

IL-31 is associated with cutaneous lymphocyte antigen–positive skin homing T cells in patients with atopic dermatitis

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Background: IL-31 is a newly discovered T-cell cytokine that, when overexpressed in mice, results in pruritus and skin dermatitis resembling human atopic dermatitis (AD).

Objective: We sought to investigate the expression of IL-31 and IL-31 receptor A (IL-31RA) in skin biopsy specimens and peripheral blood cells from patients with AD and healthy individuals.

Methods: Expression of IL-31 and IL-31RA was evaluated in skin biopsy specimens from patients with AD and healthy individuals by means of immunohistochemistry and RT-PCR. IL-31 protein production by skin-homing cutaneous lymphocyte antigen (CLA)–positive T cells was also assessed. **Results:** IL-31RA protein was expressed by keratinocytes and infiltrating macrophages in skin biopsy specimens from patients with AD. Comparisons between skin from patients with AD and healthy skin showed IL-31RA expression at higher levels on epidermal keratinocytes in AD samples. Infiltrating cells, more numerous in skin from patients with AD compared with that of healthy individuals, expressed *IL31* mRNA.

Histomorphometric analysis of these cells indicated they were of the lymphocytic lineage, with the majority of cells staining positive for CLA and CD3. *IL31* mRNA and protein expression is largely restricted to CD45RO⁺ (memory) CLA⁺ T cells in peripheral blood of patients with AD and healthy volunteers. Moreover, circulating CLA⁺ T cells from patients with AD, but not from patients with psoriasis, are capable of producing higher levels of IL-31 compared with CLA⁺ T cells from healthy individuals. However, the average levels of IL-31 were not significantly different between patients with AD and healthy individuals.

Conclusion: We provide evidence that IL-31 expression is associated with CLA⁺ T cells and might contribute to the development of AD-induced skin inflammation and pruritus. (J Allergy Clin Immunol 2006;117:418-25.)

Key words: Atopic dermatitis, pruritus, IL-31, IL-31 receptor A, keratinocytes, macrophages, skin-homing T cells

AD is a chronic inflammatory skin disease associated with marked pruritus.¹ The cause of pruritus in AD is unknown. IL-31 is a newly described 4-helix bundle cytokine that is expressed by activated T cells and is preferentially produced by T cells skewed toward a T_H2-type cytokine profile.² Overexpression of IL-31 in transgenic mice induces alopecia and chronic pruritus that ultimately results in the development of skin lesions through excoriation from excessive scratching, a condition similar to that observed in patients with AD. Histopathology of lesional skin from these animals confirms the similarities in pathology with skin lesions from patients with AD.² Other T cell–derived cytokines, like IL-4 and IL-13, have been implicated in the pathogenesis of AD, particularly because these cytokines are produced by a subset of T cells that have the distinct capacity to home to the skin through expression of the skin-homing receptor cutaneous lymphocyte antigen (CLA).³ CLA is a carbohydrate structure that is expressed on memory T cells as an epitope of a single cell-surface protein named P-selectin glycoprotein ligand 1 and facilitates binding of T cells to E-selectin, an inducible adhesion molecule expressed on vascular endothelium.^{4,5} Approximately 90% of infiltrating T cells in inflamed cutaneous sites express CLA, whereas circulating CLA⁺ T cells represent only approximately 15% of the peripheral T-cell population.⁶ In patients with AD, there is a significant increase in circulating CLA⁺ T cells, and these cells have an augmented capability to produce IL-4 and IL-13 compared with cells from control individuals³ and respond to allergens more vigorously compared with CLA[–] cells. Hence CLA⁺ T cells are probably the most relevant T-cell population in the initiation and maintenance of dysregulated immune responses to cutaneous allergen insult in these patients.⁷

In the skin cytokines released by T cells interact with cells in the local environment, including keratinocytes and local antigen-presenting cells, to induce production of chemokines, cytokines, or other proinflammatory molecules. Keratinocytes in particular can produce a panel of cytokines and chemokines that not only induce cellular infiltration^{1,8,9} but can also influence antigen-presenting cell–mediated skewing of T-cell cytokine profiles.¹⁰

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Abbreviations used

AD:	Atopic dermatitis
CLA:	Cutaneous lymphocyte antigen
HPRT:	Hypoxanthine-guanine-phosphoribosyl-transferase
IHC:	Immunohistochemistry
IL-31RA:	IL-31 receptor A
LCM:	Laser capture microdissection
MDC:	Macrophage-derived chemokine
OSMR:	Oncostatin M receptor
TARC:	Thymus and activation-regulated chemokine

Dysregulated production of these inflammatory mediators is thought to be a contributing factor to the development of AD. Primary normal human keratinocytes express both subunits of the receptor for IL-31, namely IL-31RA and oncostatin M receptor (OSMR), and respond to IL-31 stimulation by upregulating mRNA-encoding chemokines known to induce infiltration of immune cells.² Both thymus and activation-regulated chemokine (TARC; CCL17) and macrophage-derived chemokine (MDC; CCL22) mRNAs were found to be upregulated by keratinocytes after stimulation by IL-31. Both chemokines have been implicated in the recruitment of T cells into the skin of patients with AD and might contribute to the inflammatory process associated with the pathogenesis of AD.¹¹⁻¹⁴

In this study we investigate the potential association of IL-31 with the pathogenesis of AD in human subjects. We analyzed skin biopsy specimens from both patients with AD and healthy individuals for the expression of *IL31* and IL-31 receptor and measured IL-31 protein produced by peripheral blood T-cell populations, focusing on skin-homing T cells from both patients with AD and control individuals.

METHODS

Study subjects

This study was approved by the Institutional Review Board at National Jewish Medical and Research Center. Twelve patients with moderate-to-severe AD (median age, 32 years; skin involvement, 5% to 45%) according to the criteria of Hanifin and Rajka,¹⁵ 6 patients with psoriasis (median age, 56 years; skin involvement, 10% to 65%), and 12 healthy individuals (median age, 34 years) were included in the study. Informed consent was obtained from all subjects before the study commenced. None of the patients had previously received any systemic corticosteroids. All patients were off topical corticosteroids for 1 week before skin biopsy or blood draws. Two-millimeter punch biopsy specimens were taken from acute erythematous AD lesions of less than 3 days' onset and from healthy skin. The skin samples were immediately frozen at -70°C for immunohistochemistry.

T-cell subset isolation from peripheral blood

Human PBMCs from donors were isolated with standard Ficoll-gradient centrifugation. Total T cells were isolated with the T Cell Isolation Kit II (Miltenyi Biotec, Auburn, Calif), according to the manufacturer's instructions. Separation efficiency was assessed by using standard flow cytometry, and purity of samples was determined

to be greater than 95% T cells. To separate naive (CD45RA^{+}) T cells from the memory (CD45RO^{+}) T cells, the total T-cell population was incubated with anti-CD45RO magnetic microbeads (Miltenyi Biotec) for 15 minutes at 4°C and isolated with a magnetic column, according to the manufacturer's instructions. The naive and memory T-cell populations were determined to be greater than 90% pure by means of flow cytometry.

CLA^{+} T cells were isolated from total T cells by means of magnetic bead separation with a 1:50 dilution of anti-CLA-FITC antibody (PharMingen, San Diego, Calif) and anti-FITC microbeads (Miltenyi Biotec), as per the manufacturer's instructions. The labeled T cells were determined to be greater than 80% pure by means of flow cytometry, whereas the CLA-depleted T cells were more than 98% pure. Both CLA^{+} and CLA^{-} T cells were collected and cultured concurrently.

Activation of T cells

CD45RA^{+} and CD45RO^{+} T-cell subsets cells were activated with $2.0\text{ }\mu\text{g/mL}$ plate-bound anti-CD3 and $2.0\text{ }\mu\text{g/mL}$ soluble anti-CD28 (BD PharMingen) in 24-well tissue-culture plates at 37°C . Cells were plated at a concentration of 2.5×10^6 cells/mL in tissue culture media (RPMI, 5% FBS, L-glutamine, and sodium pyruvate; Gibco, Grand Island, NY). After 24 hours, cells were collected, and conditioned media was stored at -20°C .

CLA^{+} and CLA^{-} T-cell subsets were activated similarly in 48-well tissue-culture plates. The cells were activated for 24 hours at 37°C at a concentration of 6.25×10^5 cells/mL. Cells were collected, and conditioned media was stored at -20°C . For suboptimal activation, CLA^{+} T cells were cultured in plates precoated with $0.5\text{ }\mu\text{g/mL}$ anti-CD3 antibody.

Analysis of IL-31 mRNA by using quantitative real-time PCR

Expression of IL-31 mRNA was determined by using multiplex real-time TaqMan PCR with the ABI PRISM 7900 sequence detection system (PE Applied Biosystems, Foster City, Calif). Levels of mRNA were calculated relative to hypoxanthine-guanine-phosphoribosyl-transferase (*HPRT*). The following sequences are the forward, reverse, and probe oligonucleotide primers for *IL31* (forward, 5'-TGG ACC TCG CAC TAA AAT CAT TG-3'; reverse, 5'-CGA AAG GAA GAG ATG GCC TTA A-3'; probe 5'-TCT GGA GCC CAA CAG GCC ACC A-3') and for *HPRT* (forward, 5'-GAC TTT GCT TTC CTT GGT CAG G-3'; reverse, 5'-AGT CTG GCT TAT ATC CAA CAC TTC G-3'; probe, 5'-TTT CAC CAG CAA GCT TGC GAC CTT GA-3').

Bioassay for human IL-31 protein quantification

BaF3, a mouse pre-B-cell line¹⁶ expressing IL-31RA, OSMRB, and KZ134 (a signal transducer and activator of transcription-activated luciferase reporter), as previously described,² were grown to 5×10^5 and 1×10^6 cells/mL. Cells were washed and resuspended at 3×10^5 cell/mL in assay medium (RPMI 1640, 10% FBS, L-glutamine, sodium pyruvate, and Pen/Strep; Gibco). In a 96-well opaque plate, human IL-31 standards were titrated in duplicate from 600 pg/mL to 9.38 pg/mL in assay medium in $100\text{ }\mu\text{L}$ per well, 1:2 serial dilutions. Quality control standards comprising high (350 pg/mL) and low (35 pg/mL) concentrations of human IL-31 spiked in human serum were added in duplicate to the plate in $100\text{ }\mu\text{L}$. Test samples were diluted 1:2 or 1:4 and added in duplicate. One hundred microliters of the washed BaF3 cells were then added to each well for a final concentration of 3×10^4 cells/well. The plate was then incubated for 16 to 24 hours at 37°C in a 5% CO_2 incubator. Luminescence values were analyzed, and IL-31 levels were calculated and expressed as

TABLE I. Results of IHC for IL-31RA in skin biopsy specimens from patients with involved and uninvolved AD compared with healthy volunteers

Case ID	IL-31RA IHC score*†	CD3 IHC score*†
AD-1	2-3	0-1
AD-2	2-3	2
AD-3	2-3	1-2
AD-4	3	1
AD-5	2	2
UAD-1	1-2	1
UAD-2	1	0-1
UAD-5	1-2	0-1
UAD-6	2-3	ND
UAD-7	2	1
UAD-8	1	1
UAD-9	1-2	1
UAD-10	2	ND
Normal-1	1	0-1
Normal-2	0-1	0-1
Normal-3	1	0-1

UAD, Uninvolved AD; ND, not done.

*IHC signal was based on signal intensity and scored from 0 (no signal) to 4 (intense signal).

†Example shown in Figs 1 and 2.

picograms of IL-31 per 10^6 cells/mL. Samples of less than the limit of detection of the assay were designated as 0 pg of IL-31 per 10^6 cells/mL.

Immunohistochemistry for IL-31RA

Skin biopsy specimens were fixed overnight in 10% normal buffered formalin and embedded in paraffin with standard techniques. Five-micrometer sections were baked at 61°C for 30 minutes for tissue adhesion. Slides were subsequently dewaxed in 3×5 -minute incubations in xylene and rehydrated 2×2 minutes in 100% EtOH and then 95% EtOH and 1×2 minutes in 70% EtOH. After rinsing, heat-induced epitope retrieval was performed for 20 minutes under steam. Slides were cooled to room temperature in 10 mM Tris and 1 mM EDTA, pH 9.0, and loaded onto a DakoCytomation Autostainer (Dako, Glostrup, Denmark), rinsed with Tris-buffered saline Tween buffer, and prepared as recommended by the manufacturer. Endogenous biotin was blocked with a 10-minute incubation in avidin solution, followed by a 10-minute incubation in biotin solution. A protein block (phosphate-buffered saline block [PBSB]; 0.5% Blocking Powder in PBS; Perkin Elmer, Boston, Mass) was applied for 30 minutes. Primary antibodies, diluted from 665 ng/mL to 1330 ng/mL for IL-31RA, were applied for 60 minutes in ChemMate Antibody Dilution Buffer (Ventana Medical systems, Tucson, Ariz). Slides were washed twice in Tris-buffered saline Tween buffer, incubated for 45 minutes in 750 ng/mL biotinylated goat anti-rabbit antibody in PBSB (Vector Labs, Burlingame, Calif), washed twice, and then incubated with Vectastain Elite ABC Reagent (Vector Labs, Burlingame, Calif) for 45 minutes and washed twice. Signals were developed with DAB+ (DakoCytomation, Glostrup, Denmark) for 10 minutes at room temperature. Slides were counterstained in hematoxylin (Vector Labs), dehydrated, and placed in coverslips in VectorMount (Vector Labs). The scoring system for analysis of IL-31RA expression is based on signal intensity.

For double staining, tissues were incubated with primary and cell identification marker antibodies simultaneously for 60 minutes in ChemMate Antibody Dilution Buffer (Ventana Medical Systems). Tissues were then incubated for 30 minutes with Dako Envision+ and

anti-rabbit peroxidase (DakoCytomation), washed twice followed by an 8-minute incubation of tyramide: alexa555 (Invitrogen, Carlsbad, Calif), washed, and incubated for 45 minutes in either biotinylated horse anti-mouse IgG (Vector Labs) or biotinylated goat anti-rat IgG (Vector Labs). Tissues were subsequently washed in Tris-buffered saline and incubated for 30 minutes in SA:Qdot 705 (Quantum Dot Corp, Hayward, Calif), rinsed in Tris-buffered saline, counterstained with DAPI (Invitrogen) for 5 minutes, rinsed again in Tris-buffered saline and then distilled water, and mounted with Polyvinyl Alcohol mounting media with DABCO (Sigma, St Louis, Mo).

Isolation of epidermal cells and cellular infiltrates from skin biopsy specimens

Epidermal cells and cellular infiltrates were isolated from skin tissue sections on slides by using the PixCell Ite Laser Capture Microdissection (LCM) System (Arcturus Engineering, Mountain View, Calif).

Slides were dewaxed in xylene 3×5 minutes and incubated for 5 minutes each in 100%, 95%, and 70% EtOH; stained in Gill's Hematoxylin formula (Vector H-34010); washed with water; dehydrated in graded ethanol solutions (70%, 95%, and 100%) for 5 seconds each; and cleared by 3 incubations in xylene for 5 minutes each. Approximately 50 infiltrating cells or 500 epidermal cells were isolated by LCM per HS CapSure cap receptacle. Ambion's Optimum FFPE RNA isolation protocol (Ambion, Austin, Tex) was used with the following modifications: 55 μ L of Proteinase K solution was added to the cap and covered with a 500- μ L microcentrifuge tube. The tubes were inverted and incubated at 37°C overnight in a hybridization oven. After gently vortexing the cap, the tube was centrifuged at 4000g for 2 minutes. The final volume of cell lysate in the tube was adjusted to 55 μ L with sterile RNase-free water. One hundred microliters of RNA extract buffer was added, the tube was vortexed, and then 80 μ L of 100% ethanol was added. Total RNA was isolated after Ambion's FFPE protocol and DNase I treatment and eluted in 75°C RNase-free water.

RT-PCR of LCM samples

The LCM RNA was DNase treated as per the manufacturer's instructions (Ambion) and analyzed by means of RT-PCR for *IL31* and *HPRT*. cDNA was synthesized with Invitrogen's 1st Strand cDNA kit (Invitrogen). PCR amplification was performed in a 25- μ L reaction, containing 5 μ L of the cDNA mix (for *IL31*) or 1 μ L of the cDNA mix (for *HPRT*) and BD Biosciences Clontech's Advantage2 PCR Mix (BD Biosciences, San Jose, Calif). The primers for amplification of *IL31* producing a 132-bp fragment were 5' GTC TTG GTA TTT ATG GAA TGC 3' and 5' CCA GGG AGC ATT GAC AAC TCT TAG 3', and the primers used for *HPRT* producing a 101-bp fragment were 5' GAC TTT GCT TTC CTT GGT CAG G and 5' AGT CTG GCT TAT ATC CAA CAC TTC G.

Amplification proceeded for either 27 (*HPRT*) or 33 (*IL31*) cycles of 20 seconds each at 94°C, 61°C, and 70°C in the Perkin Elmer 9600 cycler. PCR products were visualized on a 3% agarose gel stained with ethidium bromide. Semiquantitative analysis of *IL31* mRNA gene expression was performed with ImageQuant 5.2 software (Amersham Biosciences, Pittsburgh, Pa).

RESULTS

Analysis of IL-31 and IL-31RA in the skin of patients with AD

Our initial studies demonstrated that IL-31 is a class I cytokine produced by activated T cells and is

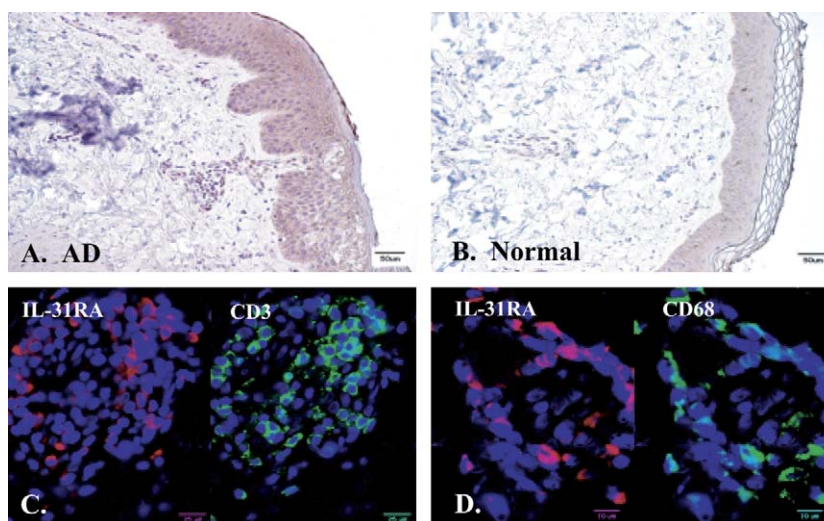


FIG 1. IL-31RA in skin biopsy specimens from patients with AD (**A**) compared with healthy individuals (**B**) was determined by means of IHC (40× magnification). Immunofluorescent costaining of infiltrating cells present in skin biopsy specimens from patients with AD with IL-31RA and CD3 (**C**) or CD68 (**D**) is shown. These data are representative of skin biopsy specimens from 5 individual patients with AD and 3 healthy individuals (Table I).

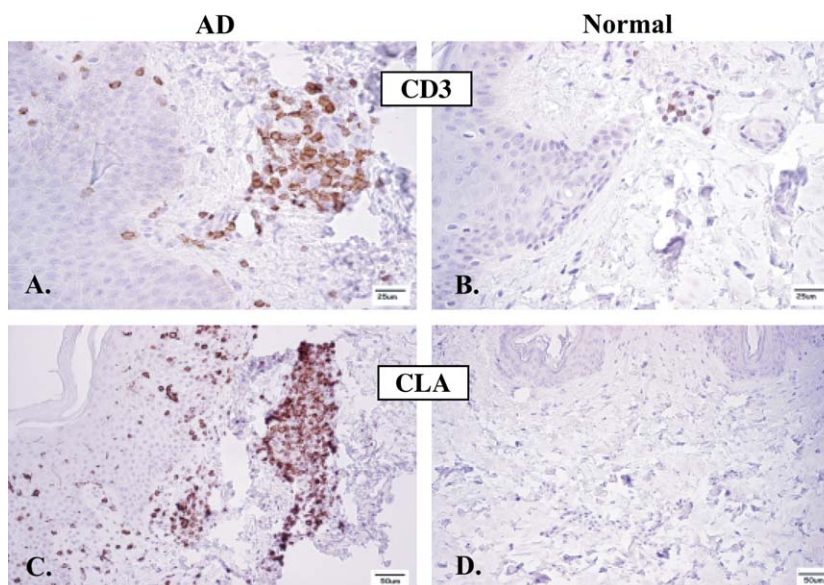


FIG 2. CD3⁺ T cells in skin biopsy specimens from patients with AD (**A**) compared with healthy individuals (**B**). CLA⁺ cells in skin biopsy specimens from patients with AD (**C**) compared with that in skin from healthy individuals (**D**; 40× magnification) are shown. These data are representative of skin biopsy specimens from 5 individual patients with AD and 3 healthy individuals (Table I).

preferentially made by T cells skewed toward a T_H2-type phenotype.² Primary keratinocytes express both receptor components for IL-31 and can respond to IL-31 stimulation through production of chemokines.² Given the potential role of keratinocytes in perpetuating a dysregulated immune response in dermatitis, we examined skin punch biopsy specimens from patients with AD for IL-31RA expression by means of immunohistochemistry (IHC). Table I lists the IHC results on the basis of signal intensity

for 5 lesional AD skin biopsy specimens, 8 uninvolved AD skin biopsy specimens, and 3 normal control samples. Collectively, these data demonstrate that IL-31RA is expressed by epidermal keratinocytes from both patients with AD and healthy control subjects. However, the levels of IL-31RA expressed on keratinocytes from AD skin biopsy specimens (Table I and Fig 1, A) were consistently higher than the levels observed in skin biopsy specimens from healthy control subjects (Table I and Fig 1, B),

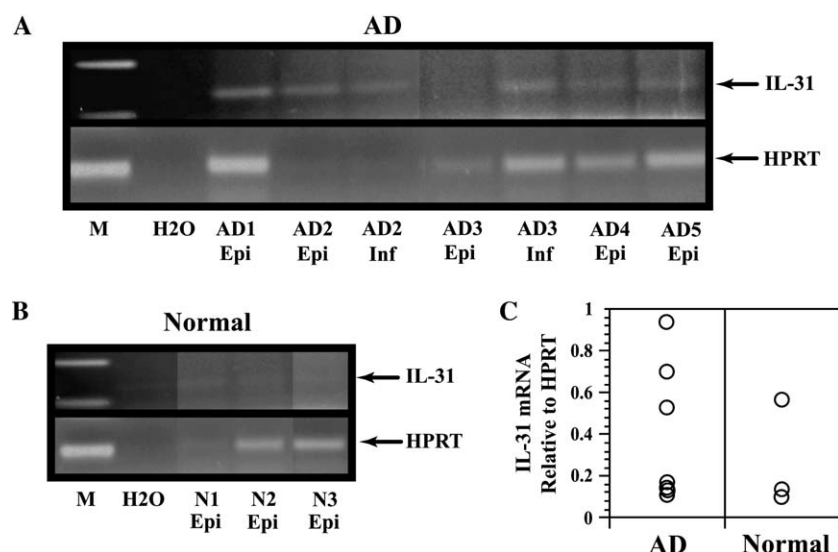


FIG 3. IL-31 mRNA in the epidermal keratinocyte layer (*Epi*) and infiltrating cells (*Inf*) of skin biopsy specimens from patients with AD (**A**) or the epidermal keratinocyte layer of healthy individuals (**B**) is shown. Water was included as a negative control. *M*, Molecular weight markers. **C**, Semiquantitative analysis of IL-31 mRNA gene expression relative to an internal housekeeping gene, HPRT (IL-31/HPRT), is shown.

TABLE II. IL-31 protein production by CD45RO⁺ and CD45RA⁺ peripheral blood T cells in healthy individuals after 24-hour stimulation with anti-CD3 and anti-CD28 (picograms per milliliter per million cells)

T-cell subset	Donor 1	Donor 2	Donor 3
CD45RO ⁺	110.4	145.6	267.1
CD45RA ⁺	13.1	12.7	14.6

indicating a potential for increased responsiveness to IL-31 in the context of AD.

IL-31RA was also found to be expressed on a subset of perivascular infiltrating cells that were more obvious in skin biopsy specimens from patients with AD compared with control skin samples (Fig 1, A and B, respectively). Skin biopsy specimens from patients with AD and healthy subjects were double stained with anti-IL-31RA antibodies and antibodies specific for cell-subset markers, including CD3 (T lymphocytes), CD68 (macrophages), or α mast cell tryptase (mast cells), to identify the cell type expressing IL-31RA. These costaining experiments showed that the perivascular infiltrating IL-31RA⁺ cells were CD68⁺ macrophages (Fig 1, D) and not T cells (Fig 1, C) or mast cells (data not shown). This observation supports previous *in vitro* studies that demonstrate that IL-31RA is expressed by macrophages.^{2,17,18}

The presence of skin-infiltrating T cells is a distinguishing feature in skin biopsy specimens from patients with AD compared with those from healthy individuals.¹⁹ Because IL-31 is a T cell–associated cytokine, we examined whether IL-31 was expressed in skin-infiltrating T cells in tissue biopsy specimens. First, we confirmed that skin tissue biopsy specimens from patients with AD showed increased numbers of CD3⁺ and CLA⁺ T cells

by means of IHC compared with those seen in biopsy specimens from healthy subjects (Fig 2, A and B, and Fig 2, C and D, respectively).^{3,20} We subsequently used LCM to specifically isolate skin-infiltrating cells for analysis of *IL31* mRNA by means of RT-PCR. Fig 3, A, shows that *IL31* mRNA was expressed by skin-infiltrating cells isolated from biopsy specimens of patients with AD. In normal tissues infiltrating cells are not usually found and therefore could not be analyzed. However, we did analyze the epidermal keratinocyte layer from both AD and normal skin for *IL31* mRNA (Fig 3, A and B). Semiquantitative analysis of *IL31* mRNA expression compared with that of an internal control gene (*HPRT*) showed that although *IL31* mRNA levels were not significantly different between samples from patients with AD and samples from healthy subjects, there was a trend toward higher *IL31* expression in skin from patients with AD (Fig 3, C).

Our data demonstrate that IL-31 is produced both locally in the skin and by skin-infiltrating cells in patients with AD compared with control subjects. Local production of cytokines in tissues by T cells is thought to be a key mechanism for disease pathogenesis in AD, and increased numbers of T cells both in circulation and in skin is thought to correlate with disease.^{3,20} Given our previous finding that IL-31 overexpression in mice results in pruritus, these data suggest that IL-31 overexpression in the skin of patients with AD might contribute to the development, maintenance, or both of disease.

IL-31 is produced by memory T cells with a skin-homing phenotype

CLA⁺ T cells, which almost always express the CD45RO⁺ memory-effector phenotype,²¹ have been implicated as a major contributor to the pathology of

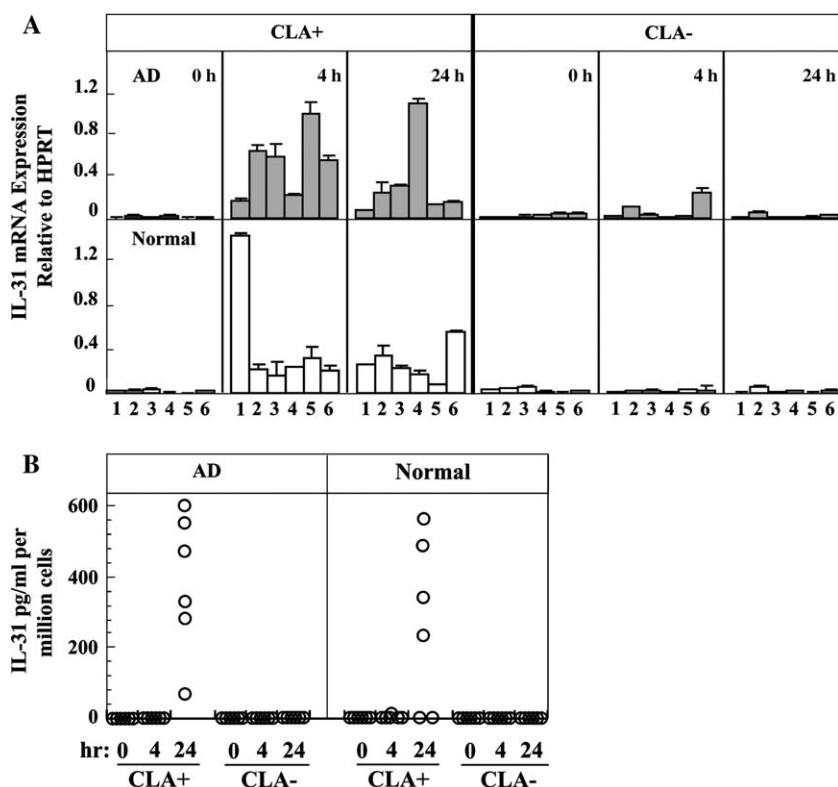


FIG 4. CLA⁺ and CLA⁻ T cells from 6 patients with AD and 6 healthy individuals were stimulated for 0, 4, or 24 hours with anti-CD3 and anti-CD28. IL-31 mRNA was assayed by means of quantitative PCR (**A**), and culture supernatants were analyzed for IL-31 (**B**). Statistical analysis was done with the nonparametric Mann-Whitney *U* test.

AD.^{3,7,22-24} Of the total T-cell population in normal human peripheral blood, we have found that IL-31 expression is largely restricted to CD45RO⁺ memory-effector cells rather than the CD45RA⁺ naive T-cell population (Table II). To determine whether IL-31 production was associated with CLA⁺ skin-homing T cells, we isolated CLA⁺ and CLA⁻ T cells from peripheral blood of patients with AD and control volunteers and compared IL-31 mRNA and protein levels after anti-CD3 plus anti-CD28 stimulation. Our results indicate that *IL31* mRNA was significantly increased in CLA⁺ T cells compared with the CLA⁻ subset from both patients with AD and healthy individuals at both 4 hours ($P = .0087$ and $P = .0022$, CLA⁺ compared with CLA⁻ subsets for patients with AD and healthy control subjects, respectively) and 24 hours ($P = .0022$, CLA⁺ compared with CLA⁻ subsets for both patients with AD and healthy control subjects) after stimulation (Fig 4, A). Analysis of IL-31 protein levels in culture supernatants confirmed that IL-31 was produced predominantly by CLA⁺ T cells because there was no detectible IL-31 in culture supernatants from CLA⁻ T cells from both patients with AD and control individuals (Fig 4, B). However, there were no significant differences in CLA⁺ T-cell IL-31 levels between patients with AD and healthy subjects. Therefore the increased IL-31 mRNA levels measured in the skin of patients with AD

is likely due to increased numbers of infiltrating CLA⁺ T cells. We also analyzed the production of IL-31 by peripheral blood T cells from healthy volunteers that express other tissue-specific homing markers, such as the gut-specific homing marker $\alpha 4\beta 7$. Comparison of the IL-31 levels produced by CLA⁺ T cells and $\alpha 4\beta 7$ ⁺ cells demonstrated that CLA⁺ T cells preferentially produce IL-31 compared with the $\alpha 4\beta 7$ ⁺ cells (average of 34.5 pg/mL and 14.42 pg/mL IL-31, respectively; data not shown).

Although both patients with AD and healthy control subjects have circulating CLA⁺ T cells that express IL-31 on activation, CLA⁺ T cells from patients with AD are reported to exist in a more activated state compared with cells from healthy individuals.²⁴ Consequently, the threshold of stimulation required for the production of IL-31 by CLA⁺ T cells might differ between patients with dermatitis and control subjects. To test this hypothesis, we stimulated CLA⁺ T cells from patients with AD and control individuals with suboptimal concentrations of anti-CD3 in the absence of anti-CD28 and analyzed the production of IL-31 in culture supernatants at 24 hours after stimulation. Our results demonstrate that circulating CLA⁺ T cells from some patients with AD produce higher levels of IL-31 compared with cells from healthy individuals, with maximum levels reaching 1200 pg/mL. By comparison, the maximal detected level of IL-31 in CLA⁺

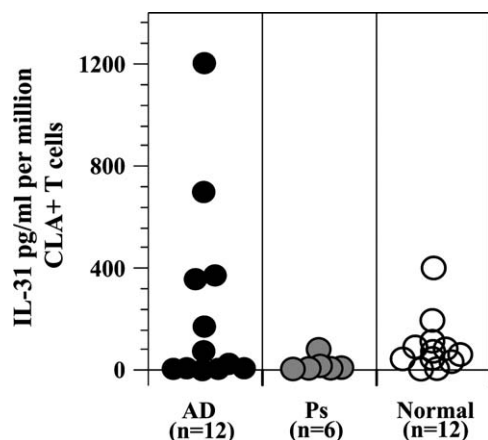


FIG 5. CLA⁺ T cells from 12 patients with AD, 6 patients with psoriasis (Ps), and 12 healthy individuals were stimulated with a sub-optimal concentration of anti-CD3 alone for 24 hours. Culture supernatants were collected, and IL-31 protein levels were assessed. Samples below the limit of detection were designated as 0 pg/ml IL-31.

supernatants from healthy individuals was only 400 pg/mL, and the maximal detected levels for patients with psoriasis was 73 pg/mL at suboptimal concentrations of anti-CD3 stimulation (Fig 5). Five of 12 patients with AD had IL-31 levels of less than the limit of detection of our assay, suggesting there might be a subset of patients with AD in which IL-31 is produced at low levels. This might reflect variations in the stage of disease of our study population. Nevertheless, half of the patients with AD showed a trend toward higher IL-31 levels compared with those seen in patients with psoriasis and healthy individuals after suboptimal stimulation with anti-CD3. Because more CLA⁺ T cells are localized in the skin of patients with AD patients compared with that of healthy individuals, our studies suggest that there is an increased potential for IL-31 activity in the AD skin microenvironment.

DISCUSSION

IL-31 is a newly characterized T cell–derived cytokine that signals through a class 1 cytokine heterodimeric receptor complex composed of IL-31RA and OSMR. Both IL-31RA and OSMR are constitutively expressed on epithelial cells, including keratinocytes, and can be induced on activated macrophages.² We have recently shown that overexpression of IL-31 in transgenic mice or administration of IL-31 protein induces severe pruritus, alopecia, and dermatitis.² Production of IL-31 by T cells *in vitro* is favored by, but not restricted to, the presence of T_H2-type skewing conditions. In human AD, allergen-specific T cells that are preferentially found in the skin-homing CLA⁺ fraction of memory cells spontaneously produce T_H2-type cytokines such as IL-4 and IL-13, and are implicated in the pathology of disease.^{7,24}

In this study we present evidence that circulating CLA⁺ T cells produce IL-31 after activation and that CLA⁺ skin-homing T cells located in the epidermis of patients with

AD express *IL31* mRNA. Moreover, skin epidermal keratinocytes and infiltrating macrophages in skin tissue sections from patients with AD show increased expression of IL-31RA, thus potentiating IL-31 responsiveness. Previous studies *in vitro* have shown that IL-31 stimulation of keratinocytes induces the production of key chemokines, such as I-309 (CCL1), macrophage inflammatory protein 1β (CCL4), thymus-expressed chemokine (CCL25), TARC (CCL17), MDC (CCL22), myeloid progenitor inhibitory factor-1 (CCL23), and Groα (CXCL1), that contribute to the recruitment of lymphocytes, monocytes, and polymorphonuclear cells to the epidermis.² Both IL-31-inducible TARC and MDC have been implicated in AD because of their ability to recruit T_H2-enriched CCR4⁺ CLA⁺ T cells to the skin.^{11-14,25} Indeed, TARC has been suggested as a biomarker for disease in AD because clinical improvement after steroid treatment is associated with a decrease in TARC serum levels.²⁶ Given that IL-31 can induce TARC and MDC to recruit CLA⁺ T cells that have the potential to produce IL-31, our data suggest that IL-31 not only contributes to inflammation but might amplify progression of disease in AD.

Although our studies suggest that circulating CLA⁺ T cells from both patients with AD and healthy individuals produce IL-31 on activation, there is a trend toward higher levels of IL-31 from cells of patients with AD. Moreover, skin from patients with AD generally contains greater numbers of CLA⁺ T cells than skin from healthy individuals.¹⁹ This suggests that activation of larger numbers of CLA⁺ T cells in skin from patients with AD will result in higher IL-31 levels in proximity to cells expressing more IL-31 receptor.

Of particular note was the finding that minimal IL-31 levels could be detected from CLA⁺ T cells of patients with psoriasis. Unlike AD, pruritus is not considered to be a characteristic of psoriasis and does not correlate with Severity Index Scores.²⁷ Our data therefore provide further evidence for the association of IL-31 with pruritic skin diseases. Combined with our previous findings,² these data provide compelling evidence that IL-31 might play a role in both the initiation and perpetuation of AD and that blocking IL-31 could be therapeutic for the treatment of AD in human subjects.

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