ocular inflammation. High SP-D levels suggest the involvement of inflammation in another organ. In addition, because SP-D levels should be unaffected by ACE inhibitors, it may be worthwhile to add measurement of serum SP-D levels for sarcoidosis in patients with suspected uveitis.

Histological examinations of the lungs, bronchial tubes, and conjunctiva and lacrimal gland biopsy specimens are now accepted in diagnosing sarcoidosis. However, as biopsy sometimes causes much discomfort and pain, use of another laboratory parameter, SP-D, may be less invasive and less expensive when screening for sarcoidosis. SP-D should be considered as a new and useful laboratory parameter for the diagnosis of sarcoidosis and differential diagnosis of various uveitis entities.

References

1. Newman LS, Rose CS, Maier LA. Sarcoidosis. N Engl J Med 1997;336:1224–1234.

2. Crick RP, Hoyle C, Smellie H. The eyes in sarcoidosis. Br J Ophthalmol 1961;45:461–481.
3. Obenauf CD, Shaw HE, Sydnor CF, et al. Sarcoidosis and its ophthalmic manifestations. Am J Ophthalmol 1978;86:648–655.
4. Jabs DA, Johns CJ. Ocular involvement in chronic sarcoidosis. Am J Ophthalmol 1986;102:297–301.
5. Uyama M. Uveitis in sarcoidosis. Int Ophthalmol Clin 2002;42: 143–150.
6. Kitaichi N, Kotake S, Sasamoto Y, et al. Prominent increase of macrophage migration inhibitory factor in the sera of patients with uveitis. Invest Ophthalmol Vis Sci 1999;40:247–250.
7. Kotake S, Kitaichi N, Ohno S. Macrophage migration inhibitory factor in uveitis. Int Ophthalmol Clin 2002;42:99–103.
8. Semenzato G, Bortoli M, Agostini C. Applied clinical immunology in sarcoidosis. Curr Opin Pulm Med 2002;8:441–444.
9. Kawaguchi T, Hanada A, Horie S, et al. Evaluation of characteristic ocular signs and systemic investigations in ocular sarcoidosis patients. Jpn J Ophthalmol 2007;51:121–126.
10. Kitaichi N, Kotake S, Shibuya H, et al. Increase of KL-6 in sera of uveitis patients with sarcoidosis. Graefes Arch Clin Exp Ophthalmol 2003;241:879–883.
11. Kitaichi N, Ariga T, Kase S, et al. Usefulness of quantifying serum KL-6 levels in the follow-up of uveitic patients with sarcoidosis. Graefes Arch Clin Exp Ophthalmol 2006;244:433–437.
12. Akiyama J, Hoffman A, Brown C, et al. Tissue distribution of surfactant proteins A and D in the mouse. J Histochem Cytochem 2002;50:993–996.
13. Fujita M, Shannon JM, Ouchi H, et al. Serum surfactant protein D is increased in acute and chronic inflammation in mice. Cytokine 2005;31:25–33.
14. Leth-Larsen R, Nordenbaek C, Tornoe I, et al. Surfactant protein D (SP-D) serum levels in patients with community-acquired pneumonia small star, filled. Clin Immunol 2003;108:29–37.
15. Madsen J, Kliem A, Tornoe I, et al. Localization of lung surfactant protein D on mucosal surfaces in human tissues. J Immunol 2000;164:5866–5870.
16. Kitaichi N, Kotake S, Mizue Y, et al. Increase of macrophage migration inhibitory factor in sera of patients with iridocyclitis. Br J Ophthalmol 2000;84:1423–1425.
17. Goto H, Mochizuki M, Yamaki K, et al. Epidemiological survey of intraocular inflammation in Japan. Jpn J Ophthalmol 2007; 51:41–44.
18. Kitamei H, Kitaichi N, Namba K, et al. Clinical features of intraocular inflammation in Hokkaido, Japan. Acta Ophthalmol 2009; 87:424–428.
19. Kase S, Kitaichi N, Namba K, et al. Elevation of serum Krebs von den Lunge-6 levels in patients with tubulointerstitial nephritis and uveitis syndrome. Am J Kidney Dis 2006;48:935–941.
20. Sorensen GL, Hjelmborg JVB, Kyvik KO, et al. Genetic and environmental influences of surfactant protein D serum levels. Am J Physiol Lung Cell Mol Physiol 2006;290:1010–1017.

eradication treatment will probably give a huge advantage in terms of social health, especially in high-risk areas.

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References

1. Leung WK, Lin SR, Ching JY, To KF, Ng EK, Chan FK, et al. Factors predicting progression of gastric intestinal metaplasia: results of a randomised trial on *Helicobacter pylori* eradication. Gut. 2004;53:1244-9. [PMID: 15306578]
2. Zhou L. Ten-year follow-up study on the incidence of gastric cancer and the pathological changes of gastric mucosa after *H. pylori* eradication in China [Abstract]. Gastroenterology. 2008;134:A233.
3. Zhou L, Sung JJ, Lin S, Jin Z, Ding S, Huang X, et al. A five-year follow-up study on the pathological changes of gastric mucosa after *H. pylori* eradication. Chin Med J (Engl). 2003;116:11-4. [PMID: 12667379]
4. Zhou LY, Lin SR, Ding SG, Huang XB, Zhang L, Meng LM, et al. The changing trends of the incidence of gastric cancer after *Helicobacter pylori* eradication in Shandong area. Chin J Dig Dis. 2005;6:114-5. [PMID: 16045599]

CLINICAL OBSERVATIONS

Granulysin as a Marker for Early Diagnosis of the Stevens–Johnson Syndrome

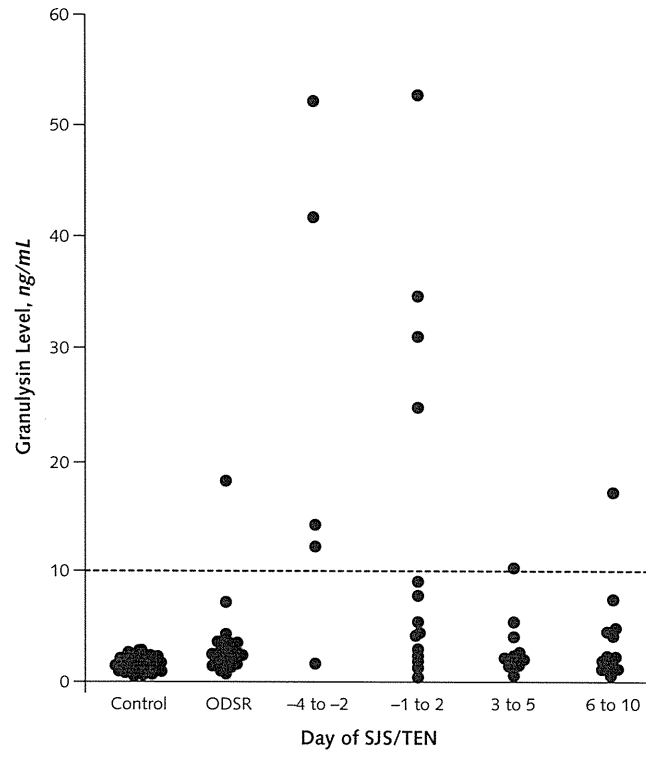
Background: The Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening adverse drug reactions characterized by massive epidermal necrosis. In the early stage, clinical presentations of SJS/TEN are very similar to those of ordinary drug-induced skin reactions (ODSRs); therefore, SJS/TEN is difficult to diagnose and the start of treatment is often delayed, resulting in high mortality rates. Other investigators (1) reported that granulysin is highly expressed in blisters of SJS/TEN and causes disseminated keratinocyte death. Because SJS/TEN progresses and spreads rapidly, the granulysin level should be increased in the serum of patients with active SJS/TEN if it is a key mediator of these diseases.

Objective: To determine whether serum granulysin levels are higher in patients with SJS/TEN than in healthy control participants or those with ODSRs.

Methods: We measured granulysin in the sera of 31 healthy control participants, 24 patients with ODSR, 13 patients with SJS, and 7 patients with TEN by using enzyme-linked immunosorbent assay (2). Disease onset in patients with SJS/TEN was defined as the day (day 1) on which the mucocutaneous or ocular lesion first eroded or ulcerated (3), and we collected sera from these patients from 4 days before to 10 days after ulceration. We used the Tukey–Kramer test to conduct multiple comparisons between groups.

Results: None of the 31 healthy control participants had a granulysin level greater than the upper limit of normal, which was 10 ng/mL (0% elevated; mean, 1.6 ng/mL [SD, 0.6]), and among 24 patients with ODSRs, only 1 patient had an elevated granulysin level (4.2% elevated; mean, 3.5 ng/mL [SD, 3.4]) (**Figure**). We obtained samples from 5 patients with SJS/TEN on day −4 to day −2, and we detected the highest granulysin concentrations (elevated in 80% of patients); mean, 24.8 ng/mL [SD, 21.2]). Granulysin levels were lower in the 14 samples collected on day −1 to day 2 (28.6% elevated; mean, 13.7 ng/mL [SD, 16.0]), and were even lower in the 10 samples collected from day 3 to day 5 (10.0% elevated; mean, 4.2 ng/mL [SD, 3.0]) and in the 13 samples collected from day 6 to day 10 (7.7% elevated; mean, 4.5 ng/mL [SD, 4.5]). When we compared granulysin levels from day −4 to day −2 among patients with SJS/TEN, patients with ODSRs, and healthy control participants, the differences were statistically significant ($P < 0.010$).

Figure. **Granulysin levels of healthy control participants, patients with ODSRs, and patients with SJS/TEN.**



ODSR = ordinary drug-induced skin reaction; SJS/TEN = Stevens–Johnson syndrome/toxic epidermal necrolysis.

Discussion: Granulysin is cytotoxic for tumor cells, transplant cells, bacteria, fungi, and parasites, in which it damages negatively charged cell membranes because of its positive charge (4). It plays an important role in the host defense against pathogens, and it induces apoptosis of target cells by using a mechanism involving caspases and other pathways (4). Its potency makes it a credible mediator of skin damage in patients with SJS/TEN. Adding to this credibility is a report (1) that granulysin is the most highly expressed cytotoxic molecule in the blisters of patients with SJS/TEN. We show that serum granulysin levels in 4 of 5 patients with SJS/TEN were elevated before skin detachment or mucosal lesions develop. Soluble Fas ligand (sFasL) shares some properties with granulysin: It contributes to keratinocyte death in SJS/TEN (3, 5), and levels are elevated in the sera of patients with SJS/TEN (3). Serum granulysin levels, however, are approximately 100 times higher than those of sFasL on day

−4 to day −2 (23.1 ng/mL [SD, 16.6] vs. 147.76 pg/mL [SD, 104.4]). Therefore, we believe it would be easier to develop bedside granulysin serum measurement, for example, by using immunochromatography, than it would be to develop a similar sFasL measurement. Monitoring serum granulysin might enable early diagnosis of SJS/TEN in patients with cutaneous adverse drug reactions that otherwise could not be distinguished from ODSRs.

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References

1. Chung WH, Hung SI, Yang JY, Su SC, Huang SP, Wei CY, et al. Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis. Nat Med. 2008;14:1343-50. [PMID: 19029983]
2. Ogawa K, Takamori Y, Suzuki K, Nagasawa M, Takano S, Kasahara Y, et al. Granulysin in human serum as a marker of cell-mediated immunity. Eur J Immunol. 2003;33:1925-33. [PMID: 12884856]
3. Murata J, Abe R, Shimizu H. Increased soluble Fas ligand levels in patients with Stevens-Johnson syndrome and toxic epidermal necrolysis preceding skin detachment. J Allergy Clin Immunol. 2008;122:992-1000. [PMID: 18692887]
4. Kaspar AA, Okada S, Kumar J, Poulain FR, Drouvalakis KA, Kelekar A, et al. A distinct pathway of cell-mediated apoptosis initiated by granulysin. J Immunol. 2001; 167:350-6. [PMID: 11418670]
5. Abe R, Shimizu T, Shibaki A, Nakamura H, Watanabe H, Shimizu H. Toxic epidermal necrolysis and Stevens-Johnson syndrome are induced by soluble Fas ligand. Am J Pathol. 2003;162:1515-20. [PMID: 12707034]

Localized Amyloidosis at the Site of Enfuvirtide Injection

Background: Enfuvirtide is the first of a new class of antiretroviral agents that block fusion of the viral particle with the host target cell. Its safety and antiviral activity have been demonstrated (1, 2). In clinical trials, injection site reactions occurred in 80% to 100% of patients (3). The most common signs and symptoms reported were induration in 94%, erythema in 91%, and subcutaneous nodules and cysts in 70% (4).

Objective: To describe a case of amyloidosis at the injection site of enfuvirtide.

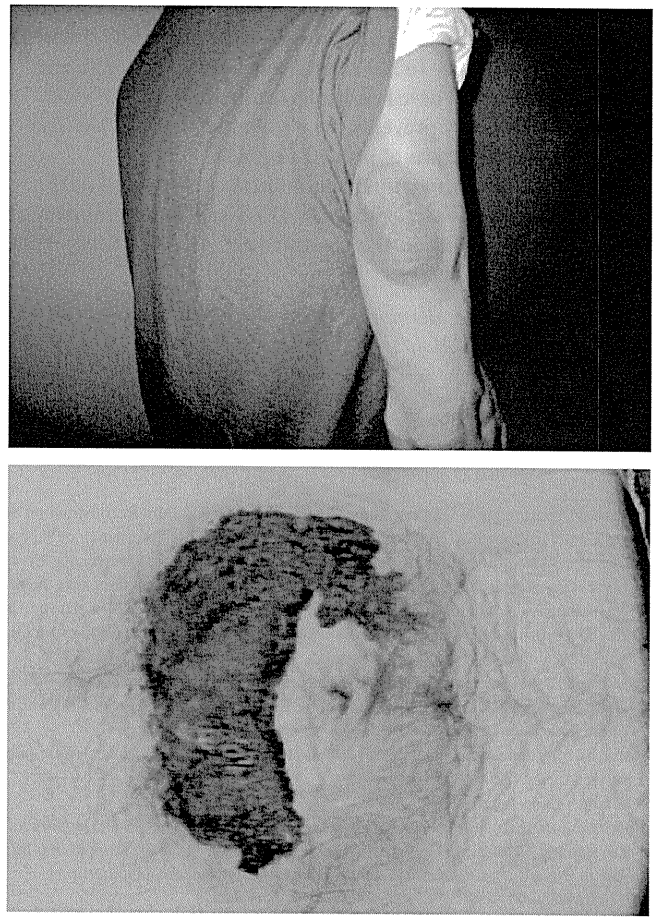
Case Report: The patient was a man aged 47 years who had a history of sexual intercourse with men and extensive treatment for HIV with a triple-class viral resistance profile. He also had long-standing leg pain thought to be secondary to HIV neuropathy and no history of intravenous drug use. There was no history of opportunistic or chronic infections.

Because of a persistently elevated viral load, enfuvirtide by subcutaneous injection was added to his highly active antiretroviral treatment regimen for 41 months; enfuvirtide therapy was then stopped in February 2007 because of intolerable injection site reactions. While he was receiving enfuvirtide, his viral loads were completely suppressed. Eighteen months after enfuvirtide therapy was stopped, large, tender, indurated reactions with fragile epithelial surfaces persisted at all injection sites (**Figure**, *top*). These reactions bled extensively into the subcutaneous tissue with minor trauma (**Figure**, *bottom*). A lesion on the triceps was excised surgically, and the wound healed without complications. Pathologic examination showed extensive deposits of proteinaceous material with intense Congo red staining that was consistent with amyloid. A lesion on the opposite arm was resected and showed similar findings. The patient had a normal leukocyte count and normal hemoglobin, blood urea nitrogen, and creatinine levels and had no evidence of plasma cell dyscrasia and no history of organ dysfunction to suggest systemic amyloidosis.

Discussion: In 7 patients receiving enfuvirtide, biopsy of injection site reactions revealed an inflammatory response consistent with a localized hypersensitivity reaction (5), and other studies (3) have reported similar findings. Other reports (6) have described 3 histologic patterns: an acute urticaria- or vasculitis-like pattern with inflammation of the fat tissue, a subacute pattern with an initial dermal sclerosis, and a long-term scleroderma-like pattern.

In our patient, surgical excision of enfuvirtide injection site reactions revealed subcutaneous nodular amyloidosis. Localized

Figure. **Lesion in right triceps area (*top*) and periumbilical site with spontaneous intradermal and subcutaneous hemorrhage (*bottom*).**



DNA vaccination against macrophage migration inhibitory factor improves atopic dermatitis in murine models

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Background: Atopic dermatitis (AD) is a common chronic inflammatory skin disease. Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that has been implicated in the pathogenesis of AD. Recently, we developed a novel DNA vaccine that generates neutralizing endogenous anti-MIF antibodies.

Objective: This study explores the preventive and therapeutic effects of this MIF-DNA vaccine in mouse models of AD.

Methods: Two different AD model mice (DS-Nh and NC/Nga) received MIF-DNA vaccination to analyze preventive and therapeutic effects, as assessed by clinical skin scores, histologic findings, and serum IgE levels.

Results: In murine models of AD, MIF-DNA vaccination prevented the occurrence of the AD skin phenotype. Furthermore, administration of MIF-DNA vaccine to mice that had already developed AD produced a rapid improvement in AD skin manifestation. There were reduced histologic signs of inflammation and lower serum IgE levels in treated mice compared with those seen in control animals. Finally, passive transfer of IgG from MIF-DNA vaccinated mice to AD mice also produced a significant therapeutic effect. These results demonstrate that MIF-DNA vaccination not only prevents the development of AD but also improves the symptoms of pre-existing AD.

Conclusion: Taken together, the induction of an anti-MIF autoantibody response using MIF-DNA vaccination appears to be a useful approach in the treatment of AD. (J Allergy Clin Immunol 2009;124:90-9.)

Key words: *Atopic dermatitis, macrophage migration inhibitory factor, DNA vaccination*

Abbreviations used
AD: Atopic dermatitis
MIF: Macrophage migration inhibitory factor
TTX: Tetanus toxin P30 T_H epitope

Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disease with significant morbidity and an adverse effect on patient well-being.[1] The prevalence of AD has increased 2- to 3-fold during the past 3 decades in industrialized countries, and it presently occurs in 10% to 20% of children and 1% to 3% of adults.[2] AD is thought to result from a dysregulation in the normal interaction between the environment, genes, defects in skin barrier function, and systemic and local immunologic responses.[3] The contribution of the immune response to the pathogenesis of AD has been largely attributed to abnormalities in the adaptive immune system, with key roles played by T_H1/T_H2 cell dysregulation, IgE production, dendritic cell signaling, and mast cell hyperactivity, leading to the pruritic inflammatory dermatosis that characterizes AD.[3]

Macrophage migration inhibitory factor (MIF) is an upstream regulator of the inflammatory response, and it is upregulated in various inflammatory disorders, including AD.[4] We previously reported that serum MIF levels in patients with AD were significantly increased compared with those seen in healthy control subjects and patients without AD.[5] In addition, circulating MIF levels in patients with AD decrease as the clinical features of the disease improve, suggesting that MIF might play a pivotal role in the inflammatory response in these patients.[5,6] Moreover, MIF promotes IL-2 and IL-2 receptor expression and memory T-cell development, and it might influence T_H1/T_H2 cell differentiation responses.[6,7] Based on these observations suggesting that MIF might be a therapeutic target in AD, we hypothesized that inhibition of MIF with neutralizing antibodies might induce beneficial therapeutic effects in patients with AD.

Monoclonal antibodies directed against proinflammatory cytokines, such as TNF-α, have been used for the treatment of rheumatoid arthritis, Crohn disease, and psoriasis,[8-10] and there have been a few reports describing the use of anti–TNF-α mAbs for the treatment of AD.[7,8] The application of mAbs to AD nevertheless might be difficult because of the requirement for frequent injections, the large quantities of immunoglobulin protein required, and the associated costs of production. Moreover, even fully humanized antibodies are potentially immunogenic and might elicit antibody responses, thereby limiting their long-term therapeutic efficacy. These limitations have led to the development of alternative neutralization strategies, including methods that aim to elicit autoantibodies against target proteins,

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such as cytokines or pathogens, by administering them in a naked or partially modified form as therapeutic vaccines.

We recently developed an MIF-DNA vaccine that breaks immunologic tolerance by introducing oligonucleotides encoding a foreign T_H cell epitope into the murine MIF cDNA sequence.[11,12] We demonstrated that this MIF-DNA vaccination elicits production of endogenous anti-MIF antibodies and showed a significant amelioration of symptoms in murine models of rheumatoid arthritis[9] and sepsis.[10]

The present study describes for the first time the preventive and therapeutic effects of this MIF-DNA vaccine in 2 different mouse models of AD.

METHODS

Animals

Six-week-old female BALB/c mice were purchased from Japan Clea (Shizuoka, Japan). Male DS-Nh mice were provided by Aburabi Laboratories, Shionogi and Co, Ltd (Shiga, Japan), and male NC/Nga mice were purchased from SLC (Hamamatsu, Japan). All mice were bred and housed under conventional conditions, and procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.

Production of DNA vaccine

We previously reported the design of the MIF/tetanus toxin P30 T_H epitope (TTX) DNA expression plasmid and our analysis of the *in vitro* expression of MIF/TTX by using this plasmid.[11] For the generation of immunologically active MIF antigen, an MIF construct harboring a T_H epitope at its second loop region was designed. For that purpose, the coding region for the second loop of the mouse MIF, amino acids 32 to 37 (GKPAQY), was deleted from the MIF cDNA and substituted with an *Eco*RI site. A complementary DNA coding for the TTX (FNNFTVSFWLRVPKVSASHL) with *Eco*RI sites at both termini was obtained by means of hybridization of partially overlapping oligo DNAs (sense, ggaattcaacaacttcaccgtgagcttctggctgcgcgtgcccaa; antisense, ggaattccaggtggctggcgctcaccttgggcacgcgcagccaga) after polymerization with the Klenow fragment of DNA polymerase. After digestion with *Eco*RI, the cDNA coding for the P30 T_H epitope was inserted into the *Eco*RI site of the MIF expression plasmid lacking the second loop, and a clone with the insert of correct orientation was selected. For vaccination, the plasmid DNA was purified by using standard methods with alkaline lysis followed by 2 rounds of CsCl density gradient ultracentrifugation.

Vaccination protocols

Gene transfer into muscle by means of electroporation was performed as described previously.[11] Briefly, mice were anesthetized with ether and shaved near their hind legs. A pair of electrode needles (5-mm gap and 0.5-mm diameter; NEPA GENE, Chiba, Japan) was then inserted into an anterior tibial muscle, and DNA vaccine (25 μg/25 μL of 0.9% saline) was injected into the portion between the needles. Electrical pulses (50 V, 50 ms, 3 times) were applied (T820 and Optimizer 500; BTX, San Diego, Calif) and followed by another 3 pulses with inverted polarity. The same injection and electroporation was applied to the other tibial muscle. Thus 50 μg of the naked plasmid was injected per mouse into the tibias. A similar vaccination was repeated 3 weeks later.

Evaluation of anti-MIF antibody titer in sera of DNA-vaccinated mice

Anti-MIF titers in plasma were determined by means of direct ELISA. Briefly, individual plasma from vaccinated mice were collected from the tail vein and diluted with 0.1% BSA/PBS/0.05% Tween 20. Small aliquots of diluted plasma (1:200) were added into 96-well flat-bottom plates precoated with recombinant MIF. Anti-MIF antibodies that reacted with the precoated recombinant MIF were detected with goat anti-mouse antibody conjugated with horseradish peroxidase, followed by color development with substrate reagent (Techne, Minneapolis, Minn).

Evaluation of clinical skin severity score

Mice were macroscopically observed and scored by 2 persons blind to the treatment protocol. Before skin conditions were scored, scratching behavior was observed for 2 minutes. A total clinical severity for AD-like lesions was defined as the sum of the individual scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of 5 signs and symptoms (itch, erythema, edema, excoriation/erosion, and scaling/dryness).[13]

Measurement of IgE and TNF-α levels in sera

Serum total IgE levels were measured by using a sandwich ELISA kit (Yamasa Shouyu, Chiba, Japan). Serum MIF levels were assayed with ELISA kits for Genetic Lab (Sapporo, Japan). The ELISA procedures were conducted according to the manufacturer's instructions. The concentration of TNF-α was determined by using the BD Cytometric Bead Array (BD PharMingen, San Jose, Calif). Flow cytometric analysis was carried out with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, Calif).

Real-time PCR analysis

Total RNA was extracted from dorsal skin to quantify cytokine mRNA expression levels in dermatitis lesions. RNA samples were analyzed with the ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, Calif). Primers and probes specific for IL-1β, IL-4, IL-6, and IFN-γ were obtained from the TaqMan gene expression assay (Applied Biosystems). Differences between the mean cycle threshold (CT) values of cytokines and those of β-actin (Applied Biosystems) were calculated as

$$\Delta CT_{sample} = CT_{cytokine} - CT_{\beta-actin},$$

and those of ΔCT for the normal adult skin were calculated as

$$\Delta CT_{calibrator} = CT_{cytokine} - CT_{\beta-actin}.$$

Final results for fetal skin sample/adult skin (as percentages) were determined as $2-(\Delta CT_{sample} - \Delta CT_{calibrator})$.

Histologic analysis

Six-micrometer-thick sections of dorsal skin were stained with hematoxylin and eosin, acidic toluidine blue (pH 4.0) for mast cells, and direct fast scarlet for eosinophils. Cells between the epithelium and panniculus carnosus were counted at a magnification of ×400 and were expressed as the total number of cells in 5 fields.

Treatment of neutralizing MIF mAbs

Neutralizing anti-MIF mAb (NIH-III.D9) was previously described.[14] Neutralizing MIF mAbs (50 μg) or control IgG (50 μg) were injected intravenously into 15-week-old NC/Nga mice with dermatitis twice a week for 3 weeks.

Adoptive transfer of autoantibodies elicited by DNA vaccines

IgG was purified from the sera of control pCAGGS plasmid– or MIF/TTX-vaccinated DS mice at 6 weeks after the vaccination by using the protein A Antibody Purification Kit (Amersham Biosciences, Piscataway, NJ). The purified IgG was tested for its ability to suppress ongoing dermatitis in an adoptive transfer experiment. DS-Nh mice with developing dermatitis were separated at 15 weeks of age into 3 equally sick groups of 3 mice each. Every 3 days, these animals were administered 50 μg per mouse of purified IgG from control pCAGGS plasmid–vaccinated DS-Nh mice, purified IgG from MIF/TTX-vaccinated DS-Nh mice, or an equal volume of PBS.

RESULTS

MIF/TTX vaccination prevents the onset of AD in DS-Nh mice

DS-Nh mice housed under conventional conditions but not in a specific pathogen free environment spontaneously exhibit AD-like skin symptoms, including erythema, edema, excoriation, erosion, dry skin, and desquamation.[15-17] Early skin symptoms appear around 9 weeks of age and continue to worsen until age 25 weeks. An increase in total serum IgE levels is detected at approximately 17 weeks of age and after the development of skin lesions.[15-17]

We first examined the potential protective effect of the MIF/TTX vaccine on dermatitis development by treating 9-week-old DS-Nh mice before the development of skin eruptions. The clinical features of the control pCAGGS plasmid–vaccinated mice were similar to those of untreated mice. At 18 weeks of age, or 9 weeks after the vaccination, both groups of mice showed severe erythema, erosions, and dry skin (Fig 1, *A*). By contrast, the MIF/TTX-vaccinated mice exhibited almost no eruptions (Fig 1, *B*). The clinical skin score of MIF/TTX-vaccinated mice was low until 21 weeks of age (Fig 1, *C*), which is a time at which the MIF/TTX-vaccinated mice showed high serum levels of anti-MIF antibodies (Fig 1, *D*). Furthermore, in the MIF/TTX-vaccinated mice the serum level of IgE was significantly decreased and the serum MIF level was only slightly decreased when compared with those seen in the control vaccinated mice (Fig 1, *E* and *F*). In addition, cytokine expression in affected skin lesions was analyzed by using real-time PCR. The T_H2 cytokine IL-4 was very slightly downregulated, and the T_H1 cytokine IFN-γ was slightly upregulated. Of note, the expression of the proinflammatory cytokines IL-1β and IL-6 was significantly suppressed in MIF-vaccinated mice compared with that seen in control mice (Fig 1, *G*). Therefore the inhibition of MIF in the atopic model mice appears to result primarily in the suppression of inflammation rather than affecting the T_H1/T_H2 cytokine balance.

Improvement of clinical skin condition with MIF/TTX vaccine also was confirmed by the observation that the lesions of mice vaccinated with MIF/TTX vaccine showed amelioration in hyperkeratosis, acanthosis, dermal edema, and infiltration of the inflammatory cells at 21 weeks when compared with the condition of mice vaccinated with control pCAGGS plasmid (Fig 2, *A*). At the affected skin sites, the numbers of eosinophils and mast cells decreased significantly in the MIF/TTX-vaccinated mice at 21 weeks when compared with those seen in the control vaccinated mice (Fig 2, *B-D*).

These data clearly show that MIF/TTX vaccination can prevent the onset of AD-like dermatitis in DS-Nh mice.

MIF/TTX vaccination improves pre-existing AD

To determine whether the MIF/TTX vaccine has any therapeutic effect in AD, we next vaccinated 15-week-old DS-Nh mice with pre-existing AD and evaluated the progression of skin changes. Mice treated with the control plasmid continued to exhibit severe dermatitis 6 weeks after vaccination (Fig 3, *A*). By contrast, the MIF/TTX vaccination significantly improved dermatitis symptoms (Fig 3, *B*). The clinical skin scores of control-vaccinated mice increased after the vaccination, whereas that of MIF/TTX-vaccinated mice began to decrease at 21 weeks of age (Fig 3, *C*). At this time, the MIF/TTX-vaccinated mice showed high levels of anti-MIF antibodies (Fig 3, *D*). Furthermore, the serum IgE and MIF levels of MIF/TTX-vaccinated mice also were lower than those of control mice at 21 weeks of age (Fig 3, *E* and *F*). In addition, serum TNF-α levels were significantly lower in the MIF/TTX-vaccinated mice when compared with those seen in the control plasmid–vaccinated mice (Fig 3, *G*).

By means of histologic analysis, the lesions of mice vaccinated with MIF/TTX vaccine showed improvement of hyperkeratosis, acanthosis, dermal edema, and infiltration of inflammatory cells at 21 weeks when compared with the control plasmid–vaccinated mice (Fig 4, *A*). In addition, the numbers of eosinophils and mast cells decreased significantly in the MIF/TTX-vaccinated mice at 21 weeks when compared with those seen in the control mice (Fig 4, *B-D*). The serum IgE level of MIF/TTX-vaccinated mice decreased at 21 weeks of age compared with that of control-vaccinated mice (Fig 4, *E*).

These data indicate that MIF/TTX vaccination leads to an improvement in already established dermatitis in the DS-Nh mice.

We further observed that MIF-DNA vaccine improved the manifestation of pre-existing AD in a second model of AD, which develops in the NC/Nga strain.[11,13] We vaccinated 15-week-old NC/Nga mice with dermatitis, and although the control pCAGGS plasmid–vaccinated mice still had severe dermatitis 6 weeks after the vaccination treatment (Fig 5, *A*), the MIF/TTX-vaccinated mice showed significant improvement (Fig 5, *B*). The clinical skin score of control-vaccinated mice increased after the vaccination, whereas that of MIF/TTX-vaccinated mice began to decrease at 21 weeks of age (Fig 5, *C*). At this time, the MIF/TTX-vaccinated mice showed high levels of anti-MIF antibodies (Fig 5, *D*). The serum IgE and MIF levels of MIF/TTX-vaccinated mice decreased at 21 weeks of age (Fig 5, *E* and *F*). These data show that MIF-DNA vaccination improves dermatitis not only in the DS-Nh strain but also in the NC/Nga mouse strains.

To confirm that anti-MIF antibodies suppress AD, we performed an additional therapeutic experiment by using a neutralizing anti-MIF mAb. Anti-MIF mAb (50 μg) or an isotypic control IgG (50 μg) were injected intravenously into 15-week-old NC/Nga mice with dermatitis twice a week for 3 weeks. Anti-MIF mAbs, as well MIF vaccination, significantly improved AD skin manifestations when compared with conditions seen in control IgG–treated mice (Fig 5, *G* and *H*).

Adoptive transfer of autoantibodies elicited by MIF/TTX-DNA vaccine suppressed AD

To better substantiate that the therapeutic action of MIF/TTX-DNA vaccination could be attributed to anti-MIF autoantibodies, we performed adoptive transfer of purified IgG from vaccinated DS-Nh mice into naive DS-Nh mice. The purified IgG was adoptively transferred into the 15-week-old DS-Nh mice that had already demonstrated skin eruptions. As shown in Fig 6, this IgG was effective in ameliorating AD, indicating that the therapeutic effect of MIF/TTX vaccination could be adoptively transferred by immune serum IgG.

DISCUSSION

In the present study we have shown that active vaccination against MIF is a novel preventive and therapeutic approach in 2 murine models of AD. We showed that mice administered a MIF/TTX-DNA vaccine did not experience the cutaneous manifestations of AD. The MIF/TTX-DNA vaccine also improved the symptoms

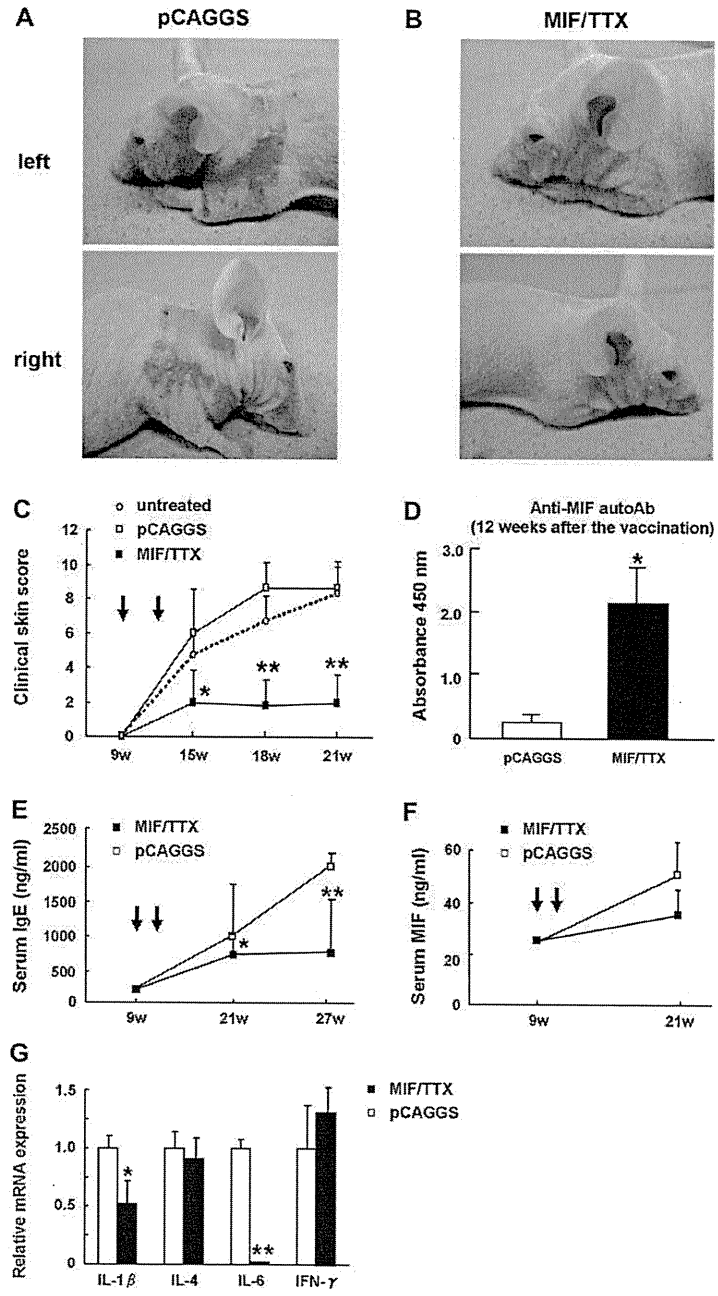

FIG 1. Prevention of the onset of AD by MIF/TTX-DNA vaccine. Nine-week-old DS-Nh mice without skin eruptions were subjected to administration of MIF/TTX or a control plasmid (pCAGGS). Clinical features of 21-week-old DS-Nh mice vaccinated with endotoxin-free pCAGGS **(A)** and MIF/TTX (**B**; 12 weeks after the vaccination) are shown. **C**, The clinical skin score of mice immunized with the MIF/TTX-DNA vaccine *(solid squares)*, immunized with pCAGGS plasmid *(open squares)*, or left untreated *(open circles)*. Results are given as means ± SEs of 5 mice in each group. $*P < .01$ and $**P < .005$ versus pCAGGS at the same time point. **D**, Serum level of anti-MIF autoantibodies *(autoAb)* at 12 weeks after vaccination. Means ± SEs are shown (n = 5). $*P < .01$. **E**, Serum IgE levels of the mice vaccinated with MIF/TTX *(solid squares)* and pCAGGS *(open squares)*. $*P < .01$ and $**P < .005$ for MIF/TTX versus pCAGGS at the same time point. Means ± SEs of 5 mice in each group are shown. **F**, Serum MIF levels of the mice vaccinated with MIF/TTX *(solid squares)* and pCAGGS *(open squares)*. **G**, Cytokine expression (IL-1β, IL-4, IL-6, and IFN-γ) in affected skin lesions was analyzed by using real-time PCR. $*P < .05$ and $**P < .001$.

of pre-existing AD in 2 different strains of AD-prone mice, the DS-Nh and NC/Nga strains. Finally, we demonstrated that the therapeutic effect of MIF/TTX vaccination could be adoptively transferred by serum IgG that contained MIF autoantibodies.

Proinflammatory cytokines are believed to be important contributors to the pathogenesis of skin inflammation in patients with AD, which might depend on the duration of the skin lesion. Patients with acute AD typically have a systemic T_H2 response

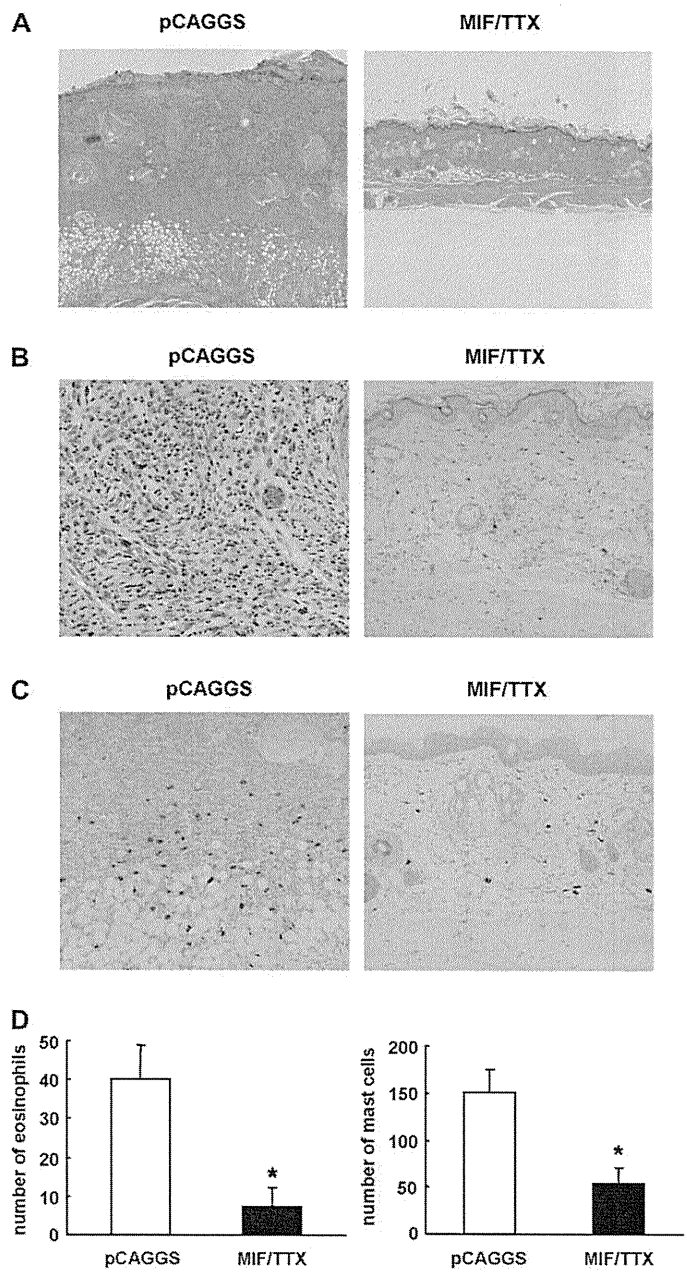

FIG 2. Histologic analysis of AD in DS-Nh mice vaccinated with MIF/TTX or pCAGGS plasmid before disease onset. Nine-week-old DS-Nh mice without eruptions were administered MIF/TTX or control plasmid. Specimens were collected from the dorsal skin 6 weeks after the first vaccination and stained with hematoxylin and eosin (**A**, original magnification ×40), direct fast scarlet for eosinophils (**B**, original magnification ×200), or toluidine blue for mast cells (**C**, original magnification ×200). **D**, The number of eosinophils and mast cells in 5 high-power fields from 4 individual skin specimens were enumerated by means of microscopy. Means ± SEs of 4 mice are shown. *$P < .001$ for MIF/TTX versus pCAGGS.

with increased serum IgE levels, eosinophilia, and a marked infiltration of T_H2 cells into acute skin lesions. The infiltrating T cells show a predominance of IL-4, IL-5, IL-10, and IL-13 expression.[12,18] In patients with chronic AD, however, there is infiltration of eosinophils and macrophages, and the disease becomes associated with an increase in the expression of IL-12, with a switch to T_H1 cellular responses.[12,18] Chronic AD skin lesions in adults with a prolonged duration of disease have been shown to manifest an increase in the expression of IL-1, IL-5, IL-12, IFN-γ, TNF-α, GM-CSF, and MIF.[12,18] This biphasic T_H1/T_H2 switch in immune response is characterized pathologically by lichenification, epidermal hyperplasia, and dermal fibrosis. MIF regulates the production of various proinflammatory cytokines, including TNF-α, and the inflammatory cytokines in response to stimulation by LPS are known to be suppressed in MIF-deficient mice. We previously reported that MIF-deficient mice have an impaired contact hypersensitivity (CH) response and that immunoneutralization of MIF effectively suppresses CH response[19]; these observations led us to speculate that MIF would be a therapeutic target for AD.

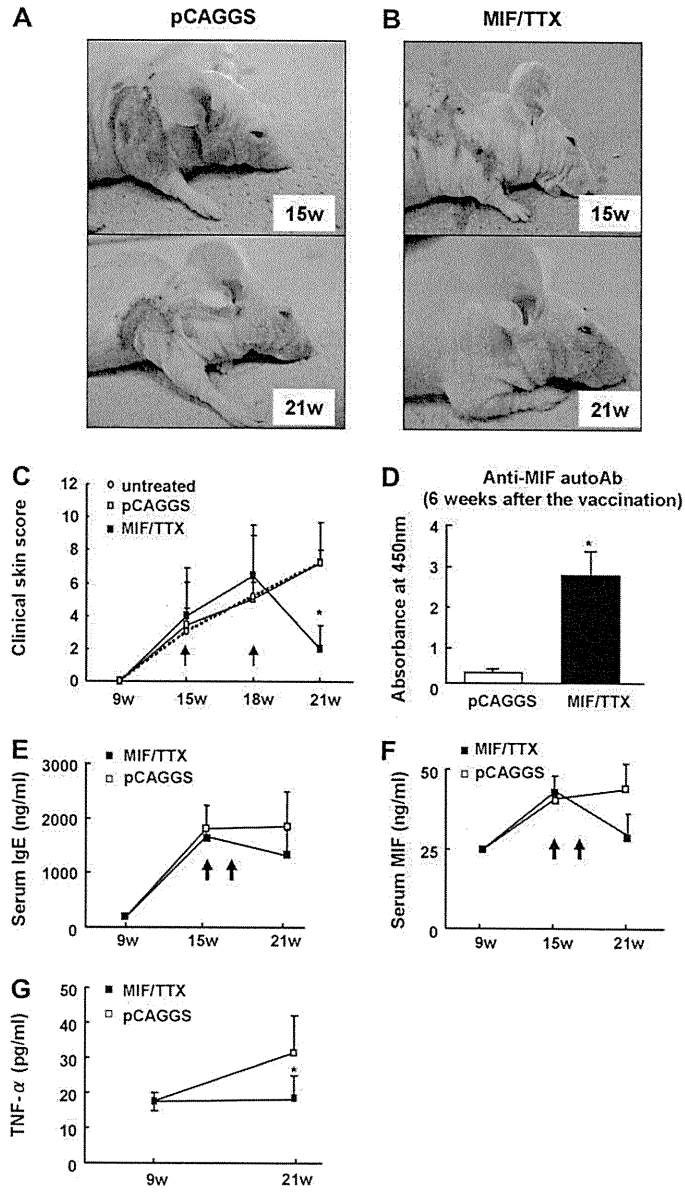

FIG 3. Therapeutic effect of MIF-DNA vaccination in DS-Nh mice with pre-existing AD. Fifteen-week-old DS-Nh mice with ongoing dermatitis were administered MIF/TTX or control plasmid (pCAGGS) or left untreated. **A**, Clinical features of DS-Nh mice vaccinated with control plasmid. **B**, Clinical features of DS-Nh mice vaccinated with MIF/TTX. **C**, Clinical skin scores of mice immunized with MIF/TTX-DNA vaccine *(solid squares)*, control plasmid *(open squares)*, or left untreated *(open circles)*. Means ± SEs of 10 mice per group are shown. $*P < .005$ for MIF/TTX versus pCAGGS. **D**, Serum level of anti-MIF autoantibodies *(autoAb)* at 6 weeks after the vaccination. Means ± SEs are shown (n = 10). $*P < .001$. Serum IgE **(E)** and MIF **(F)** levels of the mice vaccinated with MIF/TTX vaccine *(solid squares)* and control pCAGGS plasmid *(open squares)* are shown. The data shown are for 10 mice per group. **G**, The serum levels of TNF-α were decreased in MIF/TTX-vaccinated mice *(solid squares)* compared with those seen in the control (pCAGGS) plasmid–vaccinated mice *(open squares)*. $*P < .01$ for MIF/TTX versus pCAGGS.

The therapeutic aim of cytokine vaccine therapy is to induce high titers of circulating polyclonal autoantibodies to neutralize the pathologic levels of a particular cytokine. The advantages of this therapy include the potential to maintain high antibody titers, long-term efficacy, and low cost. Monoclonal antibodies directed against TNF-α have been used for the treatment of psoriasis.[10] Jacobi et al[7] recently reported a clinical trial of infliximab monotherapy for 9 patients with moderate or severe AD who showed significant improvement in all clinical parameters; however, this improvement was not sustained by maintenance of the therapy. The authors considered that the development of antichimeric antibodies could explain the lack of a durable response to infliximab maintenance therapy. A cytokine vaccine results in the production of native antibodies, and it might overcome this limitation in anti-cytokine antibody therapy.

It is unknown whether long-term inhibition of MIF activity might be safe in human subjects. A major limitation of an active immunization approach is the inability to control the outcome. However, it should be noted that serum MIF levels in the MIF-DNA–vaccinated mice were maintained at a baseline level

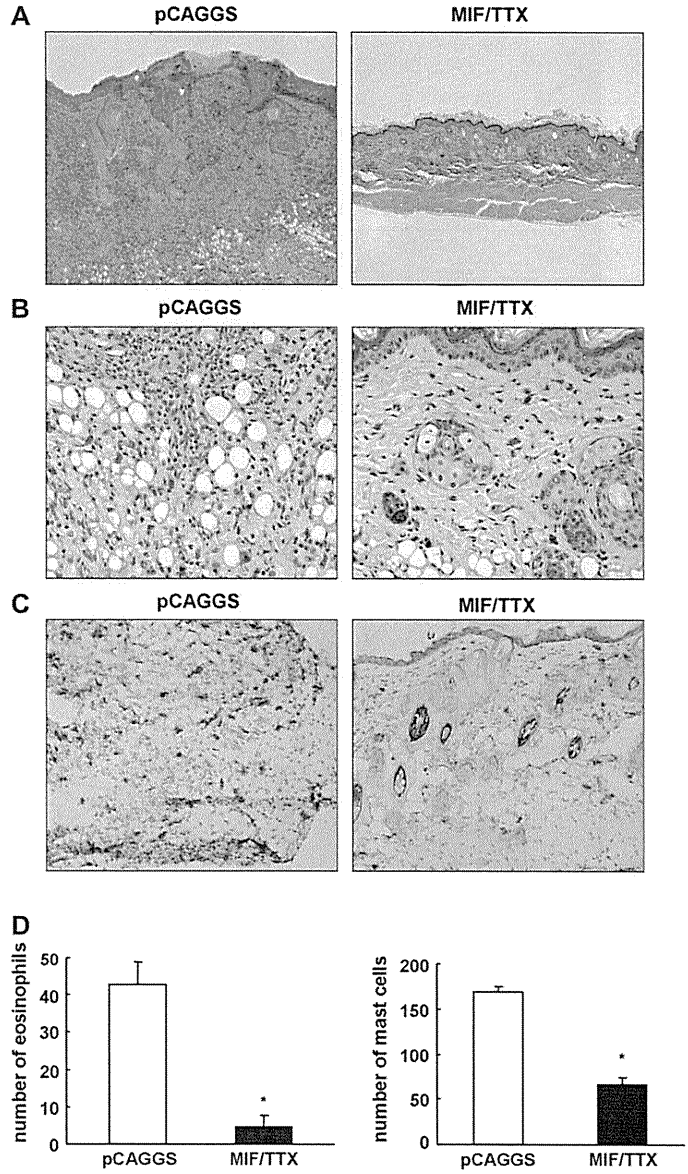

FIG 4. Histologic analysis of AD in DS-Nh mice vaccinated with MIF/TTX or control plasmid after disease onset. Fifteen-week-old DS-Nh mice with ongoing dermatitis were administered MIF/TTX or control (pCAGGS) plasmid. Specimens were collected at 6 weeks after the first vaccination and stained with hematoxylin and eosin (**A**, original magnification ×40), direct fast scarlet for eosinophils (**B**, original magnification ×200), or toluidine blue for mast cells (**C**, original magnification ×200). **D**, The number of eosinophils and mast cells in 5 high-power fields from 4 individual skin specimens were enumerated by means of microscopy. Data represent means ± SEs of 4 mice. **P* < .001 for MIF/TTX versus pCAGGS.

(Figs 2, *F*, and 4, *F*), despite an anti-MIF antibody level that remained high for 6 weeks of vaccine administration. It is possible that the present protocol of vaccination dose not induce a high enough level of anti-MIF antibody to inhibit serum MIF protein completely. There are reports that autoantibody production induced by vaccine-encoded antigens regress to baseline levels shortly after remission in acute experimental autoimmune encephalomyelitis,[15] whereas in adjuvant-induced arthritis[16] autoantibodies continue to be produced at high titer. It has been considered that targeted DNA vaccines amplify a pre-existing anti–self-regulatory response that by itself is capable of limiting, although not preventing, the emerging autoimmune condition.[15-17,20-22] In addition, we observed that MIF-DNA–vaccinated mice did not show serious side effects, such as evident infections.

Serum IgE levels were significantly decreased when MIF vaccination was used as a preventive agent, whereas the levels were not significantly decreased when it was used as a therapeutic agent. It is known, however, that IgE levels do not parallel the clinical severity of AD in human patients.[19]

The cardinal principles in the treatment of AD are to reduce symptoms, prevent exacerbations, and minimize medication side effects. This approach incorporates the use of emollients, topical corticosteroids, topical calcineurin inhibitors, antihistamines, stress management, and avoidance of allergens or

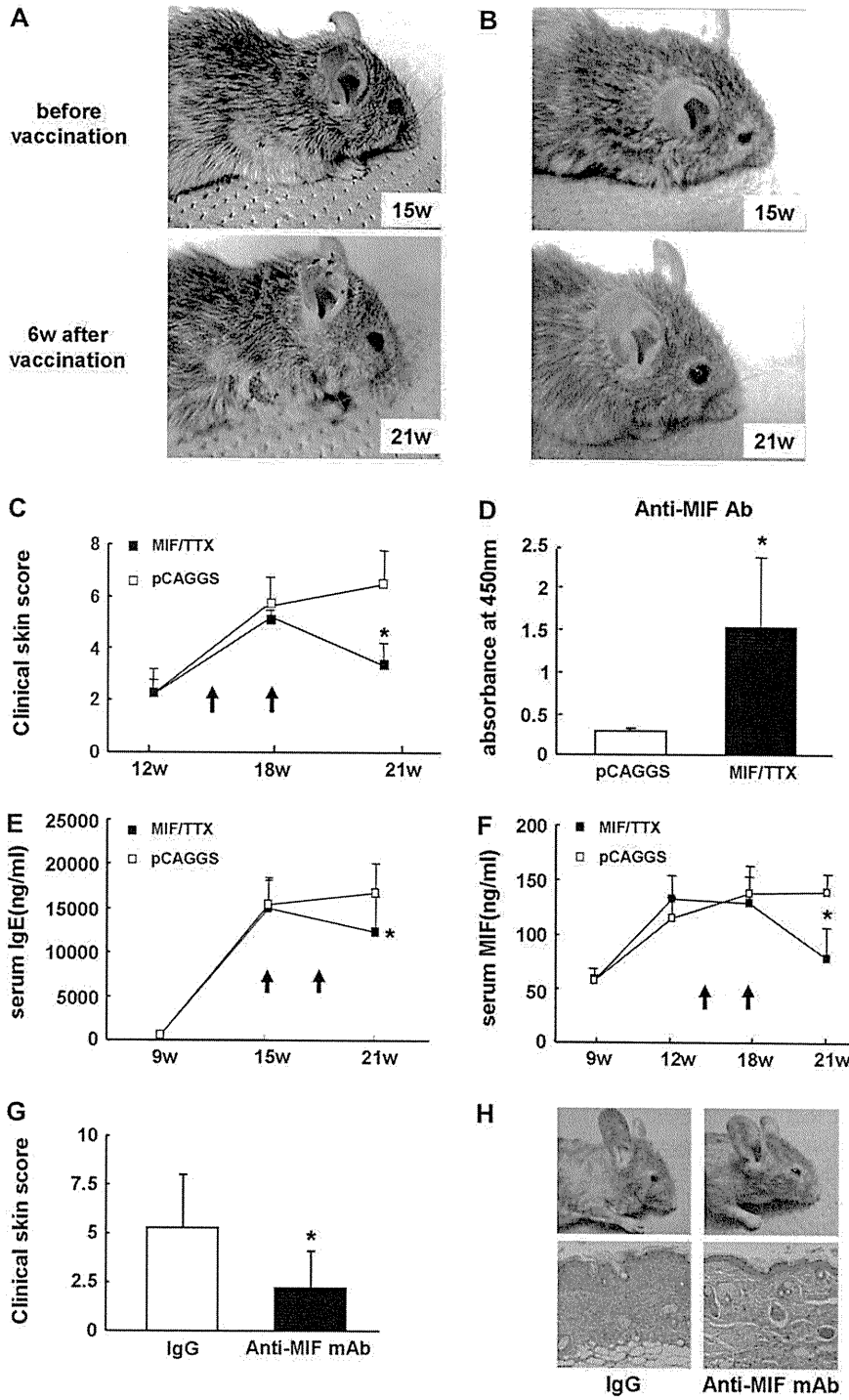

FIG 5. Therapeutic effect of MIF/TTX-DNA in vaccine for pre-existing AD on NC/Nga mice. Fifteen-week-old NC/Nga mice with dermatitis were vaccinated with control pCAGGS plasmid or MIF/TTX. **A**, Clinical features of NC/Nga mice vaccinated with control pCAGGS plasmid. **B**, Clinical features of DS-Nh mice vaccinated with MIF/TTX (*top*, vaccination). **C**, Clinical skin scores of mice vaccinated with MIF/TTX *(solid squares)* and control pCAGGS plasmid *(open squares)*. $*P < .05$ for MIF/TTX versus pCAGGS at the same time point. Each point represents means ± SEs of 10 mice in each group. **D**, Serum level of anti-MIF autoantibodies *(Ab)* at 6 weeks after the vaccination. Means ± SEs are shown (n = 10). $*P < .005$. Serum IgE **(E)** and MIF **(F)** levels of mice vaccinated with MIF/TTX vaccine *(solid squares)* and control pCAGGS plasmid *(open squares)* are shown. Means ± SEs of 10 mice in each group are shown. $*P < .0005$. **G** and **H**, Neutralizing anti-MIF mAbs (50 μg) or control IgG (50 μg) were injected intravenously into 15-week-old NC/Nga mice with dermatitis twice a week for 3 weeks. Fig 5, *G*, shows clinical skin scores ($*P < .05$). In Fig 5, *H*, the *upper panels* show clinical features, and the *lower panels* show histologic images.

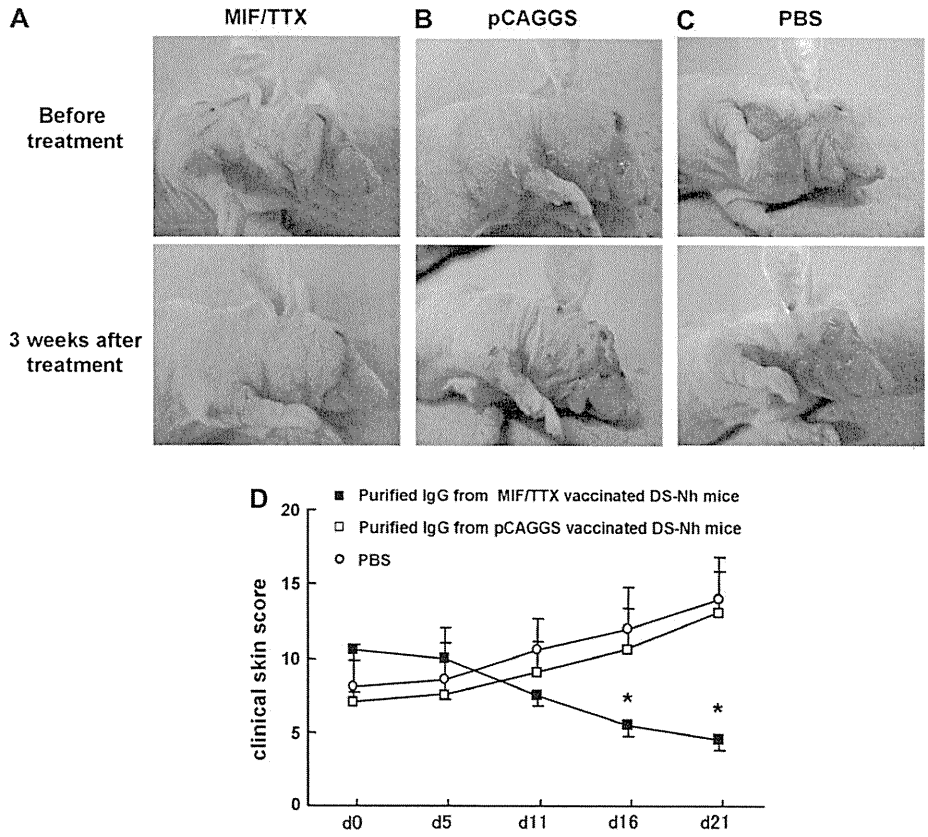

FIG 6. Adoptive transfer of autoantibodies elicited by DNA vaccines. IgG were purified from the sera of control (pCAGGS) plasmid– or MIF/TTX-vaccinated DS mice and adoptively transferred. DS-Nh mice were administered 50 μg per mouse of purified IgG from control plasmid–vaccinated DS-Nh mice, purified IgG from MIF/TTX-vaccinated DS-Nh mice, or PBS. **A-C**, Clinical features at 3 weeks after administration of purified IgG from MIF/TTX-vaccinated mice (Fig 6, *A*), purified IgG from control pCAGGS plasmid–vaccinated mice (Fig 6, *B*), and clinical features after administration of PBS (Fig 6, *C*). **D**, Clinical skin scores of DS-Nh mice administered IgG from MIF/TTX-vaccinated DS-Nh mice *(solid squares)*, IgG from control pCAGGS plasmid–vaccinated DS-Nh mice *(open squares)*, or PBS *(open circles)*. Results are shown as means ± SEs of 3 mice in each group. $*P < .05$ versus pCAGGS at the same time point.

disease triggers.[3] The fact that the treatment of AD mainly depends on the self-application of topical agents often hinders the effective and long-term treatment of the disease. Notwithstanding these conventional treatments, if a patient with severe and refractory AD requires additional therapy, cyclosporine A has been used despite systemic side effects, such as renal toxicity.[23,24] The relapsing and remitting course of AD also places a psychologic, social, and financial burden on patients and their families. New treatment options are needed to prevent the progression of AD to more severe forms of disease and to halt the so-called atopic march toward asthma. An MIF-DNA vaccine approach offers the additional advantage of requiring only a periodic booster injection, and it might allow for the potential resolution of immunopathology in those with chronic refractory disease.

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Clinical implications: MIF-DNA vaccination might be a useful preventive and therapeutic approach for AD.

REFERENCES

1. Jung T, Stingl G. Atopic dermatitis: therapeutic concepts evolving from new pathophysiologic insights. J Allergy Clin Immunol 2008;122:1074-81.
2. Morar N, Willis-Owen SAG, Moffatt MF, Cookson WOCM. The genetics of atopic dermatitis. J Allergy Clin Immunol 2006;118:24-34.
3. Leung DY, Boguniewicz M, Howell MD, Nomura I, Hamid QA. New insights into atopic dermatitis. J Clin Invest 2004;113:651-7.
4. Shimizu T. Role of macrophage migration inhibitory factor (MIF) in the skin. J Dermatol Sci 2005;37:65-73.
5. Shimizu T, Abe R, Ohkawara A, Mizue Y, Nishihira J. Macrophage migration inhibitory factor is an essential immunoregulatory cytokine in atopic dermatitis. Biochem Biophys Res Commun 1997;240:173-8.
6. Shimizu T, Abe R, Ohkawara A, Nishihira J. Increased production of macrophage migration inhibitory factor by PBMCs of atopic dermatitis. J Allergy Clin Immunol 1999;104:659-64.
7. Jacobi A, Antoni C, Manger B, Schuler G, Hertl M. Infliximab in the treatment of moderate to severe atopic dermatitis. J Am Acad Dermatol 2005;52:522-6.
8. Cassano N, Loconsole F, Coviello C, Vena GA. Infliximab in recalcitrant severe atopic eczema associated with contact allergy. Int J Immunopathol Pharmacol 2006;19:237-40.
9. Onodera S, Ohshima S, Tohyama H, Yasuda K, Nishihira J, Iwakura Y, et al. A novel DNA vaccine targeting macrophage migration inhibitory factor protects joints from inflammation and destruction in murine models of arthritis. Arthritis Rheum 2007;56:521-30.
10. Tohyama S, Onodera S, Tohyama H, Yasuda K, Nishihira J, Mizue Y, et al. A novel DNA vaccine-targeting macrophage migration inhibitory factor improves the survival of mice with sepsis. Gene Ther 2008;15:1513-22.

11. Matsumoto M, Ra C, Kawamoto K, Sato H, Itakura A, Sawada J, et al. IgE Hyperproduction through enhanced tyrosine phosphorylation of Janus kinase 3 in NC/Nga Mice, a model for human atopic dermatitis. J Immunol 1999;162:1056-63.
12. Leung DYM, Bieber T. Atopic dermatitis. Lancet 2003;361:151-60.
13. Matsuda H, Watanabe N, Geba GP, Sperl J, Tsudzuki M, Hiroi J, et al. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. Int Immunol 1997;9:461-6.
14. Calandra T, Echtenacher B, Roy DL, Pugin J, Metz CN, Hultner L, et al. Protection from septic shock by neutralization of macrophage migration inhibitory factor. Nat Med 2000;6:164-70.
15. Wildbaum G, Netzer N, Karin N. Plasmid DNA encoding IFN-gamma-inducible protein 10 redirects antigen-specific T cell polarization and suppresses experimental autoimmune encephalomyelitis. J Immunol 2002;168:5885-92.
16. Salomon I, Netzer N, Wildbaum G, Schif-Zuck S, Maor G, Karin N. Targeting the function of IFN-γ-inducible protein 10 suppresses ongoing adjuvant arthritis. J Immunol 2002;169:2685-93.
17. Youssef S, Maor G, Wildbaum G, Grabie N, Gour-Lavie A, Karin N. C-C chemokine-encoding DNA vaccines enhance breakdown of tolerance to their gene products and treat ongoing adjuvant arthritis. J Clin Invest 2000;106:361-71.
18. Leung DYM. Atopic dermatitis: new insights and opportunities for therapeutic intervention. J Allergy Clin Immunol 2000;105:860-76.
19. Abramovits W. Atopic dermatitis. J Am Acad Dermatol 2005;53(suppl):S86-93.
20. Wildbaum G, Youssef S, Karin N. A targeted DNA vaccine augments the natural immune response to self TNF-alpha and suppresses ongoing adjuvant arthritis. J Immunol 2000;165:5860-6.
21. Wildbaum G, Karin N. Augmentation of natural immunity to a pro-inflammatory cytokine (TNF-alpha) by targeted DNA vaccine confers long-lasting resistance to experimental autoimmune encephalomyelitis. Gene Ther 1999;6:1128-38.
22. Youssef S, Wildbaum G, Maor G, Lanir N, Gour-Lavie A, Grabie N, et al. Long-lasting protective immunity to experimental autoimmune encephalomyelitis following vaccination with naked DNA encoding C-C chemokines. J Immunol 1998;161:3870-9.
23. Berth-Jones J, Finlay AY, Zaki I, Tan B, Goodyear H, Lewis-Jones S, et al. Cyclosporine in severe childhood atopic dermatitis: a multicenter study. J Am Acad Dermatol 1996;34:1016-21.
24. Berth-Jones J, Graham-Brown RA, Marks R, Camp RD, English JS, Freeman K, et al. Long-term efficacy and safety of cyclosporin in severe adult atopic dermatitis. Br J Dermatol 1997;136:76-81.