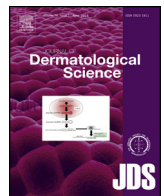




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Distribution of IL-31 and its receptor expressing cells in skin of atopic dermatitis

Atsuhiko Kato^{a,*}, Etsuko Fujii^a, Takeshi Watanabe^b, Yoshiaki Takashima^a, Hiroaki Matsushita^a, Takuya Furuhashi^c, Akimichi Morita^c

^aResearch Division, Chugai Pharmaceutical Co., Ltd., 1-135 Komakado, Gotemba-shi, Shizuoka 412-8513, Japan

^bChugai Research Institute for Medical Science Inc., 1-135 Komakado, Gotemba-shi, Shizuoka 412-8513, Japan

^cDepartment of Geriatric and Environmental Dermatology, Nagoya City University Graduate School of Medical Sciences, 1-Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya-shi, Aichi 467-8602, Japan

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ABSTRACT

Background: To understand the clinical segments of IL-31 signaling blockade therapy in pruritus of atopic dermatitis (AD), direct detection of the target proteins in the diseased tissues will provide crucial information. There is a lack of direct evidence concerning the cellular origin of IL-31 in AD skins, and data on the expression of IL-31RA in AD are inconsistent. Also, there is no available information regarding IL-31RA protein expression in human dorsal root ganglia (DRG), which mediates the sensation of itch and is the long-suspected source of the protein.

Objective: We sought to obtain direct evidence concerning the distribution of IL-31- and IL-31RA-protein expressing cells and their characteristics in AD skin samples and in human DRG.

Methods: IL-31 was detected immunohistochemically in AD skins, and representative sections were double stained with IL-31 and several immune-markers. IL-31RA was stained immunohistochemically in AD skins and normal human DRG, and representative AD skins were double stained with IL-31RA and PGP9.5 (a nerve marker).

Results: IL-31-positive cells were observed as mononuclear infiltrating cells and as CD11b co-expressing cells in severe AD samples. As for IL-31RA, positive reactions were detected in keratinocytes and nerve fibers in the dermis of AD and in the neurons of normal DRG.

Conclusion: The detection of IL-31 in infiltrating cells of severe AD skin and of IL-31RA in nerve fibers of AD dermis and normal DRG indicates IL-31 signaling may be a contributing factor in the persistence and exacerbation of AD skin lesions.

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1. Introduction

Pruritus is an essential feature and main symptom of atopic dermatitis (AD), with a high impact on patients' quality of life [1,2]. Although the pathophysiology of pruritus is still not fully understood, it is known that itch, as a cutaneous sensory perception, is excited on neuropeptide-containing unmyelinated nerve fibers in the papillary dermis and epidermis. Several mediators, such as neuropeptides, interleukins, proteases, or cytokines, have been suggested as provoking itch in AD directly or indirectly [3–5].

Interleukin-31 (IL-31) was recently discovered as a new member of the IL-6 family of cytokines, and it signals through a heterodimeric receptor composed of oncostatin M receptor (OSMR) and a IL-31 receptor alpha (IL-31RA) subunit [6,7]. There is emerging evidence that IL-31 signaling may play a role in the pruritus of AD. Transgenic mice that overexpress IL-31 develop severe pruritus and skin lesions similar to AD [8]. In an AD-like murine model (NC/Nga mice), high IL-31 mRNA expression is associated with scratching behavior [9], while an anti-IL-31 antibody reduces scratching behavior [10]. Because of this accumulating knowledge on IL-31 signaling and the pruritus of AD, IL-31 signal blocking is considered a promising therapeutic target for the disease [6,7].

On the basis of this accumulated knowledge, we developed an antibody that specifically binds to IL-31RA and inhibits its signaling as a therapeutic for pruritus of AD. When developing

* Corresponding author. Tel.: +81 550 87 8757; fax: +81 550 87 6383.
E-mail address: katoath@chugai-pharm.co.jp (A. Kato).

drugs by molecular targeting, the importance of defining patient segments using a biomarker, such as the expression of target molecules in diseased tissue samples, is widely recognized [11]. To better understand the clinical segments in IL-31 signal blocking therapy for pruritus of AD, information of such systemic or local expression levels or of the distribution of IL-31 and IL-31RA molecules in patients is essential.

In terms of the systemic expression of IL-31, serum levels were higher in AD patients than in the non-atopic healthy individuals and correlated with disease activity [12]. In addition, an IL-31 gene haplotype may be linked with genetic susceptibility to non-atopic eczema in humans [13]. As for local levels in AD skin lesions, an increase of the protein [14] and mRNA [15–17] was detected when biopsy skin samples from AD patients were compared to those of healthy control individuals. The data showing the increase of IL-31 expression levels (both in mRNA and protein levels) in AD skin lesion samples is consistent between studies; however, direct evidence that shows the cellular source of the interleukin in patient skin with immunohistochemistry (IHC) is not available.

Local expression of IL-31RA is less clearly understood than that of its ligand (IL-31) or its heterodimeric partner OSMR. Using skin samples from AD patients, some researchers describe increased IL-31RA mRNA or protein expression than that of healthy control [15], while others could not detect such clear increase [14,16,18]. Furthermore, analysis of the systemic distribution of IL-31 receptor heterodimer revealed that IL-31RA transcripts are most abundantly expressed in the dorsal root ganglia (DRG) of 63 different human tissues [17]. In mice, expression of both IL-31RA mRNA and protein was detected in neurons of DRG and primary afferent fibers projecting into the dermis [19]. Since these data suggest that IL-31 might induce pruritus by directly modulating the function of sensory neurons [7], it is crucial to know the exact distribution of IL-31RA protein within AD patient skin and in human DRG.

Because previous attempts to obtain this necessary information using IHC in formalin-fixed paraffin-embedded skin tissues of AD patients had failed to identify the cellular source of IL-31 [14], we applied IHC to frozen AD skin samples that were expected to have better antigen preservation [20]. Furthermore, we also applied double staining of several immune-cell markers with IL-31 IHC to identify the cellular origin of the protein. As for IL-31RA, because of the above-mentioned conflicting data, we produced a novel mouse antibody that specifically detects human IL-31RA, and stained not only frozen samples of AD skin (some were double-stained with nerve marker), but also normal human DRG tissues.

2. Materials and methods

2.1. Samples

Twenty-four skin biopsy samples were obtained from patients who were clinically diagnosed with AD according to the “Guidelines for Management of Atopic Dermatitis [21]” at the Nagoya City University Hospital between November 2009 and January 2012. After collecting a serum sample, a biopsy was taken of the most severe area of the skin lesions (abdomen or back) in each patient (4 mm in diameter) under local anesthesia (Xylocaine Injection 1% with Epinephrine, AstraZeneca, Osaka, Japan), divided into 4 pieces, and processed into frozen blocks with Tissue-Tek O.C.T. medium (Sakura Finetek, Tokyo, Japan).

All skin and serum samples were obtained from patients who had given their written informed consent before the sample collection. The protocol of the current study was approved by the Ethical Committee for the Study of Human Gene Analysis at Nagoya City University Medical School and the Ethics Committee concerning the Use of Human-Derived Samples at Chugai

Pharmaceutical Co., Ltd, which stipulates following the Declaration of Helsinki.

Five fresh, unfixed, completely anonymized, normal human DRG were procured from a tissue supplier (Asterand Inc., Detroit, MI, USA), and at the testing facility (Charles River Laboratories, Pathology Associates, Maryland, Frederick, MD, USA), the tissue samples were processed into frozen blocks with Tissue-Tek O.C.T. medium (Sakura Finetek) and were stored in a tissue bank (at -70°C). All DRG tissues used in this study were obtained under the guidelines of the tissue supplier, which include provisions for written informed consent and anonymity procedures to protect donor confidentiality. The study protocol was approved by the ethical committee of the Charles River Laboratories, Pathology Associates, Maryland, which stipulates following the Declaration of Helsinki.

2.2. Clinical, serum and pathological scores

As a clinical score for AD, the value of the Severity Scoring of Atopic Dermatitis (SCORAD) and the pruritus visual analog scale (VAS) score were obtained from each patient, and as serum parameters, serum IgE and thymus and activation regulated chemokine (TARC) levels were measured with fluorescence-enzyme immunoassay and enzyme linked immunosorbent assay, respectively (SRL, Inc., Tokyo, Japan).

From the AD patient skin tissue blocks, cryosections of approximately $6\text{ }\mu\text{m}$ were prepared, fixed in acetone for 10 min, and stored below -70°C prior to staining. One section from each patient was stained with hematoxylin and eosin (HE). Using the HE-stained sections, pathological findings of perivascular inflammation, spongiosis, acanthosis and parakeratosis/hyperkeratosis which are known to characterize the AD lesion [22], were scored as 0–3 along with the severity of the lesions (Fig. S1a).

2.3. IHC

For IL-31 detection, all 24 skin sections were stained by IL-31 IHC that was visualized by diaminobenzidine (DAB; Wako Pure Chemical, Osaka, Japan), and read under a light microscope (OPTIPHOT-2, Nikon, Tokyo, Japan). To identify the nature of IL-31-positive cells observed under light microscopy, we also double stained 2 representative highly IL-31-positive samples with anti-IL-31 antibody and an anti-CD3, -CD11b, or -CD11c antibody, visualized with Alexa Fluor[®] 488 (Life Technologies, Grand Island, NY, USA) and Alexa Fluor[®] 568 (Life Technologies) respectively, and observed them under confocal microscopy (C1si, Nikon). In addition, IL-31 and CD68 double staining was applied to a representative section, visualized with DAB and HistoGreen Substrate kit for peroxidase (AbCys SA, Paris France), and observed under a light microscope (OPTIPHOT-2, Nikon) for further characterization of the IL-31-positive cells.

For IL-31RA detection, all 24 skin sections were stained with IL-31RA IHC and visualized by DAB (Wako Pure Chemical). To identify the nature of the IL-31RA-positive fibrous structures observed in the dermis, we double stained 2 representative positive samples with an anti-IL-31RA and an anti-PGP9.5 antibody, visualized with Alexa Fluor[®] 488 (Life Technologies) and Alexa Fluor[®] 568 (Life Technologies), respectively.

Five normal human DRG cryosections were cut on the day of staining into slices approximately $5\text{ }\mu\text{m}$ thick and were fixed in acetone for 10 min at room temperature. Just prior to staining, the slides were fixed with 10% neutral buffered formalin (NBF) for 10 s. After fixation, the slides were stained with an anti-IL-31RA antibody, and visualized by alkaline phosphatase (Dako EnVision G2 System/AP Rabbit/Mouse (Permanent Red) kit, Dako, Carpinteria, CA, USA), because the dark brown endogenous lipofuscin

pigment within the neurons of DRG precluded evaluating the positive staining with dark brown diaminobenzidine reaction products.

For detailed procedures, the specificity of the anti-IL-31 and IL-31RA antibodies, and the primary antibodies used in this study will be found in the Supplementary Materials and Methods section and Fig. S1 and Table S1 of the supporting information.

2.4. Statistic analysis

Statistical analysis was performed using the Student's *t*-test for clinical and serum parameters, and Wilcoxon test for pathological scores (SAS version 5.0; SAS system 8.02, SAS Institute Japan Ltd., Tokyo, Japan). *p*-Values below 0.05 were regarded as significant.

3. Results

3.1. Identification of the IL-31-positive cells

IL-31-positive cells were observed as a part of the mononuclear cells infiltrating the dermis in 7 out of 24 AD patients' skin samples (Fig. 1a). Positive reactions were detected in the cytoplasm of the mononuclear cells, including foot process-like structures (Fig. 1b).

In the sections of double staining with immune cell-markers (CD3, CD11b, CD11c, and CD68), most of IL-31-positive cells were

positive for CD11b, and part of the CD11b-positive cells were positive for IL-31 (Fig. 1d and g). In addition, a very small population of weakly CD11c-positive cells was positive for IL-31 as well (Fig. 1e and h). Since the CD68-positive reaction was not evenly distributed within the cytoplasm, and hence the colocalization of the positive reactions was relatively more obscure than that of other markers, the overlap of both proteins was noted in a certain number of infiltrating cells (Fig. S1b). On the other hand, no overlap was detected between the positive reactions of IL-31 and CD3 (Fig. 1c and f).

3.2. Correlations between IL-31 positivity and disease severity

In the patients with IL-31-positive cells in their skin samples, the values of the SCORAD, serum TARC, and IgE levels were significantly increased (Fig. 2). As for the pathological scores, the known findings that characterize the AD lesion – perivascular inflammation, spongiosis, acanthosis and parakeratosis/hyperkeratosis [22] – were significantly worse in the samples with IL-31-positive cells (Fig. 2).

Twenty-one available serum samples were also measured for serum IL-31, but low levels of the protein were detected only in 2 subjects (those whose skin was negative for IL-31-positive cells), and because of the limited number of positive samples, it was not possible to determine the correlation between the

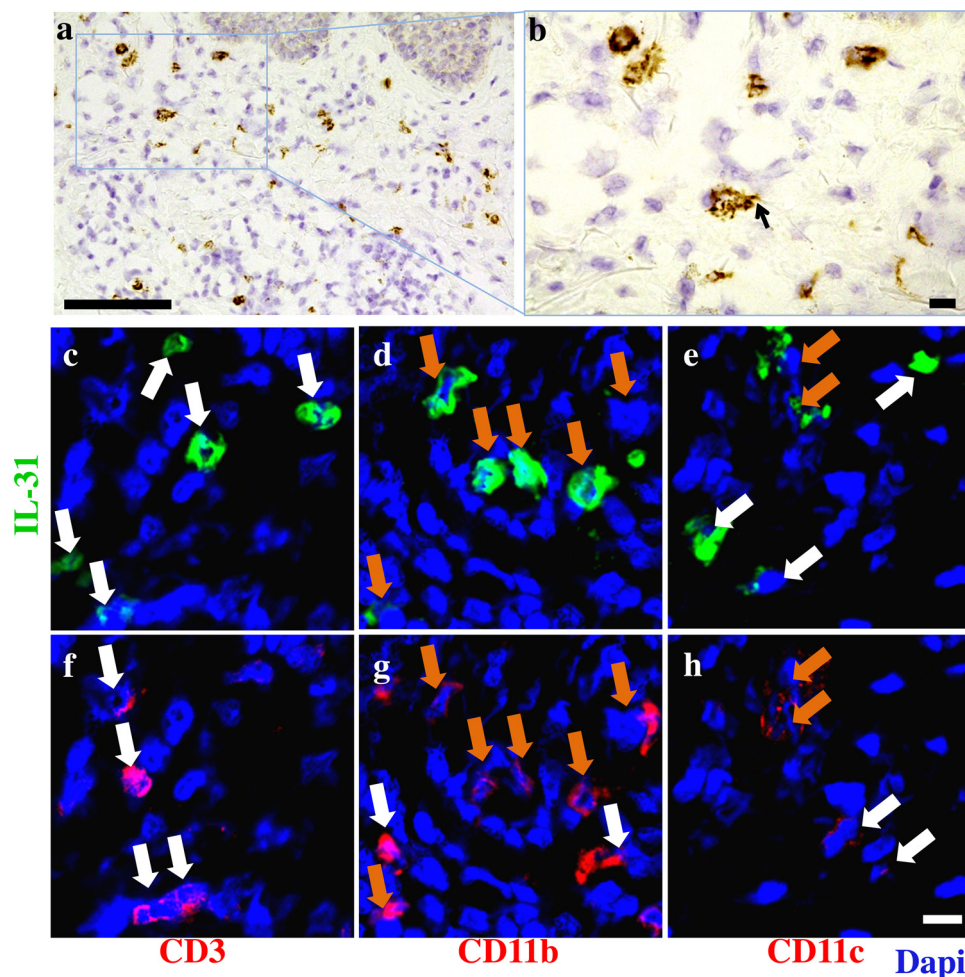


Fig. 1. Identifying the IL-31-positive cells. (a) Microphotograph of a representative case of IL-31-positive mononuclear cells infiltrating the AD lesion. Bar = 100 μ m. (b) IL-31-positive infiltrating cells at a higher magnification. Arrow: foot process-like structure. Bar = 10 μ m. (c–e) IL-31-positive cells (green) in different tissue sections of the same AD skin sample. (f–h) CD3-, CD11b- and CD11c-positive cells (red) in the same areas of the sections in c, d and e, respectively. Orange arrows: double positive cells. White arrows: single positive cells. Bar = 10 μ m.

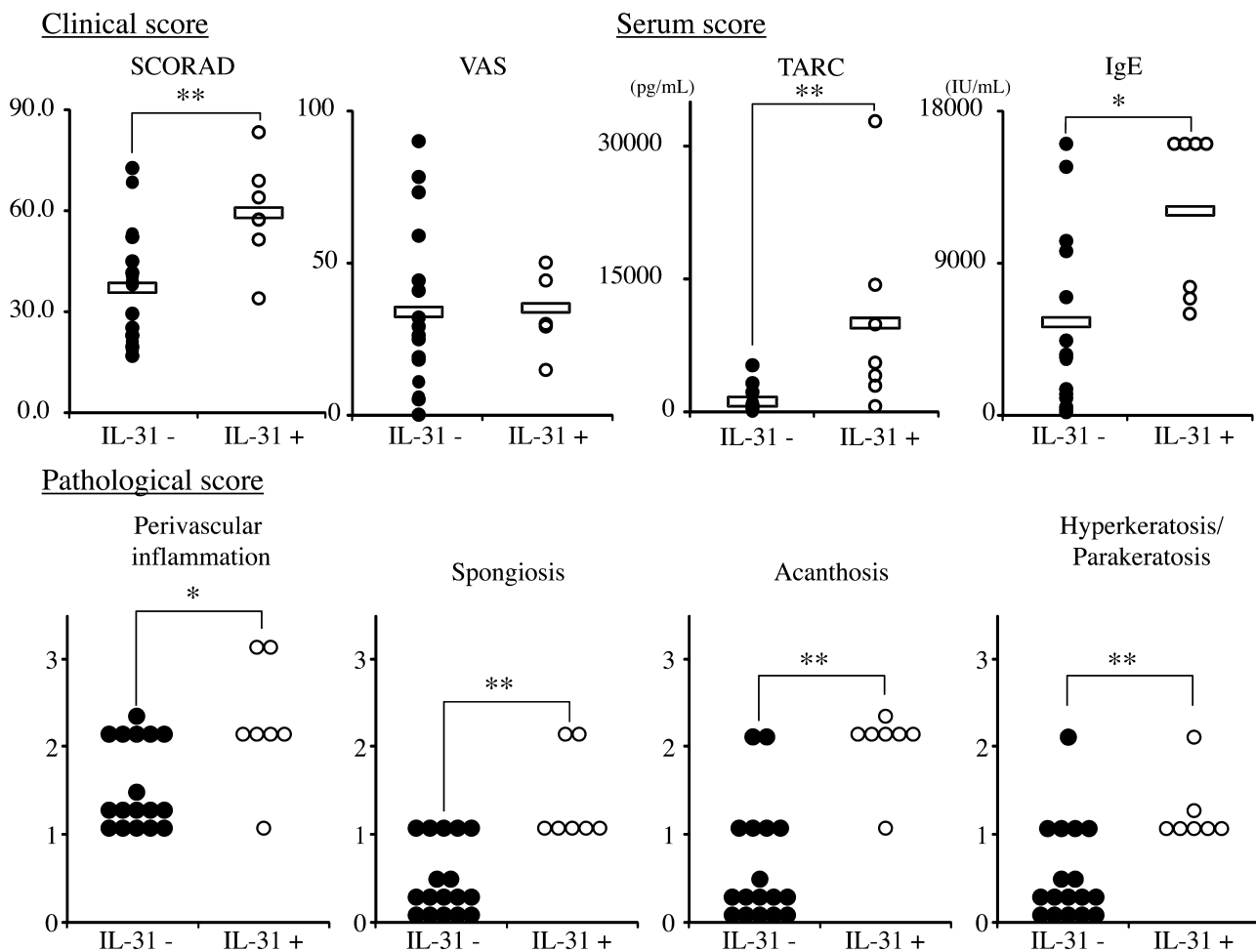


Fig. 2. Correlations between IL-31 positivity and disease severity. The values of the clinical scores (SCORAD and VAS) and levels of serum parameters (IgE and TARC) compared against the patients with or without IL-31-positive cells in their skin samples. Four pathological findings were scored from 0 to 3 and compared between the groups with or without IL-31-positive cells. Bar = average, * $p < 0.05$ and ** $p < 0.01$.

IL-31-positive cells infiltrating the skin and other parameters (data not shown).

3.3. Identification of the IL-31RA-positive cells and areas of expression

The keratinocytes of all AD samples and the fibrous structures in the dermis of 17 out of 24 AD samples showed a positive reaction with IHC (Fig. 3a) using an antibody specific to human IL-31RA (Fig. S1d). No clear correlation was noted between the IL-31RA-positive patients and the clinical, serum, and pathological parameters. In order to examine the nature of the fibrous structures in the dermis, we established a double staining system with anti-IL-31RA and anti-PGP9.5 antibodies. With this system, fibrous structures were positive for both antibodies (Fig. 3b).

In normal human DRG, some of the ganglion cells were positive for IL-31RA IHC in all 5 samples. The positive reactions were observed mainly in the cytoplasm and cytoplasmic granules of ganglion cells (Fig. 3c).

4. Discussion

Because of the knowledge that is accumulating on IL-31 signaling and pruritus of AD, IL-31 signal blocking is considered to be a promising therapeutic target for the disease [6,7]. The distribution of the OSMR molecule, the heterodimeric partner of IL-31RA, is well described elsewhere with certain consistency [14,16,17,19]; in

contrast, the cellular source of IL-31 and IL-31RA and its distribution in AD skin lesions and human DRG is obscure.

To obtain this information, which is crucial for the development of therapeutics, we first assessed IL-31 protein expression, and found a positive reaction in the mononuclear cells that infiltrate the lesions of some AD skin samples. Previous studies that analyzed IL-31 mRNA in AD patient skin tissues reported a similar patient distribution in which positive cases were limited to particular patients and other cases showed no difference to healthy cases [15–17]. Because stimulation by anti-CD3 and CD28 antibody resulted in IL-31 expression being detected in circulating skin-homing, cutaneous lymphocyte antigen (CLA)-positive T cells of AD patients, and because the cells were the major population of infiltrating cells in AD lesions, many researchers considered that the IL-31-positive infiltrating cells in AD skin samples were CLA-positive T cells [14,15].

As to the molecules that used to stimulate T cells, CD3 and CD28 are the molecules that constitute the central supra-molecular activation complexes (cSMAC) that enables productive T cell receptor (TCR) signaling from the class II major histocompatibility complex (MHC) of antigen-presenting cells (APCs) [23]. On the other hand, as another candidate of the stimulus, staphylococcal enterotoxins are well known and widely used in AD research, because more than 90% of AD patients have *Staphylococcus aureus* colonization on their skin, and more than 70% of the *S. aureus* are exotoxin-producing strains, which can secrete various exotoxins including staphylococcal enterotoxins A, B (SEB), and C and toxic

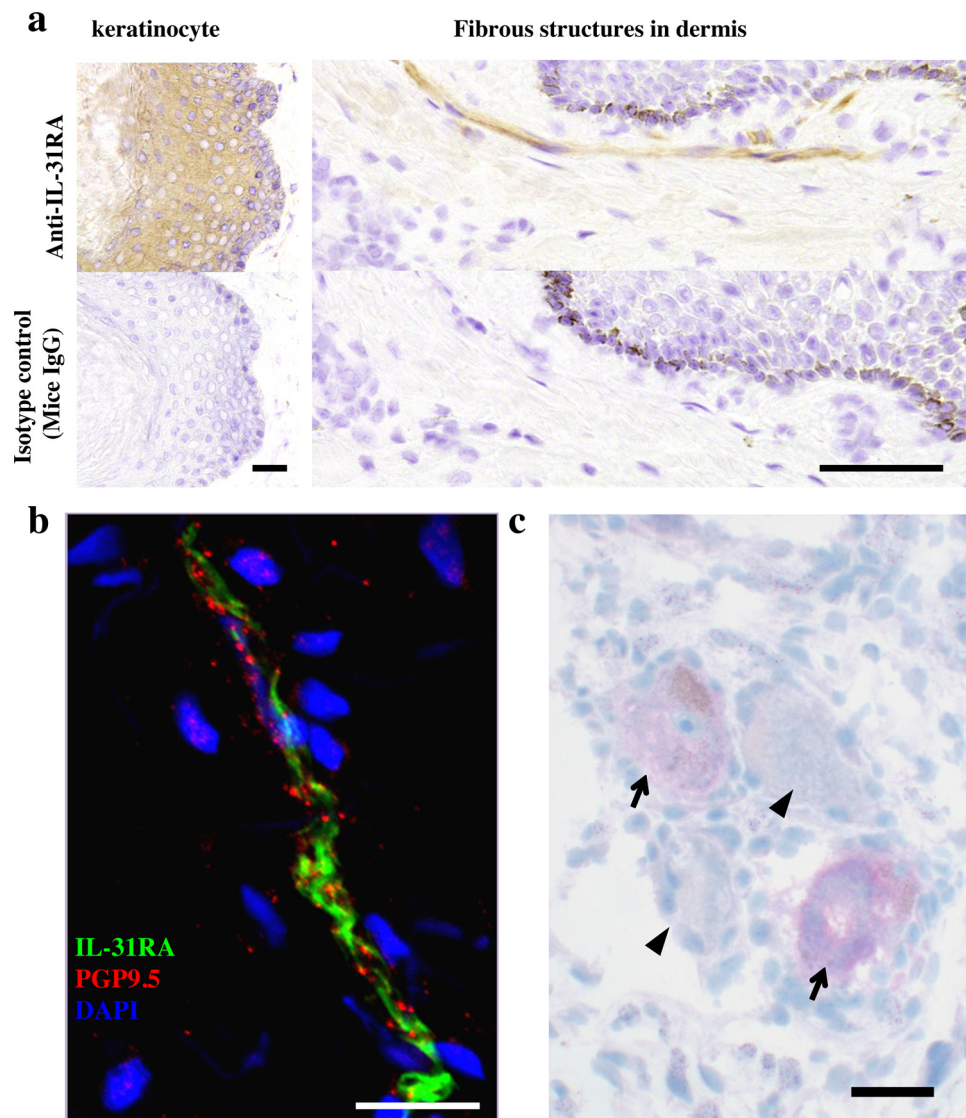


Fig. 3. Identifying the IL-31RA-positive cells and areas. (a) Microphotographs of IL-31RA-positive keratinocytes (top left) and a fibrous structure in the dermis (top right). The bottom row shows isotype-matched controls of serial sections. Bars = 50 μ m. (b) IL-31RA (green) and PGP9.5 (red) double staining of fibrous structure of dermis. Bar = 20 μ m. (c) IL-31RA-positive neurons (arrows) in normal human DRG. Arrow heads: negative cells. Bar = 20 μ m.

shock syndrome toxin-1 [24]. It is well known that these staphylococcal enterotoxins are superantigens, can activate T cells or APCs by binding directly to the variable region of the β chain of TCR (TCRV β) or the class II MHC molecules, and that they may penetrate the skin barrier and contribute to the persistence and exacerbation of allergic skin inflammation in AD [24]. Moreover, since it is also well known that AD patients had a higher percentage of T cells bearing staphylococcal superantigen-reactive TCRV β in the peripheral CLA-positive T cells than healthy subjects [24], it would be interesting to know the IL-31 expression levels of the peripheral T cells that have been stimulated by staphylococcal superantigen (such as SEB), but the information is not available so far.

Conversely, other researchers stimulated PBMC with several stimuli and showed that SEB was the most potent stimulant to elevate IL-31 expression when compared with anti-CD3 antibody, ultraviolet B radiation, and H₂O₂ exposure (herpes simplex virus, influenza virus, IFN- γ , lipopolysaccharide, IL-2, and IL-4 did not increase the IL-31 expression in PBMC) [17,25]. In the current study, we detected IL-31-expressing cells as part of CD11b- and (as a very small population) CD11c-positive cells in some AD skins

without any experimental stimulation and also confirmed that some IL-31-positive cells expressed CD68 (a macrophage maker). In addition to the expression of the CD markers, because positive cells have morphological characteristics (mononuclear cells with foot processes), it is most likely that IL-31 and CD11b double-positive cells in AD skin samples are cells of the monocytes/macrophage lineage that was recently shown to also express IL-31 upon stimulation by ultraviolet B or H₂O₂ [25]. Based on the previous information and our current result, it may be possible to consider cells of the monocytes/macrophage lineage as the prime source of IL-31 in local AD skin, and it may be possible to further speculate that the induction of IL-31 in T cells might require the co-stimulation of the APCs through the cSMAC [23]. Since current data did not deny the possibility of T-cell expression of IL-31, further detailed and larger studies are required to fully understand the nature of IL-31-positive cells in skin lesions of AD patients.

The SCORAD values, serum TARC, and IgE levels, and the pathological findings that are known to correlate to AD severity [2,22], were seen to be exacerbated in AD patients with IL-31-positive cells in their skin samples. Because these results suggest that IL-31 expression was detected in severe AD patients, it may

also support the idea that IL-31 served as a contributing factor in the persistence and exacerbation of allergic skin inflammation in AD. In addition, the VAS score did not correlate to the emergence of IL-31-positive cells in AD patient skin. Since the VAS score was a subjective parameter (dependent on the patient's own perception) [21], it may not correlate to the parameters of a snapshot analysis, such as the parameter of skin-infiltrating IL-31-positive cells in the current study, and detecting the time course (pre- and post-treatment) change in each patient may be more suitable in upcoming clinical studies of IL-31 signal-blocking therapy.

Although there were several reports describing IL-31RA mRNA and protein expression in AD skin samples, a consensus on its source has not been reached [14–16,18]. In addition, data that directly shows the expression of IL-31RA protein in human DRG has not been available so far, although mRNA levels in human DRG [17], and both mRNA and protein levels in mice have been studied [19]. To overcome this situation, we prepared a novel human IL-31RA-specific antibody, established an immunohistochemical staining system with the antibody, and assessed the distribution of the protein in both AD skin lesions and normal human DRG.

With our test system, we detected a positive reaction in keratinocytes of all AD skin samples and in fibrous structures in the dermis of the majority of samples (17 out of 24 cases), as well as in some of the neurons in all 5 normal human DRG samples. To define the nature of the fibrous structures in the dermis, we applied a double staining system with IL-31RA and a neuronal marker (PGP9.5) to the AD skin samples, and found a specific double-positive reaction in fibrous structures of AD skin lesions.

This is the first report of IL-31RA protein being detected in the nerve fibers of the dermis of AD patients and in neurons in normal human DRG. Because the sensation of itch, similar to pain, is directly mediated by unmyelinated C fibers of primary sensory neurons of DRG [3–5], our results suggest that IL-31 might induce pruritus by directly modulating the function of sensory neurons [6,7]. Alternatively, IL-31 did not directly activate sensory neurons and may exert its pruritic sensation by activating IL-31RA-expressing keratinocytes, which may induce keratinocyte-derived mediators such as thymic stromal lymphopoietin that subsequently activates unmyelinated C fibers in the skin [4,5].

We could not determine a clear correlation between an increased frequency of fibrous IL-31RA-positive structures in the dermis and any of the clinical, serum, and pathological scores of the disease. In a previous report that described an increase of PGP9.5-positive nerve fibers in allergic contact eczema, the researchers used relatively thick sections (14 μm or so) to measure the extent of the nerve fibers [26]. So, in order to obtain more quantitative information regarding the extent of IL-31RA-positive nerve fibers, we will need to establish a staining system that is applicable to relatively thick tissue sections. In addition, although one report depicted the macrophages of AD patients as a source of IL-31RA [15], we could not detect a clear positive reaction in the infiltrating cells of AD skin samples in the present study. Since the reasons for the discrepancy are not clear, studies on a larger scale with multidisciplinary evaluation may be necessary.

An important aspect of AD is scratching, which not only impairs the patient's quality of life but causes defects in the functions of the skin barrier that result in increased penetration of the skin by allergens and microbes, and further enhances the effects of allergens [6,7]. With the current results, IL-31 was detected in CD10b-positive infiltrating cells in 7 out of the 24 patients (but not all CD10b-positive cells were positive for IL-31), emergence of IL-31-positive cells correlated to the severity of the disease, and IL-31RA was concurrently detected in nerve fibers of the dermis. Hence, if the notions about the vicious itch/scratch cycle are combined, IL-31 signaling may be a contributing factor in the persistence and exacerbation of AD skin lesions [7], especially in

severe cases of the disease. If this is the case, IL-31 signal-blocking therapy may be a promising direct drug therapy that interrupts the aggravated vicious cycle of AD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jdermsci.2014.02.009>.

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Supporting Information

“Distribution of IL-31 and its receptor expressing cells in skin of atopic dermatitis”

Supplementary Materials and Methods

Production of an anti-IL-31RA mouse antibody and the specificity of the primary antibodies

An anti-IL-31RA antibody was prepared by the hybridoma method. Four female BALB/c mice (Charles River Japan, Kanagawa, Japan) were immunized with IL-31RA by DNA immunization (6 weeks old when immunization was initiated). After 5 immunizations, spleens were excised from the mice. The spleen cells were fused with mouse myeloma cells (P3X63Ag8U.1, ECACC, Salisbury, UK) by the standard method using PEG1500 (Roche Diagnostics, Tokyo, Japan). After fusion, hybridomas were selected using a HAT-containing semi-solid medium (STEMCELLS Technologies, Vancouver, Canada). The specificity of the antibodies against IL-31 and IL-31RA was confirmed by immunocytochemical staining using cell lines transfected with the corresponding antigens (for IL-31: CHO/DG44 from Biogen Idec Inc., Boston, MA, USA and for IL-31RA: Mouse pro-B cell line, Ba/F3 (RCB0805), which was provided through the National Bio-Resource Project run by MEXTof RIKEN BRC, Ibaraki, Japan) (Fig. S1b and c).

All animal experiments were approved by the Ethical Committee for Treatment of Laboratory Animals at Chugai Pharmaceutical Co., Ltd.

Detailed IHC procedure

Primary antibodies and their applied concentrations in the current study are listed in Supplementary Table S1.

IL-31 IHC in a bright field (DAB reaction; AD skin samples).

The anti-human IL-31 mouse antibody (5 µg/mL) was applied overnight at 4°C, and was detected with anti-mouse IgG HRP polymer (EnVision™+, DakoCytomation, Glostrup, Denmark). The visualization of HRP was carried out with DAB (Wako Pure Chemical, Osaka, Japan), and slides were counterstained with hematoxylin (Muto Pure Chemicals, Tokyo, Japan).

Double staining of IL-31 and CD3, CD11b, and CD11c for dark field (fluorescence; AD skin samples).

A cocktail of the anti-human IL-31 antibody (1 µg/mL) and anti-CD3, -CD11b or -CD11c antibodies was applied overnight at 4°C. The primary antibodies were detected by anti-mouse IgG HRP polymer (EnVision™+, DakoCytomation) and anti-rabbit IgG goat polyclonal biotinylated antibody (2 µg/mL, VECTOR Laboratories, Burlingame, CA, USA), respectively. The secondary reaction was visualized with Alexa Fluor®488-labeled tyramide (x100, Life Technologies, Grand Island, NY, USA) and Alexa Fluor®568-labeled streptavidin (2 µg/mL, Life Technologies), and the slides were coverslipped with ProLong with DAPI (Life Technologies).

Double staining of IL-31 and CD68 in a bright field (DAB and HistoGreen reaction; AD skin samples).

Because of intense fluorescent background CD68 staining, we established the double staining of IL-31 and CD68 with a two-color bright field visualization system described below.

First an anti-human CD68 mouse antibody was applied for 30 min at room temperature. The primary antibodies were detected by anti-mouse IgG HRP polymer (EnVision™+, DakoCytomation) and visualized with DAB (Wako Pure Chemical). After removing antibodies with a denaturing solution (Elution Step; BIOCARE MEDICAL, Concord CA, USA) for 5 min at room temperature, the anti-human IL-31 mouse antibody (5 µg/mL) was applied for 30 min at room temperature. The primary antibodies were detected by anti-mouse IgG HRP polymer (EnVision™+, DakoCytomation) and visualized with HistoGreen, Substrate kit for peroxidase (AbCys SA, Paris France) and slides were counterstained with Kernechtrot (Muto Pure Chemicals).

IL-31RA IHC in a bright field (diaminobenzidine reaction; AD skin samples).

The anti-human IL-31RA mouse antibody (5 µg/mL) was applied overnight at 4°C, and was detected with anti-mouse IgG HRP polymer (EnVision™+, DakoCytomation). The visualization of HRP was carried out with diaminobenzidine (Wako Pure Chemical), and slides were counterstained with hematoxylin (Muto Pure Chemicals).

IL-31RA IHC in a bright field (alkaline phosphatase reaction; normal DRG samples).

The anti-human IL-31RA mouse antibody (2 µg/mL) was applied at room temperature for one hour. Then, the Rabbit/Mouse (Link) reagent supplied in the Dako EnVision G|2 System/AP Rabbit/Mouse kit (DakoCytomation) was applied. Next, the slides were rinsed with tris-buffered saline with 0.05% Tween 20 and with the AP Enzyme (Enhancer) supplied in the Dako EnVision

G|2 System/AP Rabbit/Mouse kit (DakoCytomation). The slides were visualized by a Permanent Red solution supplied in the Dako EnVision G|2System/AP Rabbit/Mouse kit (DakoCytomation) and counterstained.

Double staining of IL-31RA and PGP9.5 in a dark field (fluorescence; AD skin samples).

A cocktail of the anti-human IL-31RA antibody (1 µg/mL) and anti-PGP9.5 antibody was applied overnight at 4°C. The primary antibodies were detected by anti-mouse IgG HRP polymer (EnVision™+, DakoCytomation) and anti-rabbit IgG goat polyclonal biotinylated antibody (2 µg/mL, VECTOR Laboratories), respectively. The secondary reaction was visualized with Alexa Fluor®488-labeled tyramide (x100, Life Technologies) and Alexa Fluor®568-labeled streptavidin (2 µg/mL, Life Technologies), and the slides were coverslipped with ProLong with DAPI (Life Technologies).

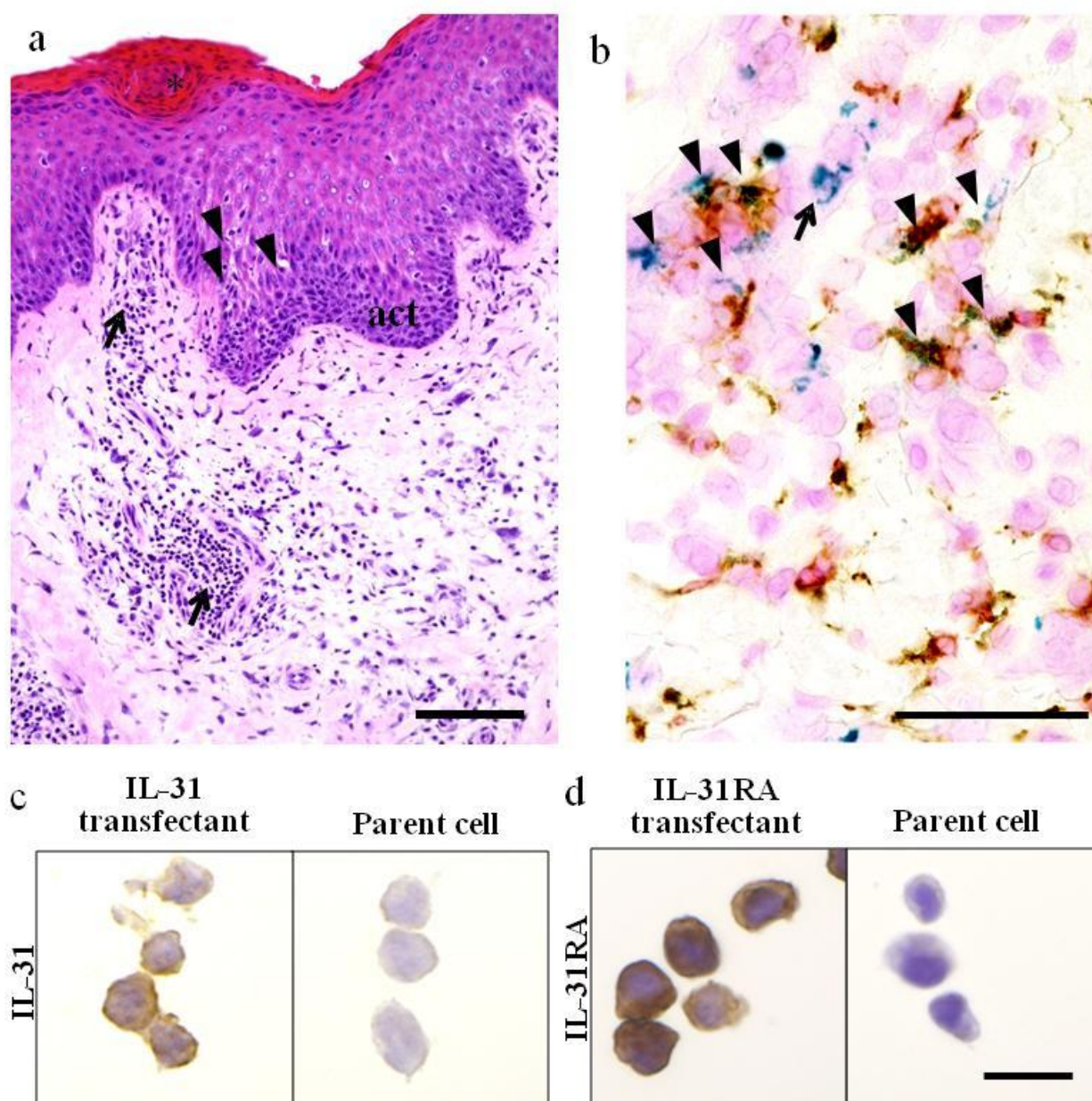
Supplementary Figure Legends

Supplementary Figure S1.

a: Four characteristic pathological findings of AD lesion were defined as follows: In dermis, inflammatory cell infiltration was observed in the perivascular area (arrows). In epidermis, spongiosis, characterized by edema seeping into the intercellular spaces of the epidermis and keratinocytes splaying apart particularly in the stratum spinosum, was observed (arrow heads). The spongiosis was accompanied by acanthosis (act), which is characterized by epithelial downgrowth, and parakeratosis/hyperkeratosis (asterisk), which is considered to be an abnormal cornification. Bar = 150 µm.

b: IHC double staining for IL-31 (blue) and CD68 (brown) was applied to a representative section. Although not all IL-31-positive reactions overlapped with CD68 (arrow), a certain number of double-positive cells were observed (arrowhead). Bar = 50 μ m.

c and d: IHC staining with anti-IL-31 and -IL-31RA antibodies were applied to the corresponding transfectant cell lines. Positive reactions were observed only in the corresponding transfectant cells and not in their parent cells. Bar = 20 μ m.



Supplementary Figure S1

Supplementary Table S1. Primary antibodies used in the study.

Antigen	Clone	Working concentration	Supplier
IL-31	308202	1 or 5 µg/mL	R&D systems, Minneapolis, MN, USA
IL-31RA	(In-house antibody)	1, 2 or 5 µg/mL	Chugai Pharmaceuticals Co., Ltd. Shizuoka, Japan
CD3	(Rabbit polyclonal)	1 µg/mL	DakoCytomation, Glostrup, Denmark
CD11b	EPR1344	x 250	NOVUS Biologicals, Littleton, CO, USA
CD11c	EP1347Y	x 100	GeneTex, Irvine, CA, USA
<u>CD68</u>	<u>PG-M1</u>	<u>1 µg/mL</u>	<u>DakoCytomation, Glostrup, Denmark</u>
PGP9.5	(Rabbit polyclonal)	x 8000	Ultraclone, Isle of Wight, UK