

Impaired Interferon- γ Production in a Subset Population of Severe Atopic Dermatitis

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Key Words

Atopic dermatitis, severe · Interferon- γ · Th1 cytokines · Th2 cytokines

Abstract

Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritic chronic eczema. The immunopathogenesis of this condition is still not well understood. We assessed the transcription and production of IFN- γ , the Th1 cytokine, and the Th2 cytokine IL-5 in peripheral blood mononuclear cells (PBMCs) from patients with severe AD. **Methods:** The subjects included 17 severe (serum IgE: 5,000–92,000 U/ml, median: 20,000 U/ml), 4 mild AD (IgE: 2–520 U/ml) and 8 nonatopic controls (IgE: <100 U/ml). The severe AD patients were classified into two groups according to the response to standard treatment with topical glucocorticoids. Individuals were classified as poorly responsive (AD-P) if the clinical score decreased less than one third after 2 weeks of hospital treatment and as responsive (AD-R) if the score decreased more than one third. PBMCs isolated from the subjects were stimulated with PHA and PMA. **Results:** The expression of IFN- γ in PBMCs in the AD-P group was much lower than that

observed in the other groups at both mRNA and protein levels. There were no significant differences in the levels of IL-5 both in mRNA and protein levels between the groups. There were no significant differences in STAT4 DNA-binding activity following PHA/IL-2/IL-12 stimulation between AD-P and controls. **Conclusions:** These results suggest that the decreased INF- γ production may account for the abnormal immunopathogenesis of severe, intractable AD.

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by eczematous skin lesions with pruritus. It is frequently seen in patients with a personal or family history of atopy [1, 2]; recent studies suggest that there is an increase in the incidence of AD [3–6]. AD is frequently associated with highly elevated serum IgE levels, which appears to be an underlying mechanism of immunopathogenesis of AD [7, 8]. Although the role of cytokines in the pathogenesis of AD or in the increased IgE has yet to be confirmed, strong evidence for such roles has been suggested.

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Table 1. Clinical features of the subjects

Patients	Sex	Age years	Total IgE U/ml	Eosinophils /μl	DAS	Ext.	Responsibility	
							DAS	Ext.
P-1	M	37	10,800	1,080	55	80	◆	◆
P-2	M	20	15,500	1,120	55	65	◆	◆
P-3	F	10	18,300	1,750	60	76	○	◆
P-4	M	24	23,700	3,090	72	81	◆	◆
P-5	M	7	44,700	1,390	41	60	○	◆
P-6	M	21	71,200	5,830	84	96	◆	◆
P-7	M	15	92,000	11,830	81	96	◆	◆
R-1	M	17	5,800	700	55	50	○	○
R-2	F	12	7,600	395	61	62	○	○
R-3	M	2	10,900	1,230	32	30	○	○
R-4	M	15	16,900	1,290	71	76	○	○
R-5	M	6	18,300	2,260	54	51	○	○
R-6	F	22	20,400	559	54	84	○	○
R-7	M	15	20,700	1,270	74	66	○	○
R-8	M	12	20,900	123	82	96	○	○
R-9	M	5	30,300	1,380	52	51	○	○
R-10	M	14	44,600	4,380	84	95	○	○
M-1	M	28	2		4	6		
M-2	F	25	48		9	10		
M-3	F	23	420		11	6		
M-4	M	25	520		4	6		
C-1	F	30	2					
C-2	F	23	9					
C-3	M	23	18					
C-4	F	22	30					
C-5	M	22	46					
C-6	M	27	72					
C-7	M	36	74					
C-8	F	23	13					

P = Poorly responding AD; R = responding AD; M = mild AD; C = control; DAS = disease activity score; Ext. = extent of skin involvement (%); ◆ = poor response; ○ = good response.

Reduced IFN- γ production in peripheral blood mononuclear cells (PBMC) from AD patients has been reported [9–15]. Whether the reduction of IFN- γ relates to an impaired production of IFN- γ per cell [9], or to a decreased number of IFN- γ -producing cells [11], or both [15] is still subject to debate. In contrast, increased IFN- γ mRNA expression has been shown in PBMC [16] and in the affected skin from AD patients [17]. Th2-like cytokines, such as IL-4 and IL-5, have also been implicated in the pathogenesis of acute and chronic AD [18, 19].

Clinicians often encounter severe AD patients who are extremely refractory to standard treatments including topical glucocorticoids and skin care. We investigated the relationship of Th1/Th2 cytokines in intractable severe AD patients compared with mild AD and nonatopic controls. In the current study, we assessed the transcription

and production of IFN- γ (the Th1 cytokine) and IL-5 (the Th2 cytokine) in PBMCs from severe AD patients after stimulation with phytohemagglutinin (PHA) and phorbol myristate acetate (PMA).

Materials and Methods

Subjects

Heparinized peripheral blood was obtained from 17 severe AD patients (mean age 15.0, range 2–37), 4 mild AD patients (mean age 25.8, range 25–28) and 8 healthy nonatopic control subjects (mean age 27.4, range 22–36). The use of topical glucocorticoid was stopped for at least 12 h prior to blood sampling. Patients with AD were diagnosed according to the criteria of Hanifin [20]. The details of the clinical features of the subjects are summarized in table 1. AD severity was scored on the basis of the modified Leicester system [21]. The extent of skin involvement was estimated using the rule of nine. The

disease activity score was defined as the sum of the individual scores at 6 body sites (head, trunk, elbow, hand, knee, and foot, graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe), for each of five parameters (erythema, edema/papulation, oozing/crusting, excoriation, and lichenification). This gave a score with a maximum of 90 points. Evaluation of the grading for AD was done by doctors who were blind to subjects' information on the basis of periodically taken photographs. The severe AD patients were classified into two groups according to their responses to the standard treatment with topical glucocorticoids. This consisted of an ointment with a topically mild potency for the face (hydrocortisone acetate is mainly used) and with a moderate one for the body (dexamethasone valerate is mainly used). Particular attention was also given to general skin care, which included bathing 3 times a day followed by hydration. Individuals were classified in the poorly responding group (AD-P, $n = 7$) if the clinical score had decreased by less than one third after 2 weeks of treatment and in the responding group (AD-R, $n = 10$) if the score had decreased by more than one third (table 1). Patients with the hyper-IgE syndrome were excluded. None of the patients had undergone treatment with systemic glucocorticoids. Informed consent was obtained from all the subjects and/or their parents.

Cells and Cell Culture

Blood sampling was performed after AD treatment. PBMCs were isolated by LCM[®] lymphocyte separation medium gradient centrifugation (ICN/Cappel, Ohio, USA), washed 3 times in phosphate-buffered saline and resuspended at 1.5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were stimulated with 5 μ g PHA and 10 ng PMA for 24 h or with IL-12 (10 ng/ml) for 48 h in 24-well plates [33]. Supernatants were harvested at 24 h, and cells were isolated by brief centrifugation and then stored in 1 ml of mixture of phenol and guanidinium isothiocyanate (Isogen[®]; Nippon Gene, Toyama, Japan) at -80°C until further processing.

Cytokines Assay

IFN- γ , IL-2, and IL-5 levels were measured using the human cytokine ELISA kit (Techne, Minn., USA; R&D Systems, Minn., USA; Amersham Life Science, Buckinghamshire, UK, respectively) with two monoclonal antibodies directed to human IFN- γ , IL-2, and IL-5 according to the manufacturers' instruction. Sensitivity of the assay was 3, 6 and 3 pg/ml, respectively. All samples were run in duplicate.

Real-Time Quantitative RT-PCR

For quantification of gene expression for cytokines, the real-time quantitative RT-PCR method was performed using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, USA). DNase-treated total RNAs were mixed with oligo (dT)₁₂₋₁₈ primer (Life Technologies, USA), and first-strand cDNAs were synthesized using Super Script II reverse transcriptase as described in the manufacturer's instruction. In each reaction mixture, 1 μ g of acetylated bovine serum albumin (Gibco BRL) was added to improve reaction efficiency [22]. Based on the band sequence, primers and a dual-labeled fluorogenic probe (TaqMan Probe[®]) were designed using the computer program Primer Express 1.0 (PE Applied Biosystems, USA). The quantitative PCR reaction was performed using the TaqMan PCR Reagent Kit according to the manufacturer's protocol (PE Applied Biosystems, USA). Sample cDNAs equivalent to 5–10 μ g of starting RNAs were examined for each reaction in a 96-well PCR plate. The plasmid DNA carrying a target sequence was used to prepare absolute standards. Levels of β -actin mRNA were measured for

each sample, and used as internal standard. The average copy number of β -actin in all samples was calculated, and a relative β -actin ratio to the average was determined for each sample. The copy number of the target sequence in each sample was normalized by dividing this value by the value obtained for β -actin. Primer sequences for the internal control, β -actin, were 5'-TCACCCACACTGTGCCCATCTACGA-3' for the upstream primer, 5'-CAGCGGAACCGCTCATTGCCAATGG-3' for the downstream primer, and 5'-ATGCCCCCCCCATGCATCC-3' for the TaqMan probe. Primer sequences for IFN- γ were 5'-TCCAACGCAAAGCAATACATG-3' for the upstream primer, 5'-CGCTTCCCTGTTTTAGCTGC-3' for the downstream primer, and 5'-TCCAAGTGATGGCTGAACTGTGCGCC-3' for the TaqMan probe. Primer sequences for IL-5 were 5'-GAGGATGCTTCTGCA-TTTGAG-3' for the upstream primer, 5'-CGAACTCTGCTGATAGCCAATG-3' for the downstream primer, and 5'-CCCACAAGTG-CATTGGTGAAAGAGAC-3' for the TaqMan probe.

Flow Cytometry

PBMCs, seeded at 3×10^6 /ml in RPMI 1640 supplemented with 10% fetal calf serum, were stimulated with 5 μ g/ml of PHA. After 48 h, purified cells were stained with 1 μ g anti-CD3-FITC and anti-IL-12 receptor-PE monoclonal antibodies specific for $\beta 1$ subunit (Fujisawa PharMingen, Tokyo, Japan) in PBS on ice for 30 min, and washed. The IL-12 receptor expression was analyzed using FACScan (Becton Dickinson), gating on CD3-positive events.

Electrophoretic Mobility Shift Assay

To induce IL-12 responsiveness, PBMCs were cultured for 3 days in RPMI 1640 medium/10% fetal calf serum/PHA at 2 μ g/ml (Sigma), diluted 1:1 with fresh medium, and then cultured for an additional day with IL-2 at 10 ng/ml [23], followed by the addition of IL-12 at 10 ng/ml for 15 min. Nuclear proteins were extracted and electrophoretic mobility shift assays were done using a ³²P-end-labeled double-stranded oligonucleotide (5'-GAGCCTGATTTC-CCCAGAAATGATGAGCTAG-3') corresponding to the binding site for STAT4 (Santa Cruz Biotechnology, Calif., USA). Briefly, nuclear protein extracts were incubated with a labeled probe in binding buffer [4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.05 mg/ml poly(dI-dC)-poly(dI-dC)] for 15 min at 4°C before electrophoresis on 6% polyacrylamide gels and autoradiography. Competitive inhibition was performed by incubating a 100-fold excess of unlabeled STAT4 consensus oligonucleotide.

Statistics

A two-factorial ANOVA with repeated measurement was used to determine the significance of data using the computer program SPSS 10.0J (SPSS Japan).

Results

Reduced IFN- γ Secretion by PBMCs in the AD-P Group

As shown in figure 1a, the mean PHA/PMA-induced IFN- γ secretion by PBMCs from the AD-P group (mean $\pm$ SE = 23.4 ± 21.8 pg/ml) was much lower than that observed in the AD-R group (492 ± 151 pg/ml; $p <$

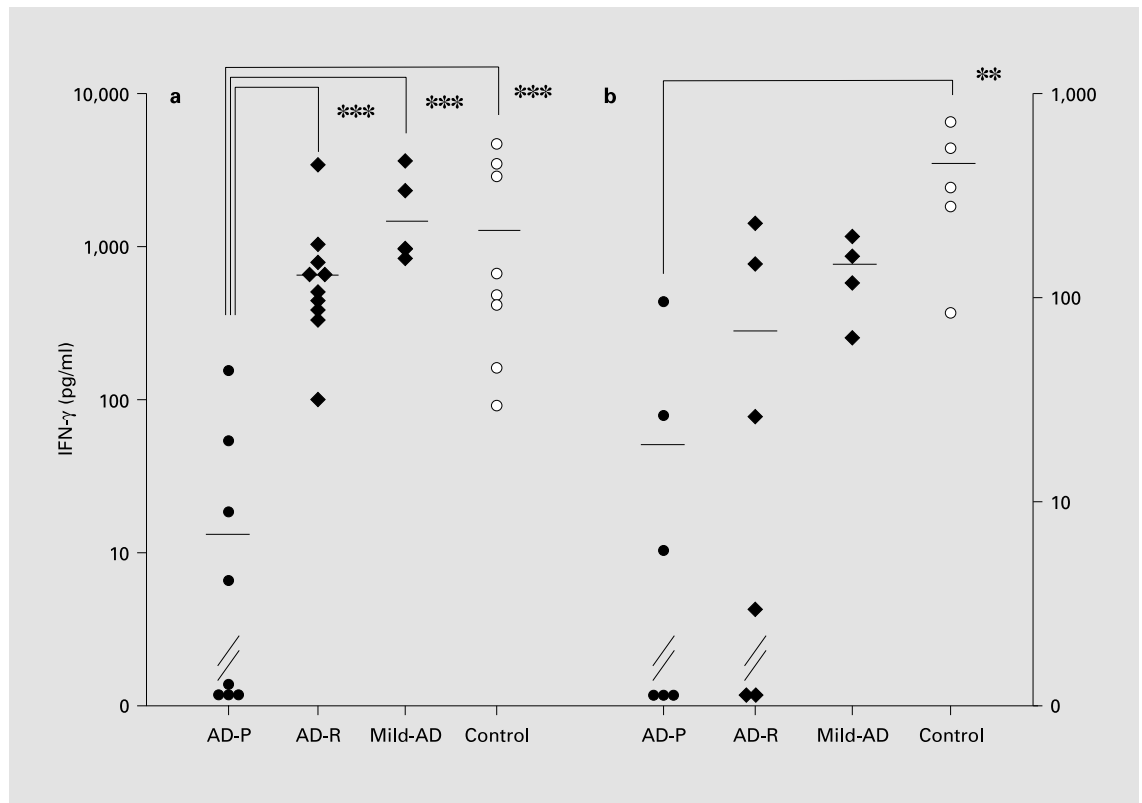


Fig. 1. **a** PHA/PMA-induced IFN- γ secretion by PBMCs from the AD-P group was much lower than that observed in the AD-R group ($p < 0.001$), the mild AD group ($p < 0.001$), and in the nonatopic controls ($p < 0.001$). **b** The IL-12-induced IFN- γ secretion by PBMCs from the AD-P group was also much less than that from controls ($p < 0.01$). ** $p < 0.01$; *** $p < 0.001$.

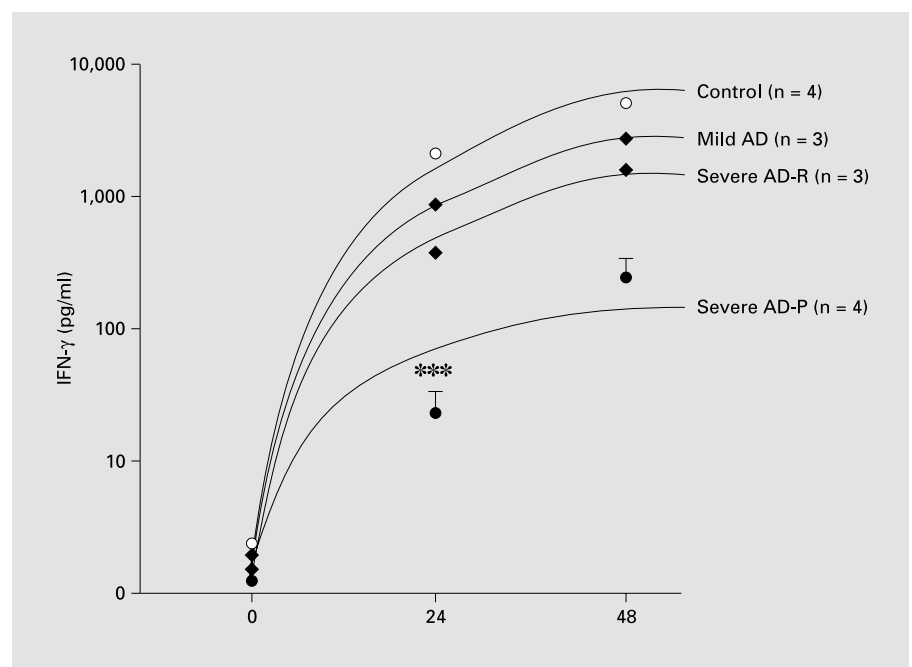


Fig. 2. Time course of PHA/PMA-induced IFN- γ secretion by PBMCs from AD-P. At any time point, the secretion was lower than that observed in the other groups. *** $p < 0.001$.

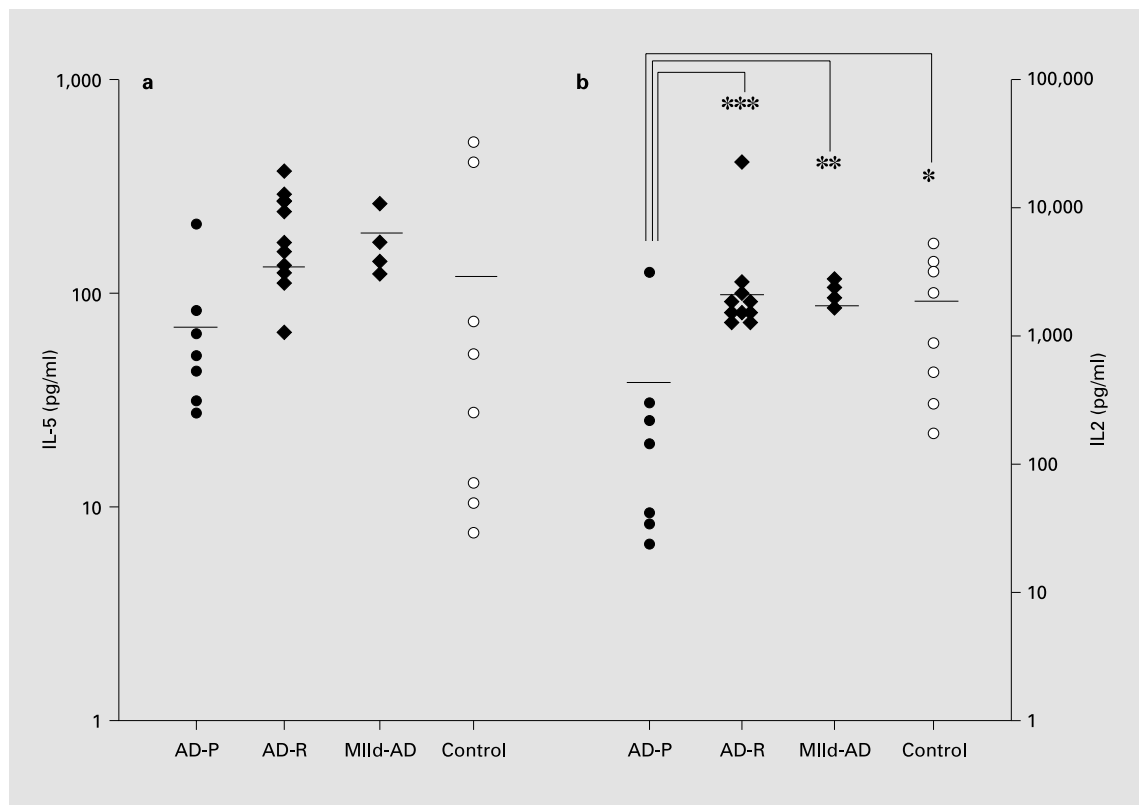


Fig. 3. **a** There were no significant differences in IL-5 levels secreted by PBMCs between AD-P, AD-R, mild AD, or controls. **b** IL-2 secretion by PBMCs in the AD-P group was lower than that seen in AD-R, mild AD, or in control groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

0.001), the mild AD group ($1,249 \pm 302$ pg/ml; $p < 0.001$), and in the nonatopic controls (883 ± 302 pg/ml; $p < 0.001$). Even when PBMCs were stimulated with PHA/PMA for a longer period, IFN- γ secretion by PBMCs from AD-P was much lower than that observed in controls (fig. 2). Furthermore, when PBMCs were stimulated with IL-12, those from AD-P produced significantly less IFN- γ secretion (28.8 ± 22.7 pg/ml) than those in the nonatopic controls (584 ± 150 pg/ml; $p < 0.01$) (fig. 1b).

As shown in figure 3a, there were no significant differences in IL-5 levels secreted by PBMCs between AD-P (mean $\pm$ SE = 77.6 ± 24.2 pg/ml), AD-R (167 ± 23.4 pg/ml; $p > 0.1$), mild AD (155 ± 45.8 pg/ml; $p > 0.1$), or controls (109 ± 45.4 pg/ml; $p > 0.1$). IL-2 secretion by PBMCs in the AD-P group (mean $\pm$ SE = 570 ± 484 pg/ml) was lower than that seen in the AD-R ($3,119 \pm 940$ pg/ml; $p < 0.001$), mild AD ($2,400 \pm 209$ pg/ml; $p < 0.01$), or control groups ($2,439 \pm 1,000$ pg/ml; $p < 0.05$) (fig. 3b).

Decreased Expression of IFN- γ mRNA in the AD-P Group

As shown in figure 4a, the abundance of IFN- γ mRNA in the AD-P group (mean $\pm$ SE = 50 ± 36 copies/ng RNA) was much less than that observed in AD-R ($2,944 \pm 1,157$ copies/ng RNA; $p < 0.001$), mild AD ($2,188 \pm 500$ copies/ng RNA; $p < 0.001$), or nonatopic control groups ($7,236 \pm 1,519$ copies/ng RNA; $p < 0.001$). There were no significant differences in the expression of IL-5 mRNA between AD-P (264 ± 71 copies/ng RNA), AD-R (275 ± 78 copies/ng RNA), mild AD (542 ± 455 copies/ng RNA), and control groups ($2,912 \pm 1,817$ copies/ng RNA) (fig. 4b).

IL-12 Receptor Expression on CD3+ Cells from AD-P

PBMCs obtained from 4 of the 7 AD-P and 4 controls were tested for IL-12 receptor expression after PHA stimulation. IL-12 receptor-expressing CD3+ cells in AD-P amounted to $39.9 \pm 9.4\%$ (mean $\pm$ SE), while in controls it amounted to $60.5 \pm 11.4\%$ ($p > 0.1$), suggesting that

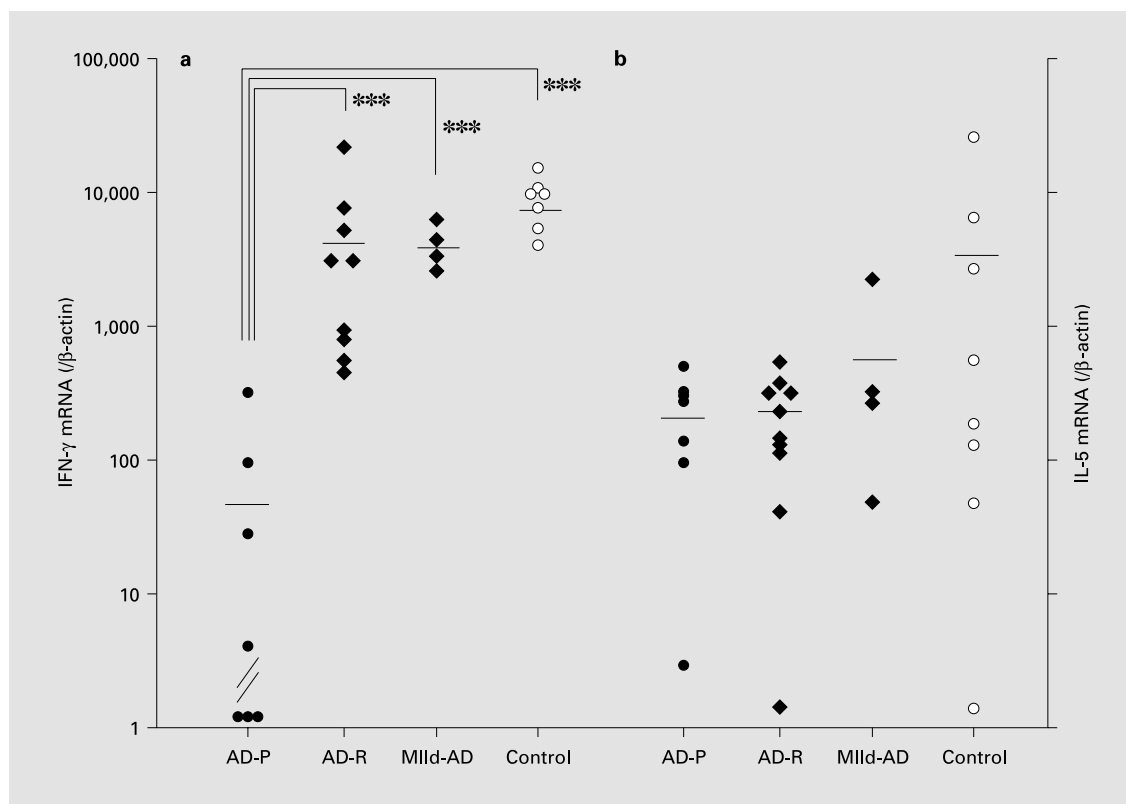
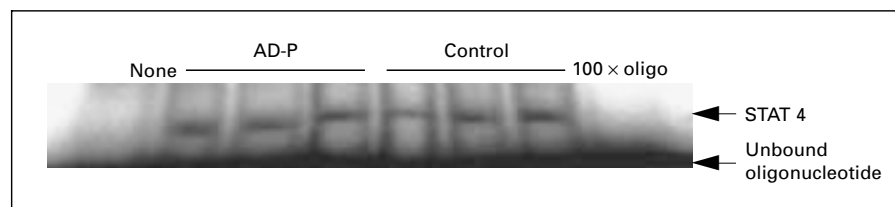


Fig. 4. **a** The abundance of IFN- γ mRNA in the AD-P group was much less than that observed in AD-R ($p < 0.001$), mild AD ($p < 0.001$), or nonatopic control groups ($p < 0.001$). **b** There were no significant differences in the expression of IL-5 mRNA between AD-P, AD-R, mild AD, and control groups. *** $p < 0.001$.

Fig. 5. There were no significant differences in STAT4 binding activity at the consensus oligonucleotide following the stimulation with PHA/IL-2 and IL-12 between AD-P and controls.



there were no significant differences in the number of Th1 cells after stimulation between AD-P and controls.

STAT4/Consensus Oligonucleotide Binding

As shown in figure 5, there were no significant differences in STAT4 binding activity at the consensus oligonucleotide following the stimulation with PHA/IL-2 and IL-12 between AD-P (patients 1, 3, 7) and controls.

Discussion

Very severe AD cases who are significantly resistant to standard AD treatment with topical glucocorticoids can be observed by clinicians. To clarify the underlying immunological mechanisms in the patients with severe AD, we investigated the transcription and production of IFN- γ and IL-5 in PBMCs from severe AD patients resistant to treatment. Although each patient received identical treatment, they could be classified according to their response to treatment during a 2-week admission (table 1). These

were the AD-P and AD-R group. They are not 'steroid-resistant', because they did not receive systemic glucocorticoid administration. This impaired response to topical treatment observed in AD-P might be due to a higher disease activity which subsequently leads to a lack of efficacy of steroids. Although the response to a topical immunomodulator such as tacrolimus would be a major concern, the drug was not applied to the subjects, because it had not been available in Japan at the time of treatment.

A significant finding of the present study is that IFN- γ transcription and secretion by PBMCs from AD-P were much lower compared with nonatopic controls, mild AD, and even with AD-R. This suppression of IFN- γ productions in AD-P was also observed at later time points, as well as after IL-12 stimulation. Thus, the decreased transcription and secretion of IFN- γ with the imbalance of Th1 and Th2 responses (Th1 $\ll$ Th2) were specifically observed in severe AD. The differences in the findings between groups might be due to the fact that in AD-R the severity of inflammation would have been lower than in AD-P because of efficient treatment at the time of blood sampling. Topical glucocorticoids may also have affected the cytokine(s) production after long-term application, even though their use was discontinued at least 12 h prior to the collection of the blood. However, this is unlikely, since there were no significant differences in the basal endogenous cortisol levels between AD-P ($10.7 \pm 2.9 \mu\text{g/dl}$) and AD-R ($8.6 \pm 2.4 \mu\text{g/dl}$, $p > 0.1$).

Reduced IFN- γ production in PBMCs from AD patients has previously been demonstrated by a number of

studies [9–15], which suggest a decreased number of IFN- γ -secreting Th1-type cells [2, 11, 14] rather than an impaired production of IFN- γ per cell [9] as a cause of low IFN- γ production. However, in the present study, the decreased number of Th1-type cells is unlikely to be the cause for the decreased transcription and secretion of IFN- γ in AD-P, since there were no significant differences in the number of IL-12 receptor-expressing CD3+ cells after PHA stimulation between the AD-P and control groups. CD3+ cells expressing IL-12 receptor were recognized as Th1-type cells [25]. Although IL-2 secretion by PBMCs from AD-P was lower than that observed in controls, the differences were not as significant as the findings for IFN- γ . These findings suggest that the impaired ability to produce IFN- γ in PBMCs from AD-P is mainly due to Th1-type cell dysfunction, which is particularly associated with IFN- γ production.

The molecular mechanisms by which IFN- γ gene transcription is regulated involves a wide variety of transcriptional factors [26–31]. This study addressed the role played by STAT4 (signal transducers and activators of transcription 4) [31] as parts of the mechanism underlying the reduced production of IFN- γ in severe AD patients. There were no significant differences in STAT4 DNA binding activity between AD-P and control groups following PHA/IL-2 and IL-12 stimulation. Further molecular investigation focusing on IFN- γ promoter will be required to clarify the mechanism(s) underlying the lowered rate of IFN- γ or the other Th1 cytokines production observed in AD-P [32].

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