A Th_2 Chemokine, TARC, Produced by Keratinocytes May Recruit CLA^+CCR4^+ Lymphocytes into Lesional Atopic Dermatitis Skin

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Atopic dermatitis is an inflammatory skin disease in which the inflammation is characterized by the influx of lymphocytes into the dermis. It is generally believed that atopic dermatitis is a Th_2-type disease, i.e., the T lymphocytes produce interleukin-4, interleukin-5, interleukin-10, and interleukin-13, although it has become evident in recent years that the cytokine profile in the skin changes during the course of the disease towards a Th_1-Th_2 mixed cytokine profile (interferon-γ, tumor necrosis factor α, and interleukin-2). The lymphocytes that home into the skin express cutaneous lymphocyte-associated antigen, and it has recently been shown that most of the lymphocytes in this population express the chemokine receptor CCR4. CCR4 is the receptor for the CC chemokine TARC (thymus and activation regulated chemokine), and this chemokine is expressed predominantly by keratinocytes in the basal layer of the epidermis of lesional atopic dermatitis skin in mice. In humans, however, it was shown to be expressed in the endothelial cells of the dermis. We have examined the peripheral blood mononuclear cells of atopic dermatitis patients for the expression of cutaneous lymphocyte-associated antigen and CCR4 and compared them with peripheral blood mononuclear cells from normal controls. We found that the proportion of CLA^+CCR4^+ lymphocytes is upregulated in atopic dermatitis patients. In addition we have examined skin biopsies of lesional and nonlesional skin from atopic dermatitis patients and found that the keratinocytes, but not the endothelial cells, produce TARC in the lesional but not in the nonlesional skin. To gain insight in the stimulatory mechanisms for TARC production in keratinocytes, as previously observed in mice, we cultured HaCaT cells and found that interferon-γ and tumor necrosis factor α work synergistically to induce TARC production. These observations suggest that the induction of TARC production in keratinocytes plays an important role in the late phase skin invasion by $CCR4^+CLA^+$ Th_2-type lymphocytes in atopic dermatitis. ***Key words: chemokine receptors/IFNγ/immunohistochemistry/lymphocyte recirculation/TNFα. J Invest Dermatol 115:640–646, 2000***

Atopic dermatitis (AD) is a pruritic inflammatory skin disease affecting approximately 20% of the population, and of these 85% are affected before the age of 5 y (Rudikoff and Lebwohl, 1998). The disease is a clinical syndrome and the diagnosis is based on major and minor criteria as defined by Hanifin and Rajka (1980). AD is generally thought to be a genetic immune disorder with a family history of atopic diseases (Cooper, 1994; Rudikoff and Lebwohl, 1998), although other mechanisms for the development of AD, such as decreased ceramide content of the skin (Imokawa *et al*, 1991) or a primary ectodermal defect (Thestrup-Pedersen *et al*, 1997), have been suggested. The level of serum IgE is increased in most, but not all, patients suffering from AD (Rudikoff and Lebwohl, 1998). The skin lesions are histologically characterized by an infiltration of lymphocytes, monocytes/macrophages, fully granulated mast cells (Soter, 1989), eosinophils (Uehara *et al*, 1990), dermal dendritic cells, and epidermal Langerhans cells (Bos *et al*, 1986; Taylor *et al*, 1991). In addition, acanthosis, parakeratosis, and hyperkeratosis may be seen (Soter, 1989). The lymphocytes invading the lesional skin are mainly Th_2-type lymphocytes, e.g., they produce the cytokines interleukin-4 (IL-4), IL-5, IL-10, and IL-13 (Grewe *et al*, 1998). There seem to be variations in the expression levels of these cytokines, however, as the number of IL-4 mRNA expressing cells is significantly higher in skin from acute AD than in skin from chronic AD, and the opposite is the case for the number of IL-5 expressing cells (Hamid *et al*, 1994). In contrast to the Th_2 model of AD, interferon-γ (IFN-γ), a Th_1-type cytokine, has been reported to be expressed in late stage AD (Grewe *et al*, 1994, 1998; Werfel *et al*, 1996; Rudikoff and Lebwohl, 1998). Treatment of the patients that resulted in improvement of their lesions was correlated with down-regulation of IFN-γ expression in the skin, but not of IL-4 (Grewe *et al*, 1994).

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Abbreviations: AD, atopic dermatitis; CLA, cutaneous lymphocyte-associated antigen; PBMC, peripheral blood mononuclear cells; TARC, thymus and activation regulated chemokine.

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The T cells invading the lesional skin in AD are also distinguished by their surface markers (Herz *et al*, 1998) such as CD4, CD3, cutaneous lymphocyte-associated antigen (CLA) (Picker *et al*, 1990), and CD45R0 (Bos *et al*, 1989). CLA defines the subset of skin-homing T cells (Berg *et al*, 1991; Santamaria Babi *et al*, 1995) that binds to E-selectin, an adhesion molecule expressed by endothelial cells in inflamed tissues (Berg *et al*, 1991) during the first step of leukocyte extravasation (Springer, 1994).

Chemokines are small proteins that play a major role in controlling leukocyte trafficking (Rollins, 1997; Baggiolini, 1998). They are divided into subgroups according to a cystein-cystein motif with varying numbers of amino acids between the cystein residues; thus there are four subgroups: CC, CXC, CX_3C, and C (Rollins, 1997; Luster, 1998). The receptors for the chemokines are subdivided in the same manner, as CCR1–9, CXCR1–5, and CX_3CR1 (Luster, 1998). The CC chemokine TARC (thymus and activation regulated chemokine) is an 8 kDa chemokine (Imai *et al*, 1996), which has been found to be expressed by dendritic cells differentiated from monocytes with granulocyte-macrophage colony stimulating factor, IL-3, and IL-4 (Hashimoto *et al*, 1999). TARC is a functional ligand for CCR4 (Imai *et al*, 1997), which expressed on Th_2-type lymphocytes (Imai *et al*, 1999), and a study in mice has shown that addition of monoclonal antibodies against TARC decreases the Th_2 response in bacteria-induced fulminant hepatitis (Yoneyama *et al*, 1998). Indeed, it has been shown in a recently published study that, in peripheral blood, most of the lymphocytes that express CLA also express CCR4 (Campbell *et al*, 1999). This, taken together with another recent study in which it was shown that in a murine model of AD TARC is produced abundantly in the basal keratinocytes of lesional skin (Vestergaard *et al*, 1999), and the perception of AD as a Th_2-type disease, led us to hypothesize that the number of CLA^+CCR4^+ lymphocytes would be upregulated in the peripheral blood of AD patients. It also led us to examine the production of TARC in the skin of AD patients, as well as the regulation of the production of TARC *in vitro* by a keratinocytic cell line (HaCaT cells; Boukamp *et al*, 1994).

MATERIALS AND METHODS

Cell cultures A human keratinocytic cell line, HaCaT cells, was cultured in 5 ml Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) in a 20 ml culture bottle (Nunc, Roskilde, Denmark) at 37°C and 5% CO_2 until confluence, after which the cells were trypsinated, washed, diluted 10 times in DMEM with 10% FBS, and moved into medium culture bottles in a volume of 12.5 ml. When confluence was again achieved, the cells were trypsinated, diluted 10 times, and 2 ml was moved to 5 ml wells in a six-well tray and cultured until confluence, yielding approximately 1×10^6 cells per ml. The cells were then washed and 2 ml DMEM without FBS was added to each well, and the various cytokines were added. The cytokines used for stimulation were rhIFN-γ (cat. no. 285-IF-100, R&D Systems, Minneapolis, MN) and tumor necrosis factor α (TNF-α) (cat.no. 210-TA, R&D systems) in concentrations of either 10 ng per ml or 100 ng per ml either alone or in combination. The cells were then cultured for 1, 3, 6, 12, 24, and 48 h, after which the supernatant was removed and stored at −80°C until further analysis. As a negative control cells cultured in DMEM without any stimulation were used as well as cells cultured for 24 h stimulated with both rhIFN-γ and TNF-α and added neutralizing antibodies against IFN-γ (2.5 μg per ml) (cat. no. MAB285, R&D systems) and TNF-α (0.2 μg per ml) (cat. no. MAB210, R&D systems) (concentrations were calculated from ND_{50} values).

In order to examine if IFN-γ and TNF-α had the same effect on keratinocytes normal adult human keratinocytes were obtained by trypsinization of skin samples from patients undergoing plastic surgery as previously described (Kragballe *et al*, 1985). First-passage keratinocytes were grown in serum-free keratinocyte growth medium (cat. no. 10785-019, Life Technologies, Copenhagen, Denmark) supplemented with 5 ng per ml human recombinant epidermal growth factor and 50 mg per ml bovine pituitary extract (cat. no. 37000-015, Life Technologies). Cells were grown at 37°C in 5% CO_2 to 50% confluency with less than 10% of the cells differentiated, as judged by light microscopy.

TARC enzyme-linked immunosorbent assay (ELISA) ELISA was performed in 96 well maxisorp plates (cat. no. 00442404 A IMMUNO F96 Maxi, Life Technologies). As capturing antibody, monoclonal mouse antihuman TARC (cat. no. MAB364, R&D systems) were used. The antibodies were dissolved in a coating buffer consisting of 0.5 M carbonate/bicarbonate, pH 9.6, to a final concentration of 1.5 μg per ml, and 100 μl were added to each well, after which the plate was incubated at 4°C overnight. The wells were then washed three times with phosphate-buffered saline (PBS) and 0.05% Tween (cat. no. 170-6531, Biorad), and stored at 4°C until use for up to 1 wk. The samples were diluted 1:1 with assay buffer [8% bovine serum albumin (BSA), A 6793, Sigma], and 100 μl were incubated in each well overnight on a shaking table at room temperature. The wells were then washed with PBS and 0.05% Tween three times after which they were incubated with 300 μl blocking buffer (2% BSA in PBS, pH 7.4) for 1 h and washed again three times with PBS and 0.05% Tween. The second antibody, a polyclonal goat antihuman TARC antibody (cat. no. AF364, R&D systems), was added, dissolved in assay buffer at a concentration of 1 μg per ml for 1 h at room temperature. The wells were washed three times and 100 μl of horseradish peroxidase antigoat-IgG antibody was added (code no. P0449, Dako, Glostrup, Denmark) and incubated for 1 h at room temperature. The result was visualized by adding 100 μl of substrate buffer, which was made up of 12.5 ml citrate (4.67 g per liter, cat. no. 1.00244.0500, Merch) and phosphate (7.3 g per liter, cat. no. 21.988-6, Aldrich), pH 5.0, added to 10 μl of 30% H_2O_2 and 10 mg OPD substrate (cat. no. p-8287, Sigma), and incubated for 30 min, after which the reaction was stopped with 1.6 N H_2SO_4. The result was read with an ELISA reader (Lab systems iEMS Reader MF) at 490 nm. As standard curve, recombinant TARC (cat. no. 364-DN-025, R&D systems) was used in the following concentrations: 0, 15, 31, 62, 125, 250, 500, and 1000 μg per ml. All measurements were performed in duplicate.

Reverse transcriptase polymerase chain reaction (RT-PCR) and PCR The mRNA from the HaCaT cell cultures were isolated after stimulation using RNA Isolator (Genosys Biotechnologies, Cambridge, U.K.) according to the manufacturer's instructions. Briefly, the cells were incubated with 1 ml RNA Isolator per 10 cm^2 for 5 min at room temperature, after which 0.2 ml of chloroform per ml RNA Isolator was added and the mixture was shaken thoroughly for 15 s and incubated at room temperature for 10 min. The samples were then centrifuged for 15 min at 12,000 × *g* at 4°C. The aqueous phase was isolated and the mRNA was precipitated using 0.5 ml of isopropanol per 1 ml of RNA Isolator used in the initial isolation. The mRNA was then washed using 75% ethanol and stored at −20°C until use, at which point it was resuspended in water. The concentration of RNA was determined by the A_{260} value of the sample. The first-strand DNA synthesis was performed using First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Briefly, 3 μg of mRNA in 8 μl water was heated to 65°C for 10 min, after which it was chilled on ice for 2 min and added to 5 μl of Bulk First Strand cDNA Mix, 1 μl of dithiothreitol solution, and 1 μl of $pd(N)_6$ primers (random hexadeoxynucleotides) in a 0.02 μg per ml solution. The solution was mixed thoroughly and heated to 37°C for 1 h. Of the resulting solution 1 μl was mixed with 14.6 μl water and added to 1 μl of forward primer and 1 μl of reverse primer, both in a solution of 0.02 μg per μl, and 2 μl of 10XPCR buffer (Amersham Pharmacia Biotech) and 0.4 μl of *Taq* DNA polymerase (Amersham Pharmacia Biotech). The primers used were as follows: TARC forward primer, 5′-CTT CTC TGC AGC ACA TCC-3′; TARC reverse primer, 5′-AAG ACC TCT CAA GGC TTT G-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer, 5′-GAG TCA ACG GAT TTG GTC GT-3′; and GAPDH reverse primer, 5′-GGT GCC ATG GAA TTT GCC AT-3′. The thermal cycling conditions were set to 95°C for 2 min, followed by 95°C for 30 s, 55°C for 45 s, and 72°C for 45 s, repeated 30 times and followed by 72°C for 10 min. The results were visualized on a 1% agarose gel using SyBr green and ultraviolet light, and quantitated using a digitized picture of the gel and the ImageQuant version 3.3 from Molecular Dynamics (Sunnyvale, CA). As negative controls one sample of isolated RNA, on which no RT-PCR was performed, and one sample of water were used.

Blood samples and flow cytometry Peripheral blood was collected in heparinized tubes from seven patients suffering from moderate to severe AD as determined by the criteria of Hanifin and Rajka (1980); they were three females and four males, with serum IgE ranging from 1157 to 4348 kU per ml, mean 2763 kU per ml, except in one case (115 kU per ml). Three of the patients suffered from asthma and all had a positive immediate-type skin test. As negative controls blood from five normal

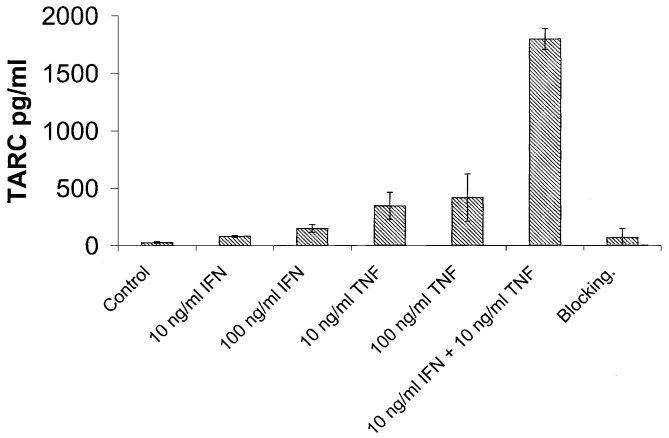

Figure 1. Induction of TARC in HaCaT cells. The concentration of TARC, as determined by ELISA, in the culture supernatants of HaCaT cells (1×10^6 cells per ml) after stimulation with TNF-α and IFN-γ. The stimuli are described on the *X* axis, and the concentration of TARC on the *Y* axis (each value represents n = 4, and each measurement was performed in duplicate). The error bars show the standard deviation.

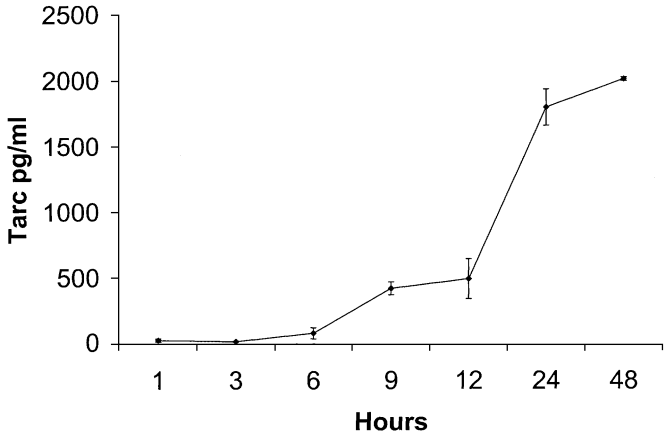

Figure 2. Time-related production of TARC. A time-course study of TARC production in the supernatants of HaCaT cells (1×10^6 cells per ml) as determined by ELISA, after stimulation with 10 ng per ml IFN-γ and 10 ng per ml TNF-α. The hours after stimulation are shown on the *X* axis and the TARC concentrations on the *Y* axis (each value represents n = 4, and each measurement was performed in duplicate). The error bars show the standard deviation.

subjects, one woman and four men, was used. None of these subjects suffered from any known atopic disease, and consequently no IgE or immediate-type test results were known.

The peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep (prod. no. 1053980, Nycomed Pharma, Oslo, Norway) according to the manufacturer's instructions. The cells were stored in RPMI 1640 with Glutamax-I (cat. no. 61870–010, Gibco, Life Technologies, Paisley, Scotland) and 10% dimethylsulfoxide (cat. no. D-2650, Sigma-Aldrich, Irvine) in liquid nitrogen until further analysis.

The cells were thawed carefully, washed in Hank's solution, and resuspended in PBS with 1% murine serum (code no. X0910, Dako) to a final concentration of 1×10^6 cells per ml. The cells were then incubated with mouse antihuman CCR4 antibody (Imai *et al*, 1999), as the primary first antibody, for 45 min in the dark at room temperature, after which the cells were washed with Hank's solution twice. The cells were resuspended in PBS with 1% goat serum (code no. X0907, Dako) and stained with the second antibody, phycoerythrin (PE) conjugated goat antimouse antibodies (code no. R0480, Dako), and incubated for 45 min in the dark at room temperature. The cells were washed in Hank's solution again, resuspended in PBS with 1% murine serum, and stained with the secondary antibody, fluorescein isothiocyanate (FITC) conjugated mouse antihuman CLA antibody (cat. no. 3582X, PharMingen, San Diego, CA). As negative controls an FITC conjugated mouse IgG (code no. X0927, Dako) or the PE conjugated goat antimouse antibody but without the primary antibody were used. The control antibodies were used alone, in combination, or in combination with the noncontrol antibodies. In order to check whether the freezing procedure changed the expression of either CCR4 or CLA, PBMC from three normal donors were isolated and divided into two groups. One group was stained immediately according to the protocol, whereas the other group was stored at –80°C according to the protocol and then stained.

The cells were analyzed on a Coulter EPICS XL-MCL Flowcytometer equipped with Coulter system II software. The color compensation was performed using Cyto-Comp Cell Kit (cat. no. PN6607023, Coulter, Luton, U.K.) and Cyto-Comp Reagent Kit (cat. no. PN 6607021, Coulter), and the negative regions were aligned using the negative controls. The cells were gated for lymphocytes and analyzed for the appropriate antibodies. The listmode data were saved and reanalyzed using WinMDI shareware (provided by Joe Trotter of the Scripps Institute, La Jolla, CA; copy from CytonetUK, HTTP://cf.ac.uk/uwcm/hg/hoy).

Skin biopsies and immunohistochemistry Skin biopsies were obtained from seven AD patients, selected for the clinical severity of the skin lesions, one from lesional skin and one from nonlesional skin, each 5 mm in diameter. The biopsies were snap frozen in liquid nitrogen and stored at –80°C until use. The samples were cut in a cryostat and mounted on glass slides for immunostaining. They were fixed in acetone for 10 min, and endogenous peroxidase activity was quenched by incubating in 0.6% hydrogen peroxide in methanol for 20 min (all incubations in this procedure were performed at room temperature). The samples were then rinsed in distilled water and placed in Tris-buffered saline (TBS) for 5 min, after which they were incubated for 20 min with rabbit serum (Dako, code no. X0902). The samples were then incubated for 45 min with the first antibody, mouse antihuman TARC antibody (10 μg per ml) in TBS, and placed for 5 min in TBS. Next, they were incubated with biotinylated goat antimouse antibodies (Dako, code no. E0433) and incubated at room temperature for 45 min, after which they were placed in TBS for 5 min. They were incubated with avidin/horseradish peroxidase (RPN 1231, Amersham Lifescience) for 30 min and washed in TBS for 5 min. They were then stained with Dako AEC substrate system (code no. K0697, Dako, Carpenteria, CA) according to the manufacturer's instructions, counterstained with Mayer's hematoxylin, and mounted on slides for microscopy. As negative controls samples were stained with the second antibody only. In addition serial sections from three AD patients were collected and stained with either mouse antihuman CD3 antibodies or mouse antihuman CCR4 antibodies, according to the protocol described above. As second antibody the Envision system (code no. K1396, Dako, Glostrup, Denmark) was used according to the manufacturer's instructions.

RESULTS

Induction of TARC production in HaCaT cells When the HaCaT cells were cultured until confluence and stimulated (n = 4 in all experiments) with either IFN-γ or TNF-α at concentrations of 10 ng per ml or 100 ng per ml for 24 h (**Fig 1**), a moderate production of TARC was induced compared with nonstimulated cells (all measurements were performed in duplicate). The levels of TARC production were significantly higher when the cells were stimulated with TNF-α than when stimulated with IFN-γ ($p < 0.01$ for both 10 ng per ml and 100 ng per ml stimulations). When stimulated with both IFN-γ (10 ng per ml) and TNF-α (10 ng per ml) the level of TARC production after 24 h (1794.03 ± 91.79 pg per ml) was significantly higher ($p < 0.001$ in both cases) than that after stimulation with IFN-γ (78.63 ± 8.34 pg per ml) and TNF-α (277.14 ± 26.07 pg per ml). Cell cultures stimulated with IFN-γ (10 ng per ml) and TNF-α (10 ng per ml) as well as blocking antibodies against these cytokines showed TARC production at the same level as nonstimulated cell cultures. A time-course study of the production of TARC by HaCaT cells after stimulation with TNF-α (10 ng per ml) and IFN-γ (10 ng per ml) was also carried out (**Fig 2**) and revealed a sigmoid concentration curve leveling out at 24 h.

To examine whether the higher concentration of TARC in the culture supernatant of the cells after stimulation with IFN-γ and TNF-α was due to newly produced TARC, cells were stimulated for 1, 2, 3, 6, and 9 h (n = 2) after which the mRNA from the cells

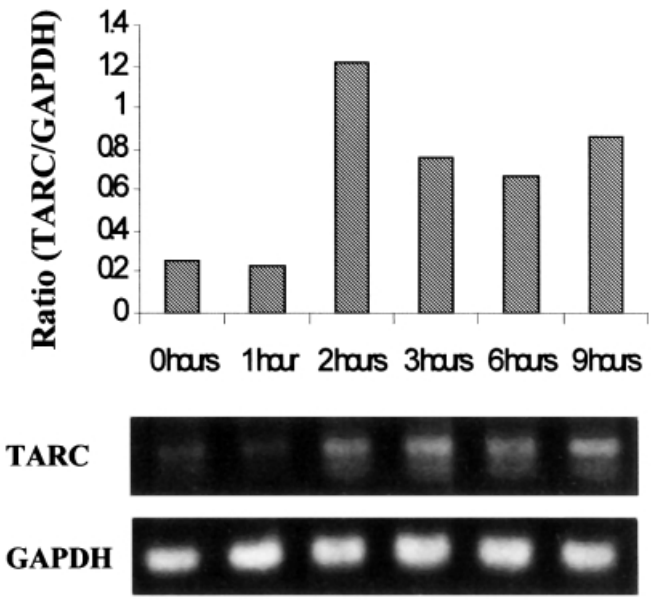

Figure 3. Induction of TARC mRNA. The upper panel shows the densitometric analysis, performed with the Image Quant software from Molecular Dynamics, of the RT-PCR gels, performed on HaCaT cells stimulated with 10 ng per ml IFN-γ and 10 ng per ml TNF-α, shown in the lower panel (n = 2). The ratio shown on the *Y* axis is the ratio between the densitometric values of TARC and GAPDH. In the lower panels the gels of the RT-PCR of TARC and GAPDH are shown. The use of densitometric analysis does not yield a quantitative result but only semiquantitative in the shape of ratios using GAPDH as an internal reference value. It is assumed that the GAPDH expression is the same in both the stimulated and nonstimulated cells, although this may not be entirely true. The induction of TARC mRNA is very clear, however.

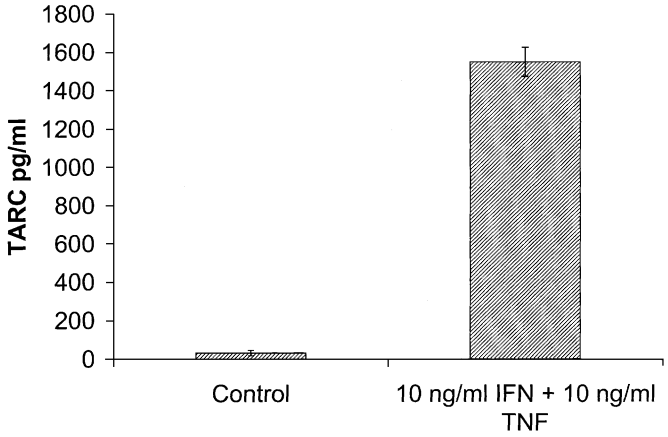

Figure 4. Induction of TARC in normal keratinocytes. Keratinocytes isolated from normal adult human patients undergoing skin surgery were cultured for 24 h without stimulation or stimulated with IFN-γ (10 ng per ml) and TNF-α (10 ng per ml). The result demonstrates that TARC production is induced by IFN-γ and TNF-α in keratinocytes. The error bars indicate the standard deviation (each value represents n = 4, and each measurement was performed in duplicate).

were isolated and RT-PCR was performed. The results (**Fig 3**) revealed that after 2 h the expression level of TARC mRNA reached a maximum, which was almost a 6-fold increase in the expression of TARC mRNA compared with that at 0 h, as determined by densitometric analysis. Within the 9 h the experiments lasted, however, the level of TARC expression did not return to normal levels.

Induction of TARC production in normal human keratinocytes Normal adult human keratinocytes obtained from patients undergoing plastic surgery were grown in cultures either without stimulation or stimulated with IFN-γ (10 ng per ml) and TNF-α (10 ng per ml). The result (**Fig 4**) showed that IFN-γ and TNF-α stimulation induced TARC production at a level similar to that of HaCaT cells (n = 4).

TARC is expressed in the keratinocytes in skin biopsies from AD patients Skin biopsies from seven AD patients were examined. From each patient biopsies of lesional and nonlesional skin were obtained and snap frozen in liquid nitrogen. The biopsies were stained with a monoclonal antibody against TARC, and counterstained with Mayer's hematoxylin. Of the seven patients four patients stained positive for TARC in the keratinocytes (**Fig 5*A***). The TARC-positive cells in the skin were found to be distributed through the different layers of the epidermis, but especially in the basal layers, in a pattern similar to the distribution of MCP-1 in psoriasis skin (Deleuran *et al*, 1996). In the cells TARC seemed to be localized perinuclearly. The biopsies were 3–4 mm in depth; however, we did not observe any TARC expression in the endothelial cells of the vessels of the skin (**Fig 5*B***), or in any other cells. The biopsies of the nonlesional skin did not reveal TARC expression (results not shown).

Lymphocytes invading the skin are CCR4 positive The skin biopsies from three AD patients were collected in serial sections and stained with CD3 and CCR4. The result revealed that the CD3-positive cells are also CCR4 positive (**Fig 5*C*, *D***). The cells are localized mainly along the dermal-epidermal border in the dermis. Some $CD3^+CCR4^+$ cells could also be observed in the epidermis. The cells were identified as lymphocytes according to the CD3 reactivity and the morphology of the cells.

The expression of CCR4 is increased in lymphocytes from the peripheral blood of AD patients PBMC were isolated from the peripheral blood of seven patients with AD, chosen as described in *Materials and Methods*, and five controls who did not suffer from any skin diseases. The cells were stained, after isolation of the PBMC with a mouse anti-human CCR4 antibody, which in turn was stained with a PE-conjugated goat antimouse antibody and with an FITC-conjugated mouse anti-human CLA antibody. The cells expressing CLA were gated and their expression of CCR4 was examined (**Fig 6**). The percentage of the CLA^+ cells expressing CCR4 was significantly ($p < 0.05$) higher in AD patients than in normal controls (90.8% ± 6.2% *vs* 82.7% ± 3.7%), although the number was found to be very high in both AD patients and normal controls. CCR4 was expressed on 42.8% of the CLA lymphocytes (gated according to the forward/side scatter) in AD patients, whereas 25.2% of the CLA^- lymphocytes in normal controls expressed CCR4 (**Fig 7**). This result was not significant, however ($p = 0.053$). To check whether the freezing procedure changed the expression of CCR4 and CLA, PBMC from three normal donors were examined according to the staining protocol before and after freezing. The two groups showed no significant difference in the expression of either CCR4 or CLA. A change in the forward/side scatter pattern, however, was observed but the different groups of cells were still discernible (result not shown).

DISCUSSION

AD is an inflammatory skin disease characterized by invasion of the skin by lymphocytes, monocytes/macrophages, mast cells, and eosinophils (Soter, 1989; Uehara *et al*, 1990). The lymphocytes invading the skin during AD are, at least in the acute phase, Th_2-type lymphocytes, i.e., they produce the cytokines IL-4 and IL-5, as opposed to Th_1-type lymphocytes that produce cytokines such as IL-2 and IFN-γ (Grewe *et al*, 1998; Herz *et al*, 1998). It has been shown, however, that the cytokine-producing pattern during the course of the disease changes from a Th_2-dominated pattern to a

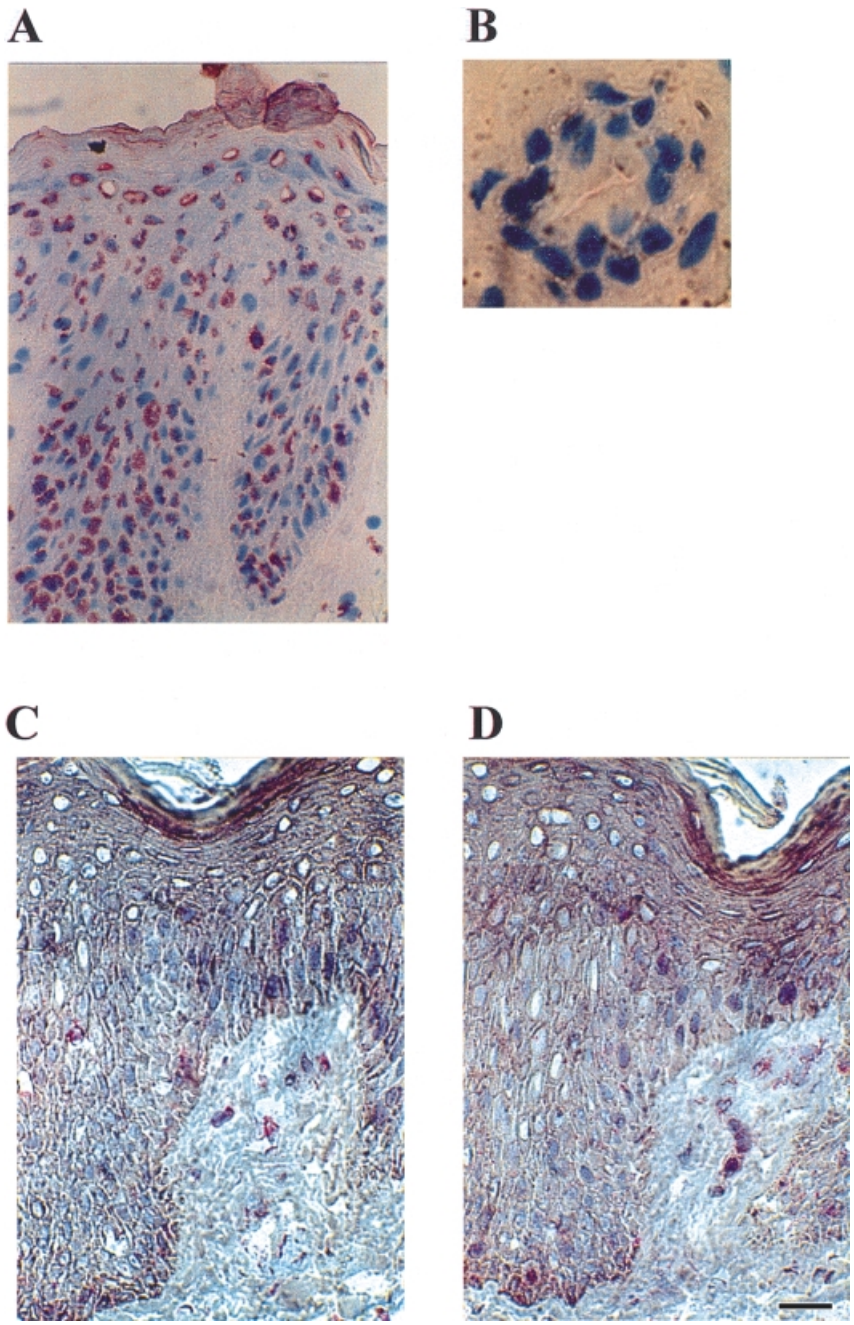

Figure 5. Immunohistochemical stainings of TARC and CCR4. (*A*) TARC staining of a skin biopsy from an AD patient. The red brown staining indicates the binding of anti-TARC antibody. The positive cells are scattered throughout the epidermis, but with a higher concentration in the basal layer. The TARC staining in the keratinocytes is localized perinuclearly. (*B*) TARC staining of a vessel from the dermis of an AD patient. No significant staining of TARC could be found in any of the vessels in the skin from the seven patients examined in this study. (*A*) and (*B*) are representative for four out of seven AD patients (see text). (*C*) CCR4 staining of a skin biopsy from an AD patient. The CCR4-positive cells are red and are seen in both the dermis and the epidermis. Morphologically the cells resemble lymphocytes. (*D*) A skin biopsy from the next serial section (approximately 5 μm) of the same sample as (*C*). The staining is CD3 and the positive cells are seen as red cells. (*C*) and (*D*) are representative of three AD patients. *Scale bars:* (*A,C,D*) 20 μm; (*B*) 10 μm.

more mixed pattern of cytokine production (Ohmen *et al*, 1995; Thepen *et al*, 1996). In particular it has been shown that, if there is no IFN-γ production in the skin after treatment of AD, relapse of the disease is very unlikely (Grewe *et al*, 1994), indicating an important role for the Th_1-type cytokines in AD. Recently it has been demonstrated that the chemokine receptors are expressed in a specific manner between Th_1-type and Th_2-type lymphocytes, with CCR5 and CXCR3 preferentially being expressed on Th_1-type lymphocytes and CCR4 and CCR3 on Th_2-type lymphocytes (Bonecchi *et al*, 1998; Sallusto *et al*, 1998); indeed CCR4 has been suggested to be a specific marker for Th_2-type lymphocytes (Imai *et al*, 1999), although others have reported CCR4 to be expressed on activated Th_1 cells (D'Ambrosio *et al*, 1998).

The lymphocytes circulate through three different types of compartment in the human body that can be divided into the primary, the secondary and the tertiary lymphoid organs. In order to arrive in these organs the lymphocytes express adhesion molecules specific for the target organ. The factors regulating the expression of these homing receptors are largely unknown (Picker and Butcher, 1992). Conversely, in the case of CLA the expression has been found to be regulated through superantigen induction of IL-12 (Leung *et al*, 1995). The effect of IL-12 is mediated through α1,3-fucosyltransferase VII, which is critical in the fucosylation needed for the functional E-selectins (Wagers *et al*, 1998). The mechanism of lymphocyte invasion into the tissues is thought to resemble that of monocytes and neutrophils, being a multistep process involving attachment and rolling through selectin–carbohydrate interactions, activation through chemoattractant–receptor interactions, and firm adhesion through intergrin–immunoglobulin family interactions (Springer, 1994). In this study we have found that in the peripheral blood of AD patients there is a significant upregulation of CLA^+CCR4^+ cells compared with normal controls. It has been shown that in peripheral blood the lymphocytes expressing CCR4 include all the cells expressing CLA and a subset of other systemic memory lymphocytes, but not intestinal T lymphocytes ($\alpha4\beta7^+$) (Campbell *et al*, 1999). In

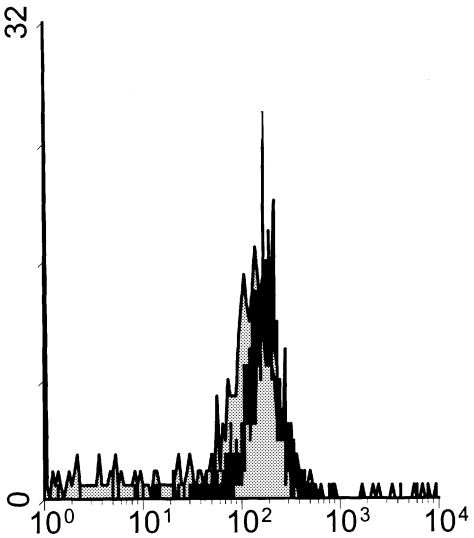

Figure 6. CCR4 expression of the lymphocytes gated positive for CLA expression. It can be seen that the number of CCR4-positive cells in AD patients is higher than in normal controls (gray). The numbers of events from the two patients are the same ± 500. The results are representative for seven AD patients and five controls.

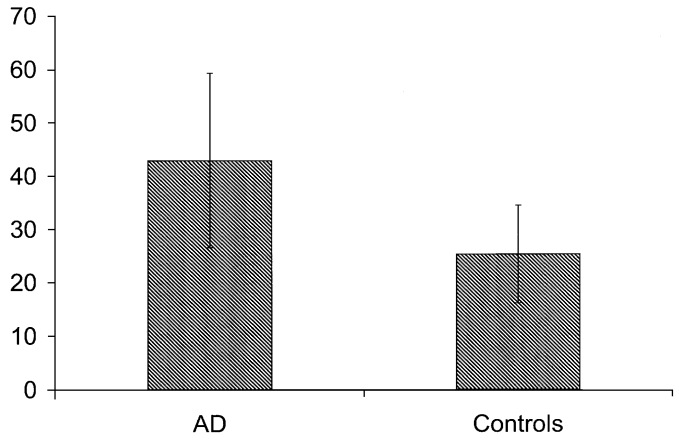

Figure 7. CCR4 expression of CLA⁻ lymphocytes in the peripheral blood of seven AD patients and five controls. CCR4 is expressed on a higher percentage of CLA⁻ lymphocytes in the blood of AD patients than in the normal controls. The result is not statistically significant, however ($p > 0.05$, see text). The *Y* axis shows the percentage of $CCR4^+CLA^-$ lymphocytes. The error bars represent the standard deviation.

addition the staining of serial sections of skin biopsies from AD patients demonstrated that the lymphocytes invading the skin, both the dermis and the epidermis, are $CCR4^+$. As TARC induces chemotaxis of $CCR4^+$ cells (Imai *et al*, 1999), these results indicate that TARC may be an important chemoattractant for the CLA^+CCR4^+ cells and thereby skin-homing lymphocytes in AD. Conversely, other chemokines and chemokine receptors may be involved in the chemotaxis and activation of CLA^+ T cells in the skin. A recently described CC chemokine, cutaneous T-cell-attracting chemokine, which is produced in the skin of both humans and mice, attracts a specific, yet undefined, subset (2%–24%) of the CLA^+ memory T cells (Morales *et al*, 1999). The CLA^+ subset of memory T cells also expresses CCR6, the receptor for MIP-1α, which thereby could act as a chemotactic signal (Liao *et al*, 1999). Moreover it has been shown that IL-8 induces transendothelial migration of CLA^+ T cells (Santamaria Babi *et al*, 1996). These results demonstrate that the process of attracting CLA^+ T cells into the skin is complicated and complex and that overlapping signals rather than a single signal could be responsible for the attraction of T cells - among these TARC and CCR4.

Interestingly, not only did the CLA^+ T cells of the AD patients have an increased expression of CCR4 compared with the normal controls, but so did the CLA^- T cells. The importance of this is unclear but it could be speculated that it is due to a general "Th_2 activation" of the lymphocytes in the blood of AD patients.

It has been shown that TARC is produced in endothelial cells of the venules of chronically inflamed human skin (Campbell *et al*, 1999); however, it has also been shown that TARC is produced by basal keratinocytes in a murine model of AD (Vestergaard *et al*, 1999). We therefore examined skin biopsies from AD patients for expression of TARC. We found expression of TARC in the keratinocytes of lesional skin in four out of seven patients suffering from AD; however, we did not find any expression of TARC in the venules of the skin. This does not mean that endothelial cells cannot produce TARC in AD skin as the production of cytokines in the course of AD is thought to be a sequential line of events (Grewe *et al*, 1998; Herz *et al*, 1998). This could also explain why TARC is not found in all patients. Endothelial cells have the ability to present cytokines at sites of inflammation (Middleton *et al*, 1997; Utgaard *et al*, 1998) and TARC mRNA has been reported to be found in endothelial cells (Campbell *et al*, 1999). The fact that the keratinocytes produce TARC is not surprising, as this has already been shown in mice (Vestergaard *et al*, 1999), and it is known that keratinocytes are able to produce other inflammatory cytokines (Barker *et al*, 1991). The pattern of expression in the keratinocytes was found to be similar to that of MCP-1 in keratinocytes of lesional psoriasis (Deleuran *et al*, 1996). Earlier it was shown (Vestergaard *et al*, 1999) that IFN-γ and TNF-α could induce TARC production in murine basal carcinoma cells. In order to examine stimulatory mechanisms for TARC production in human keratinocytes, we initially used human HaCaT cells as a model for human keratinocytes (Boukamp *et al*, 1994), to find suitable concentrations of the stimuli and to study the mechanism of induction. This was done as keratinocytes are very difficult to maintain in a nondifferentiated state in culture. The suitable concentrations of the stimuli found in the experiments from the HaCaT cells were then used in keratinocyte culture experiments. We observed as in murine basal carcinoma cells that IFN-γ and to a larger degree TNF-α could induce TARC production in both HaCaT cells and keratinocytes. When these cytokines were used for stimulation in combination they had a synergistic effect on TARC production, as could be seen by both TARC mRNA (only performed in the HaCaT cells) and protein production. Many studies have shown that IFN-γ is produced in later stages of AD (Grewe *et al*, 1994; Thepen *et al*, 1996; Akdis *et al*, 1999), although it is a Th_1-type cytokine and AD is generally thought to be a Th_2-type disease. We have shown in this study that IFN-γ is able to induce a Th_2 lymphocyte chemokine (Imai *et al*, 1999), TARC. TNF-α has been reported to be upregulated in AD lesional skin (Ackermann and Harvima, 1998), and to induce adhesion molecules in the endothelial cells of the skin (de Vries *et al*, 1998), which, taken together with the results presented here, suggests that TNF-α has a dual role, as suggested in the murine model of AD (Vestergaard *et al*, 1999), as an inducer of two of the three steps in the multistep model of cell migration (Springer, 1994).

In summary, we have shown that both human HaCaT cells and keratinocytes produce TARC in response to IFN-γ and TNF-α, which are known to be upregulated in lesional AD skin. In addition we have shown that TARC is produced in the lesional skin of four out of seven AD patients, that a larger population of CLA^+CCR4^+ lymphocytes is circulating in the peripheral blood of AD patients than in normal controls, and that the lymphocytes invading the skin are $CCR4^+$, suggesting that TARC produced by keratinocytes in lesional AD skin in certain stages of AD may be an important chemotactic signal for the skin-homing lymphocytes in AD.

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