

## INVESTIGATIVE REPORT

# Cytokine Expression of Skin T-lymphocytes from Patients with Atopic Dermatitis\*

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**We analysed the cytokine profile of skin T cells by establishing 11 T-cell lines from adult patients with moderate-to-severe atopic eczema using T-cell growth factors interleukin-2 and interleukin-4. We compared T-cell lines from lesional skin of atopic dermatitis patients with those from non-atopic skin of patients with other skin diseases, observing that T-cell lines of patients with atopic dermatitis in unstimulated cultures expressed a Th1 profile. After stimulation with anti-CD3 and anti-CD28 monoclonal antibodies, the cytokine expression showed rapid initial upregulation of Th2 followed by a Th1 profile. Furthermore, strong upregulation of interleukin-10 was observed after 24 h stimulation. Our findings suggest that skin T-lymphocytes from atopic dermatitis patients seem to consist of a heterogenous population of Th1 and Th2 or Th0 cells and the results for secreted cytokines indicate that T-cell lines from each inflammatory skin disease showed the corresponding disease-specific original cytokine profile. Key words: IL-4; IL-8; IL-10; IFN- $\gamma$ ; T-cell lines.**

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The prevailing view of atopic dermatitis (AD) is that Th2 cytokines predominate relative to Th1 cytokines, thus explaining the increased occurrence of IgE-mediated allergic reactions, which are seen in one- to two-thirds of patients (1, 2). The production of interferon (IFN)- $\gamma$  by peripheral blood mononuclear cells (PBMC) from patients with AD is low in contrast to normal or elevated interleukin (IL)-4 production (3–5). Mitogen or phorbol ester stimulation also leads to reduced release of IFN- $\gamma$  (6–10), although one study found a normal release (11).

In studies on allergen-specific PBMC (5, 12, 13) or skin-infiltrating T cells in patch tests induced by allergens, it was observed that a significant population of lymphocytes produced Th2 cytokines (14, 15). The proportion of allergen-specific T-cell clones (TCCs) was, however, not >6% (16). Recent studies have suggested a time-dependent switch by which an early Th2 response at 24 h changes into a predominant Th1 response at 48 h (17–19). TCCs from eczematous skin express a Th1 profile (17), and a Th2 pattern is only seen among TCCs from allergen-induced skin lesions

(20). CD4+ and CD8+ T-lymphocytes purified from peripheral blood of patients with AD and showing positivity for cutaneous lymphocyte-associated antigen (CLA) were found to contain IL-5 and IL-13, but not IL-4 or IFN- $\gamma$ , by flow cytometric analysis (21).

We decided to study the cytokine profile of the total population of skin T cells of patients with AD by establishing T-cell lines (TCLs) from eczematous skin lesions, a culture condition under which cytokine expression has not yet been studied. These TCLs are cytokine-driven using the T-cell growth factors recombinant human IL-2 (rhIL-2) and rhIL-4 without addition of antigen (22). Our findings show that a majority of unstimulated skin T cells in AD have a Th1 cytokine profile. When stimulated via T-cell receptor, these cells, which seem to consist of a heterogenous population of Th1 and Th2 or Th0 cells, show an early Th2 response followed by a Th1 response, although prominent expression and secretion of IL-10 also takes place. Thus the present results on secreted cytokines indicate that TCLs from the respective inflammatory skin diseases show the corresponding disease-specific original cytokine profiles.

## MATERIAL AND METHODS

### Patients

Punch biopsies (4 mm) were taken from active non-treated eczema regions of 11 patients (5 males, 6 females; mean age 31.2 years; range 19–50 years) with moderate-to-severe AD. Total serum IgE ranged between 481 and 20,000 kU/l (normal range <150 kU/l). Three patients had asthma and all had type I allergies. No allergen testing was performed on the skin prior to biopsy. The diagnosis was made according to the criteria of Hanifin & Rajka (23).

Punch biopsies were also taken from positive patch tests of patients with allergic contact dermatitis (3 females; age range 38–72 years), from positive reactions towards purified protein derivative of tuberculin (PPD) in non-atopic control subjects (2 males; ages 24 and 25 years) and from patients with untreated active psoriasis vulgaris (2 males, 1 female; age range 48–68 years). Informed consent was obtained prior to inclusion in the study, which was approved by the Ethical Committee of Aarhus County.

### Preparation of cultured TCLs from skin biopsy specimens

After removal, the biopsy specimens were immediately washed in physiological saline and cultured for 4 weeks in RPMI-1640 with L-alanyl-L-glutamine (Life Technologies, Paisley, UK) supplemented with 10% human AB serum, 10 IE/ml penicillin G, 100  $\mu$ g/ml streptomycin and 25  $\mu$ g/ml gentamicin in the presence of 1,000 U/ml rhIL-2 (Chiron, Amsterdam, The Netherlands) and 250 U/ml rhIL-4 (Schering-Plough, Kenilworth, NJ) at 37°C in an atmosphere containing 6.2% CO<sub>2</sub>. After growth, the cells were stored in dimethylsulphoxide-containing medium in liquid nitrogen before

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use. The phenotypes of TCLs were CD3+ >90%, CD4+ >90%, CD45RO+ >99% and TCR+ 100%, with ≈50% CLA-positive cells (22).

#### Cell stimulation and cytokine measurements

TCLs ( $6 \times 10^6$  cells) were cultured in medium as above in the presence or absence of 1 µg/ml immobilized anti-CD3 monoclonal antibody (anti-CD3 mAb; Research Diagnostics, Flanders, NJ) and 1 µg/ml anti-CD28 monoclonal antibody (anti-CD28 mAb; Cymbus Bioscience, Hants, UK) for either 6 or 24 h, at which times we measured the cytokine levels. These time points were chosen because transcriptional events occur at about 6 h after a stimulus and protein synthesis, with secretion occurring over the ensuing 18 h period. We measured IL-2, IL-4, IL-8, IL-10 and IFN-γ in order to estimate the levels of Th1 and Th2 type cytokines, pro-inflammatory cytokine (IL-8) and anti-inflammatory cytokine (IL-10).

#### RNA extraction and RT-PCR

Total RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method (24). The pellet was washed 3 times in 80% ethanol and dissolved in RNase-free water. Samples (8 µl) of total RNA were heated to 65°C for 10 min and cooled on ice. Reverse transcription of RNA was carried out using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Copenhagen, Denmark) for 1 h at 37°C in 15 µl volumes with 5 µl bulk first strand reaction mix (Moloney murine leukemia virus reverse transcriptase, RNaguard, RNase/DNase-free BSA, dATP, dCTP, dGTP and dTTP in aqueous buffer), 1 µl pd(N)<sub>6</sub> primer (random hexadeoxynucleotides at 0.2 µg/µl in aqueous solution) and 1 µl DTT solution (200 mM aqueous solution) per sample. cDNA dilution series were made from each cDNA reaction mixture, and 1 µl of original or diluted cDNA was used for each PCR. PCR was performed with 1 µl of reaction mixture, Taq polymerase (0.5 units per reaction; Advanced Biotechnologies, Surrey, UK), 50 µM of each pair of gene-specific primers and 200 µM dNTP in a reaction buffer [75 mM Tris-HCl, 1.5 mM MgCl<sub>2</sub>, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% (v/v) Tween 20 (Advanced Biotechnologies)].

Primer sequences were 5'TAC ATG CCC AAG AAG GCC AC, 3'CGA CTA CTC TGT CGT TGG TA for IL-2 (expected product size 225 bp), 5'CTT CCC CCT CTG TTC TTC CT, 3'GAC TTT GCC GAG CTG TCC TT for IL-4 (expected product size 316 bp), 5'ACG AAT TCC TAG GAC AAG AGC CAG GAA G, 3'CTA ACT CTC ACC TGG TGT GAC TTA AGT G for IL-8 (expected product size 253 bp), 5'ATG CCC CAA GCT GAG AAC CAA GAC CCA, 3'ACC CTA TCG ACT GGG TCG GGG AAC TCT for IL-10 (expected product size 351 bp), 5'AGT TAT ATC TTG GCT TTT CA, 3'TTC GAC TGA TTA ATA AGC CA for IFN-γ (expected product size 330 bp) and 5'GAG AAT TCG AGT CAA CGG ATT TGG TCG T, 3'TAC CGT TT AAG GTA CCG TGG CTT AAG CG for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (expected product size 174 bp). All primers were purchased from Amersham Pharmacia Biotech.

PCR was performed in a DNA engine thermocycler (MJ Research, Watertown, MA) with 45 cycles (35 cycles in the case of GAPDH). Amplification was performed using an initial cycle of 94°C (5 min), 55°C (1 min) and 72°C (1 min), followed by 33 or 43 cycles at 93°C (25 s), 55°C (50 s) and 72°C (45 s) and a final cycle of 93°C (25 s), 55°C (50 s) and 72°C (5 min). The reaction products were visualized by electrophoresis in a 2% NuSieve, 1% agarose gel and by ultraviolet light illumination after staining with SYBR Green I (Molecular Probes, Leiden, The Netherlands).

#### Secreted cytokine assays

IFN-γ, IL-4, IL-8 and IL-10 were measured with ELISA kits in accordance with the manufacturer's instructions. Each sample was tested in duplicate. IFN-γ was measured using a Quantikine kit with a sensitivity of 15.6 pg/ml (R&D systems, Abingdon, UK). IL-4 was

determined using a DuoSet kit with a sensitivity of 15.6 pg/ml (R&D systems). IL-8 was determined using flat-bottomed microtitre 96-well plates (Nunc, Roskilde, Denmark) that were coated at 4°C overnight with 1.5 µg/ml monoclonal anti-human IL-8 Ab (WS-4; Dainippon Pharmaceutical, Osaka, Japan) in 0.05 M carbonate/bicarbonate buffer (pH 9.6) at 100 µl/well. After the plates were washed, they were incubated for 1 h with blocking buffer (2% BSA plus PBS), washed again, supplemented with standard and cell-free supernatant, incubated for 24 h at 4°C, and then supplemented with polyclonal anti-human IL-8 (1 µg/ml; R&D systems). A similar procedure was done using anti-human IL-10 (R&D systems).

Following incubation of the plates for 1 h at room temperature on a rotating table, horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin G antibody (IgG Ab) was added (3:1,000; Dako, Copenhagen, Denmark). After washing, the plates were developed using 10 µl of 30% H<sub>2</sub>O<sub>2</sub> and 0.8 mg/ml of o-phenylenediamine dihydrochloride (Sigma, St. Louis, MO). After 30 min, 4.5 N H<sub>2</sub>SO<sub>4</sub> was added to each well to stop the reaction. Optical density was read at 490 nm using a microplate reader (Struers, Roedovre, Denmark).

#### Statistical analysis

We used Wilcoxon's signed rank test for paired data. The RT-PCR results were analysed using Fisher's exact probability test. *p*-values <0.05 were considered to indicate significance. All results are presented as the mean ± standard error of the mean (SEM).

## RESULTS

#### mRNA expression of cytokines in TCLs

The skin T-lymphocytes from patients with AD grown in rhIL-2 and rhIL-4 without further stimulation showed a Th1 cytokine profile, as IL-2 was found in 9/11 TCLs and IFN-γ was expressed in all TCLs (11/11) (24 h results; Fig. 1, Table I). There was no upregulation of IL-4 or IL-10 in unstimulated culture at either 6 h or 24 h. IL-8 was expressed to a variable degree (6/11).

When TCLs were stimulated with anti-CD3 mAb and anti-CD28 mAb, IL-4 and IL-10 were expressed at 6 h post-stimulation of culturing in all cell lines studied (*p* < 0.01). IL-4 mRNA was downregulated in half of the cell lines studied at 24 h, whereas IL-10 upregulation continued. In contrast, IFN-γ was expressed in half of the cell lines at 6 h after stimulation, but IL-2 was not (*p* < 0.03). These Th1

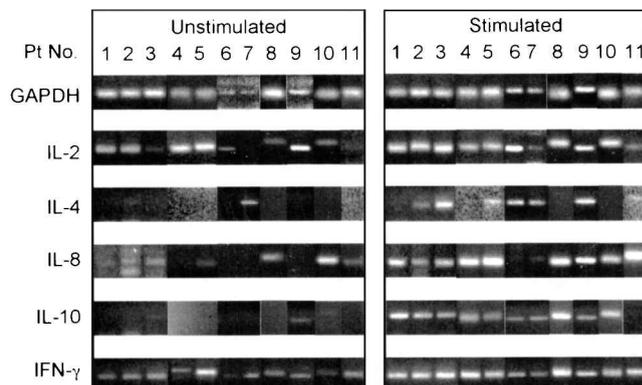


Fig. 1. Cytokine mRNA expression of T-cell lines from atopic dermatitis (*n* = 11) investigated by RT-PCR. The expression of each cytokine mRNA in all T-cell line was compared with expression of GAPDH mRNA (house-keeping gene). Unstimulated: medium alone for 24 h. Stimulated: stimulation with anti-CD3 mAb and anti-CD28 mAb for 24 h.

Table I. Expression of cytokine mRNA in T-cell lines from patients with atopic dermatitis at different times after stimulation<sup>a</sup>

mRNA	6 h (n=5)		24 h (n=11)	
	Unstimulated	Stimulated	Unstimulated	Stimulated
IL-2	1/5	5/5*	9/11	11/11
IFN- $\gamma$	1/5	3/5	11/11	11/11
IL-4	0/5	5/5**	1/11	6/11†
IL-10	0/5	5/5**	1/11	10/11††
IL-8	0/5	4/5*	6/11	9/11

\* $p < 0.03$  vs. unstimulated culture for 6 h. \*\* $p < 0.01$  vs. unstimulated for 6 h. † $p < 0.05$  vs. unstimulated culture for 24 h. †† $p < 0.01$  vs. unstimulated culture for 24 h. <sup>a</sup>For experimental details see Fig. 1 and Methods.

cytokines, however, were expressed in almost all cell lines at 24 h (Fig. 1, Table I). Based on these results, we conclude that T-cell receptor stimulation leads to an early Th2 response followed by a Th1 response. IL-8 was expressed in 9/11 cell lines after 24 h of stimulation.

T-lymphocytes from positive patch tests, PPD skin reactions or psoriasis showed a similar pattern of a Th1 type among unstimulated cells (Fig. 2A–C). However, upregulation took place in all cytokines studied after stimulation with anti-CD3 mAb and anti-CD28 mAb, except in the 3 TCLs from positive patch tests, in which IL-2 was downregulated. IL-4 mRNA expression was not detected in TCLs from positive patch tests even after stimulation for 24 h.

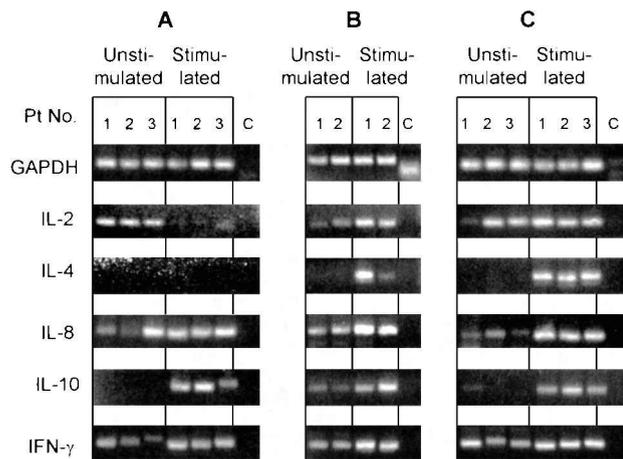


Fig. 2. Cytokine mRNA expression of T-cell lines from (A) allergic contact dermatitis ( $n = 3$ ), (B) positive reaction by purified protein derivative of tuberculin in non-atopic control subjects ( $n = 2$ ) and (C) psoriasis vulgaris ( $n = 3$ ) investigated by RT-PCR. C = PCR control.

Table II. Cytokine production (pg/ml) from T-cell lines of patients with atopic dermatitis measured by ELISA (mean  $\pm$  SEM)

		IL-8	IFN- $\gamma$	IL-4	IL-10	IFN- $\gamma$ :IL-4 ratio
6 h (n=5)	Unstimulated	186.4 $\pm$ 159.9	30.6 $\pm$ 20.7	0	3.8 $\pm$ 1.7	–
	Stimulated	2,228 $\pm$ 1470.2	747 $\pm$ 602.1	774.4 $\pm$ 622.1	52.2 $\pm$ 31.7	1.1 $\pm$ 0.3
24 h (n=4)	Unstimulated	368.5 $\pm$ 127.2	33.1 $\pm$ 13.3	0	7.7 $\pm$ 7.2	–
	Stimulated	4,086.4 $\pm$ 1395.3	5,478.2 $\pm$ 3681.6	1,003.8 $\pm$ 402.6	13,905.5 $\pm$ 12617.5	12.4 $\pm$ 4.8

\* $p < 0.05$ , \*\* $p < 0.01$ .

### Secretion of cytokines from TCLs

Unstimulated cultures of AD TCLs secreted very low but detectable amounts of cytokines, except IL-8, at both 6 and 24 h (Table II). Stimulation with anti-CD3 mAb and anti-CD28 mAb for 24 h induced significant cytokine release in all TCLs compared with unstimulated cells (Table II, Fig. 3). TCLs from patients with AD released all cytokines, and these cells released more IL-4 than IFN- $\gamma$  at 6 h. At 24 h, however, IFN- $\gamma$  release was increased 12-fold relative to IL-4 release. IL-10 showed the highest increase between 6 and 24 h.

TCLs from positive patch tests showed a cytokine excretion pattern similar to that of AD (Fig. 3AB). However, IL-10 production in TCLs from positive patch tests was higher than IL-10 production in AD, but not statistically significant. Although IL-4 mRNA expression was not detected in TCLs stimulated for 24 h, IL-4 secretion was found in TCLs stimulated for 24 h from positive patch tests. PPD cell lines showed the highest secretion of cytokines (Fig. 3C), whereas TCLs from psoriatic skin lesions had low secretion levels (Fig. 3D).

### DISCUSSION

The focus on skin T cells in AD has mostly been on allergen-specific TCCs from lesional skin or allergen-induced skin reactions (15, 25). Here we have taken advantage of all skin T cells without prior selection. We studied consecutive patients who had type I allergies and increased total serum IgE, and who were therefore considered to show a Th2 cytokine profile (1). We did not perform patch tests in the patients prior to skin biopsy, but rather took our samples from active, chronic eczema lesions.

It is quite clear that the spontaneous mRNA expression in AD TCLs at 24 h showed a distinct Th1 pattern, whereas mRNA for IL-4 or IL-10 were present in one cell line (Table I). Using 45 cycles of our RT-PCR technique, it was possible to pick up even minute fractions of Th2 cells. Stimulation of the cells via their T-cell receptors (CD3 and CD28 surface molecules) clearly led to a quick upregulation of IL-4 (6 h), followed by partial downregulation of IL-4 (24 h) and upregulation of IL-2 and IFN- $\gamma$ . This is supported by our ELISA studies, in which the excretion ratio of IL-4 relative to that of IFN- $\gamma$  was high at 6 h (IFN- $\gamma$ : IL-4 ratio = 1.1), followed by a strong upregulation of IFN- $\gamma$  (IFN- $\gamma$ : IL-4 ratio = 12.4) at 24 h. Thus according to our RT-PCR and ELISA results it seemed that TCLs of eczematous skin lesions from patients with AD consisted of a heterogeneous population of Th1 and Th2 or Th0 cells. It is noteworthy that the upregulation and secretion of IL-10 became very pronounced at 24 h. This was consistent with our observations

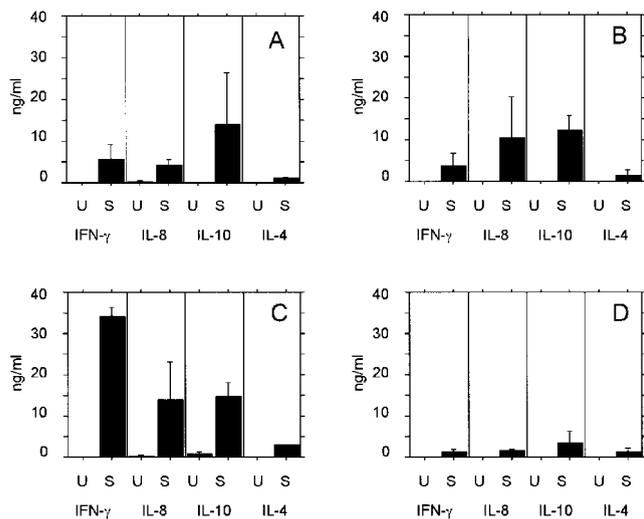


Fig. 3. Cells in T-cell lines from patients with (A) atopic dermatitis ( $n=11$ ), (B) allergic contact dermatitis ( $n=3$ ), (C) positive reaction by purified protein derivative of tuberculin in non-atopic control subjects ( $n=2$ ) and (D) psoriasis vulgaris ( $n=3$ ) were stimulated for 24 h with anti-CD3 mAb and anti-CD28 mAb (S) or were cultured for 24 h without stimulation (U). Concentrations of IFN- $\gamma$ , IL-8, IL-10 and IL-4 in harvested supernatants were determined using ELISA.

on skin biopsies from atopic eczema, in which mRNA was found to be highly upregulated. In addition, PBMC excreted high levels of IL-10, although Ohmen et al. (26) found that the IL-10 came from CD14<sup>+</sup> cells, i.e. monocytes. It should be noted that our cultures contained only T-lymphocytes, with no dendritic or monocytic cells (22).

TCLs from other skin conditions showed a Th1-type pattern of cytokine mRNA expression and secretion at 24 h of culturing. We were unable to measure mRNA expression at 6 h for logistical reasons. Tuberculin skin reactions had the highest cytokine release—and the highest IFN- $\gamma$  in particular—indicating a relative stronger Th1 profile. It is unclear why TCLs from positive patch tests showed a downregulation of mRNA for IL-2. However, we excluded technical errors, as our IL-2 primers worked in the same PCR reaction performed on other samples in the same run. In addition, although IL-4 secretion was detected by ELISA, IL-4 mRNA expression was not detected in TCLs stimulated for 24 h from positive patch tests. This might be attributed to the fact that, after stimulation for 24 h, the transcription of IL-4 was already finished in TCLs from positive patch tests. This suggests that very short and early IL-4 upregulation was seen in TCLs from positive patch tests. Intriguingly, our ELISA results indicated that TCLs from each inflammatory skin disease showed a disease-specific original cytokine profile (Fig. 3).

Our results support those of previous reports in which a majority of TCCs from eczematous skin samples expressed a Th1 profile (16, 20). Like Virtanen et al. (16), we were unable to establish any correlation between the levels of Th2 cytokine expression and total serum IgE. In addition, we found that the cytokine profile of TCLs from AD indicated a short Th2 upregulation (6 h) followed by a prominent Th1 response (24 h). However, we were unable to prove that switching from a Th2 to a Th1 profile occurred, as previously observed by Grewe et al. (17) and by Thepen et al. (19) using

immunohistochemistry in positive allergen patch tests. However, this may be because we did not use allergen stimulation and it should be considered that the kinetics of cytokine mRNA and protein expression depend on the type of cytokine (27). Rather, our results would suggest that a Th0 profile is predominant following T-cell receptor stimulation. Although we did not investigate this matter here, it is likely that a Th2 profile will predominate when allergen-specific T cells are stimulated (20).

Some may argue that our *in vitro* system does not reflect what happens in the skin as, in our culture system, the cells go through several population doublings *in vitro* under the influence of large amounts of IL-2 and IL-4. However, these T cells are derived from the skin, and growth factors are mandatory for establishing TCLs (22). Neither antigens, mitogens nor feeder cells were added to our culture system, and it has been shown that a T-cell will not change its original cytokine profile if cultured in a growth-promoting medium without T-cell receptor stimulation (28). In addition, recent studies have shown that IL-4 is able to support IL-2 short-term proliferation of human T cells (29). IL-4 has been reported to promote a Th2 profile in certain cells (30), but this was obviously not the case in our TCLs, in which no IL-4 mRNA was detected. Recently, Akdis et al. (31) reported similar findings of low to non-detectable levels of IL-4 on skin tissue and in short-term cultures of skin-homing lymphocytes in AD.

Taken together, our results indicate that the cytokine profile of non-stimulated skin T-lymphocytes from patients with AD is of a predominantly Th1 type. However, a physiological stimulus via the T-cell receptor leads to an instant, short Th2 upregulation followed by a prominent Th1 response. Thus, our skin TCLs from patients with AD consist of a heterogeneous population of Th0 cells, although we cannot completely exclude the possibility that they consisted of a mixture of Th1 and Th2 cells (18). Nonetheless, the anti-inflammatory cytokine, IL-10, was highly secreted, and this secretion might be interpreted as an attempt to dampen the induced immune inflammation. Our ELISA data indicated that TCLs from the respective inflammatory skin diseases show the corresponding disease-specific original cytokine profiles. Thus our cytokine analysis of TCLs may reflect cytokine profiles *in vivo*, and it may be possible to investigate the function of skin T-lymphocytes from various inflammatory skin diseases using this culture system.

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