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Functional effects of interleukin 31 in human primary keratinocytes

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Abstract

Background: Interleukin (IL)-31 is a T-cell cytokine acting through a heterodimeric receptor composed of IL-31RA and OSMR which is expressed on epithelial cells including keratinocytes. A major function of IL-31 in atopic dermatitis (AD) is the induction of pruritus in the skin. Inflammatory effects of IL-31 in human primary keratinocytes (HPKs) still remain unclear. We investigated expression, regulation of the IL-31 receptor as well as functions of IL-31 in HPKs.

Methods: Human primary keratinocytes were stimulated with TLR-2 ligands (Pam3Cys, lipoteichoic acid and peptidoglycan), or Th1 and Th2 associated cytokines (IFN-γ and IL-4), respectively. IL-31R expression and regulation as well as functional effects of IL-31 stimulation were then investigated at both the mRNA and protein level and compared with HPKs from patients with AD. The STAT signalling pathway and TLR-2 expression were investigated using Western blot and Immunohistochemical stainings, respectively.

Results: Pam3Cys or IFN-γ significantly up-regulated IL-31RA and OSMR expression. IL-31 activated STAT-3 phosphorylation in HPKs which was augmented after preactivation with Pam3Cys or IFN-γ. IL-31 enhanced the secretion of CCL2 after up-regulation of the receptor with Pam3Cys or IFN-γ. However, this was not observed in keratinocytes from AD patients where an impaired TLR-2 expression was found.

Conclusions: Together, our findings show a functional role of IL-31 in HPKs and provide a new link between TLR-2 ligands and IL-31 which might be dysregulated in AD. Altered function of IL-31 may have implications for cutaneous inflammation in eczema where skin colonization with *Staphylococcus aureus* and dysregulation of TLR-2 have been described.

Abbreviations
AD, atopic dermatitis; CCL2, chemokine (C-C) motif ligand 2; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; HPKs, human primary keratinocytes; IFN-γ, interferon γ; IL, interleukin; IL-31R, interleukin 31 receptor; IL-31RA, interleukin 31 receptor alpha; LTA, lipoteichoic acid; MCP-1, monocyte chemoattractant protein-1; OSMR, oncostatin M receptor; $Pam_3Cys\text{-}SK_4$, N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine; PGN, peptidoglycan; *S. aureus*, *Staphylococcus aureus*; SEB, Staphylococcal enterotoxin B; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor; VEGF, vascular endothelial growth factor.

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease characterized by eczematous skin lesions with 'lichenification', pruritic excoriations and an enhanced susceptibility to bacterial skin infections, particularly with *Staphylococcus aureus* (1, 2). Reduction of itching and pruritus as one of the hallmarks of AD is the most effective therapeutic strategy for improving the quality of life and for preventing aggravation of skin lesions (3). Although much effort has been made to identify the major pruritogen in AD, the mechanisms of itch have not been fully elucidated (4). Skin-infiltrating T cells have been suggested to play a major role through production and release of different mediators (4, 5). Among these mediators, interleukin-31 (IL-31), a newly

characterized T cell-derived cytokine has recently been discovered (6). The activity of human IL-31 is mediated through a heterodimeric receptor composed of IL-31 receptor A (IL-31RA) and oncostatin M receptor (OSMR). IL-31 has recently been demonstrated to act through three signalling pathways: Janus kinase (Jak)/signal transducer and activator of transcription (STAT) factor (Jak/STAT) pathway, phosphoinositide-3-kinase/Akt (PI3K/AKT) pathway and mitogen-activated protein kinase (MAPK) pathway (6, 7). IL-31 plays an important role in skin inflammation and AD and has been shown to induce skin inflammation, pruritus and severe dermatitis in IL-31 over-expressing transgenic mice (6, 8). IL-31 is significantly up-regulated in pruritic (e.g. AD) but not in nonpruritic (e.g. psoriasis) forms of chronic skin inflammation (8). Recently, we provided evidence that serum IL-31 levels were significantly higher in patients with AD and correlated with disease activity in AD (9). A major function which has been attributed to IL-31 is the induction of pruritus in the skin via IL-31 receptors on sensory nerve cells (6). In humans, IL-31 has been detected in the skin mainly in acute eczema (10). Target cells for IL-31 expressing IL-31RA are activated macrophages, epithelial cells including keratinocytes as well as eosinophils in the skin (6, 8, 11, 12).

There is growing evidence supporting keratinocytes as enhancer cells of the inflammatory response in AD (5, 13). Keratinocytes play a crucial role in innate immunity by expressing Toll-like receptors and producing antimicrobial peptides in response to invading microbes. Therefore, keratinocytes may represent the first line of defence against pathogens in the skin (5). Human keratinocytes are known to express TLR-1 to 10 (14, 15). In this context, TLR-2 has emerged as a principle receptor in combating Gram-positive bacteria, especially *S. aureus* (16) which colonizes more than 90% of skin lesions from patients with AD (17). *Staphylococcus aureus* colonization plays a pivotal role as complicating and exacerbating factor in AD and is therefore of extraordinary clinical interest (18). TLR-2 forms homodimers and heterodimers with TLR-1 and TLR-6 to interact with a rather broad spectrum of ligands. So far, no selective TLR-2 ligand is known. Studies using knockout mice identified TLR-1 as the co-receptor required for the recognition of triacylated lipoproteins and lipopeptides such as Pam3Cys and TLR-6 for the recognition of diacylated lipoproteins such as lipoteichoic acid (LTA) (19).

It has been described previously that *S. aureus*-derived superantigens play an important role in the regulation of IL-31 production (8). In addition, we recently demonstrated that staphylococcal exotoxins (SEB or α-toxin) significantly up-regulate IL-31RA expression on monocytes and macrophages at both the mRNA and the protein level (20).

As staphylococcal components have been previously shown to play an important role for IL-31 regulation and keratinocytes represent one of the potential targets for IL-31, we stimulated human primary keratinocytes (HPKs) with TLR-2 ligands: Pam3Cys that acts via TLR-2/TLR-1 heterodimers, LTA that acts via TLR-2/TLR-6 and peptidoglycan (PGN) which acts via TLR-2 and Nod (19, 21, 22), Th1 or Th2 cytokines (IFN-γ or IL-4) in this study. Although an enhanced expression of IL-31RA has been described in inflammatory skin diseases, the regulation and function of IL-31R in HPKs still remains unclear. In an attempt to further understand the role of IL-31 in pruritic skin inflammation, particularly within AD, we investigated IL-31R expression and regulation as well as functions (i.e. regulation of the STAT signalling pathway and cytokine secretion) of IL-31 in HPKs following stimulation with TLR-2 ligands and Th1 or Th2 associated cytokines (IFN-γ or IL-4), respectively.

Materials and methods

Preparation of cells

Human primary keratinocyte isolation and culture

Human primary keratinocytes were prepared either from foreskin of children undergoing surgery (23) or from outer root sheath cells from plucked human hair follicles (24) and cultured in keratinocyte medium (Keratinocyte Growth Medium 2 kit; PromoCell GmbH, Heidelberg, Germany) as described previously (25). Further information is provided in Online Repository (Data S1).

Stimulation of cells

Cells were left either unstimulated or were stimulated for various periods of time with PGN from *S. aureus* (10 μg/ml; Invivogen, Toulouse, France) which acts via TLR-2 and NOD, LTA from *S. aureus* isolated as previously described (26) (10 μg/ml; kind gift from the University of Konstanz, Germany) which acts via TLR-2/TLR-6 and $Pam_3Cys\text{-}SK_4$ (10 μg/ml; EMC microcollections, Tübingen, Germany) which acts via TLR-2/TLR-1, recombinant human IFN-γ (10 ng/ml; ImmunoTools, Friesoythe, Germany), IL-4 (20 ng/ml; R&D-Systems, Minneapolis, Minnesota, USA) or recombinant human IL-31 (100 ng/ml; Peprotech, NJ, USA). LPS was not detected in any reagent, as determined by the Limulus amebocyte assay (Haemochrom Diagnostika, Essen, Germany).

FACS analysis

Prior to flow cytometric analysis, adherent cells (HPKs) were detached by the addition of 0.025% EDTA for 10 min and HyQTase (Perbio, Bonn, Germany) for 10 min. Expression of IL-31RA was assessed using a polyclonal biotinylated anti-human IL-31RA antibody (R&D-Systems) or the appropriate goat IgG biotin isotype control (Abcam, Cambridge, UK) which were indirectly conjugated with Streptavidin–Allophycocyanin (BD Biosciences, Heidelberg, Germany) as described previously (20). Expression of OSMR was measured using a Phycoerythrin (PE) monoclonal anti-human OSMR antibody (eBioscience, San Diego, California, USA), mouse IgG1a-PE antibody (BD Biosciences). Stained cells were measured by flow cytometry using a FACS Calibur and were analysed using the CellQuest Pro software (BD Biosciences).

mRNA isolation, RT, light cycler PCR

RNA isolation, reverse transcription and quantitative real time PCR was performed as previously described (20). Further information is provided in OR.

Cytokine assessment

Human primary keratinocytes were grown in a 24 well plate (Nunc Inc., Wiesbaden, Germany) in a 60–90% cell density and were stimulated as indicated. Cell-free supernatants were harvested and analysed for CCL2, CCL20, CCL22, VEGF, MMP-9, IL-1β, IL-6 and IL-8 (Duo Set; R&D-Systems) using a commercially available enzyme-linked immunosorbent assay (ELISA) system following the manufacturer's instructions.

Western blot

A total of 5×10^5 HPKs were prestimulated with Pam3Cys (10 μg/ml) or IFN-γ (10 ng/ml) for 24 h and stimulated with or without IL-31 (100 ng/ml) for 15 or 60 min, respectively. Cell extracts were subjected to Western blot analysis as described previously (25). Further information is provided in OR.

Immunohistological staining

Punch biopsies from normal and AD skin were fixed in formaldehyde, thereafter they were embedded in paraffin and 2 μm sections were prepared. For TLR-2 staining, the fixed sections were treated with Target Retrieval Solution (DakoCytomation, Hamburg, Germany); polyclonal rabbit anti-human TLR-2 (Abcam) and as isotype control rabbit IgG (DakoCytomation) at 5 μg/ml were used. Staining was performed according to the manufacturer's instructions using the EnVision G/2 Singlestain System (DakoCytomation).

Statistical analysis

Statistical analyses were performed using the Student's *t*-test. *P*-values below 0.05 were regarded as significant. $P < 0.05$ is depicted with *. The program GRAPHPAD PRISM version 3.02 (GraphPad Software Inc., San Diego, California, USA) and the software SIGMA STAT for Windows (Systat Software, San Jose, USA) were used for statistical analysis.

Results

Expression and regulation of IL-31RA and OSMR in human primary keratinocytes

Interleukin 31 receptor alpha and OSMR expression on HPKs were investigated at the mRNA level as determined by quantitative RT-PCR and at the protein level using flow cytometry.

Up-regulation of IL-31RA was observed in a time dependent manner with a significant maximum after 24 h following Pam3Cys stimulation in HPKs at the mRNA level (Fig. S1). This could be confirmed at the protein level: Flow cytometric analysis revealed that Pam3Cys as well as IFN-γ significantly up-regulated IL-31RA and OSMR expression on HPKs (Figs 1 and S2). IL-31RA and OSMR expression were neither regulated by IL-4 (Fig. 1) nor by LTA or PGN in HPKs at both the mRNA and the protein level (data not shown).

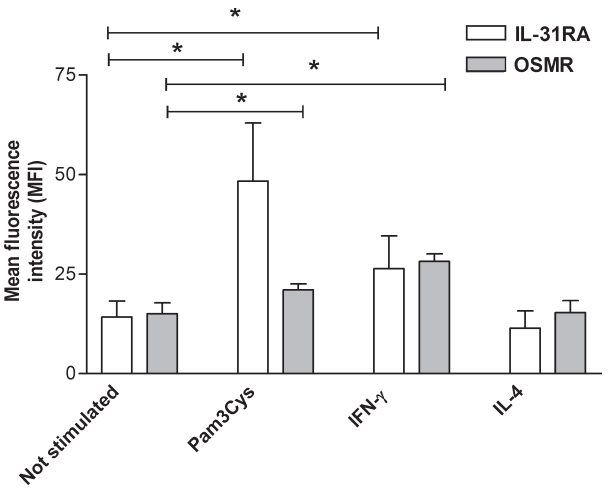

Figure 1 Expression and regulation of IL-31R on HPKs at the protein level. Regulation of IL-31RA and OSMR on HPKs following 24 h Pam3Cys (10 μg/ml), IFN-γ (10 ng/ml) or IL-4 (20 ng/ml) stimulation. Mean fluorescence intensity of IL-31RA and OSMR positive cells + SEM of $n = 5$ experiments is shown. *$P < 0.05$ compared with medium control.

Signalling pathway of IL-31 in human primary keratinocytes

In other cell types such as human intestinal or bronchial epithelial cells, it has been shown that IL-31 can activate Jak1 and Jak2 signalling molecules after binding to its receptor complex and, once activated, Jaks are known to stimulate the phosphorylation of downstream signalling molecules such as STAT-3, STAT-5 and to a lesser degree STAT-1 (7). In human intestinal epithelial cells, IL-31 mediates extracellular signal-regulated kinase (ERK-1/2) and Akt activation as well (11).

Therefore, we investigated STAT signalling pathways as well as ERK1/2 activation of IL-31 after up-regulation of the IL-31 receptor in HPKs. IL-31 strongly activated STAT-3 phosphorylation following up-regulation of its receptor with Pam3Cys or IFN-γ in foreskin (Fig. 2) as well as hair-derived (Fig. S3) HPKs, but not STAT-1 or STAT-5 phosphorylation (data not shown). There was no difference in ERK1/2 phosphorylation upon IL-31 stimulation, neither in rested nor in activated HPKs (data not shown). According to the manufacturer's instruction, extracts from HeLa cells untreated or treated with interferon-α (IFN-α) were used as a negative and positive control for STAT-3 phosphorylation, respectively. Extracts from NIH/3T3 cells untreated or treated with UV light and platelet-derived growth factor (PDGF) were used as a negative and positive control for ERK1/2 phosphorylation, respectively.

Functional effects of IL-31 on cytokine secretion in HPKs

Based on previous studies, there are several cytokines and chemokines induced by IL-31 in other cell types which are targets for IL-31 (7, 11, 12, 20, 27). Therefore, we investi-

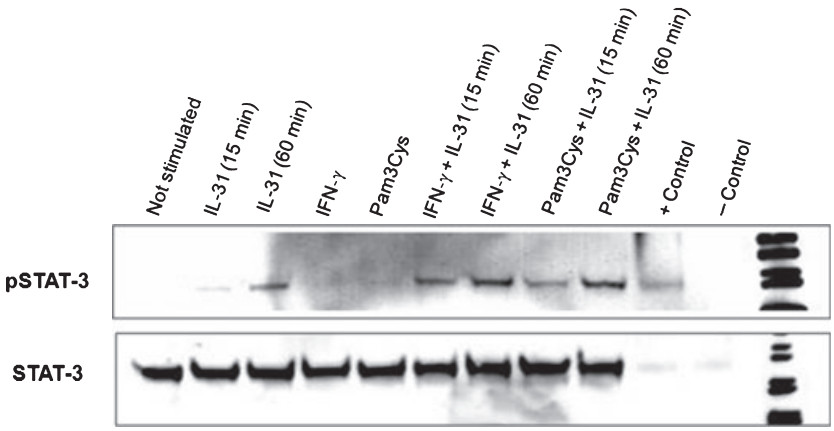

Figure 2 STAT-3 activation in HPKs. Phospho-STAT (pSTAT)-3 and total STAT-3 levels were determined by means of Western blot analysis 15–60 min after IL-31 stimulation. Approximately 5×10^5 HPKs derived from foreskin were left either unstimulated or were prestimulated with Pam3Cys (10 μg/ml) or IFN-γ (10 ng/ml) for 24 h to up-regulate the IL-31 receptor and with and without IL-31 (100 ng/ml) for 15 or 60 min. Cellular extracts were normalized on the basis of protein determination. One representative experiment of three independent experiments from different donors is shown.

gated gene and protein expression of cytokines and chemokines of our interest in HPKs with functional impact on AD. Our data show that IL-31 significantly increased the secretion of monocyte chemoattractant protein-1 (MCP-1/CCL2) following up-regulation of the IL-31 receptor with Pam3Cys or IFN-γ in keratinocytes at the protein level (Fig. 3). Moreover, stimulation with IL-31 significantly induced moderate secretion of vascular endothelial growth factor (VEGF) from HPKs following up-regulation of the IL-31 receptor with Pam3Cys for 48 h at the protein level (induction of VEGF from a mean of 949.817 pg/ml in Pam3Cys treated cell to 1270.89 pg/ml in IL-31 and Pam3Cys treated cells) (data not shown). Furthermore, IL-31 significantly increased CCL22 expression following up-regulation of the IL-31 receptor with Pam3Cys for 4 h at the mRNA level. However, this effect could not be confirmed at the protein level (data not shown). Other genes of our interest such as CCL17, CCL20, epidermal growth factor (EGF), VEGF, matrix metalloproteinase-9 (MMP-9), IL-1β, IL-6 and IL-8 expression which had been shown to be up-regulated by IL-31 in the literature in other cell types (7, 11, 12, 20, 27) were not regulated by IL-31 in Pam3Cys or IFN-γ prestimulated HPKs at the mRNA level (data not shown). Moreover, we investigated these mediators at the protein level as well and found no regulation by IL-31 in 24 and 48 h Pam3Cys or IFN-γ prestimulated cells (data not shown).

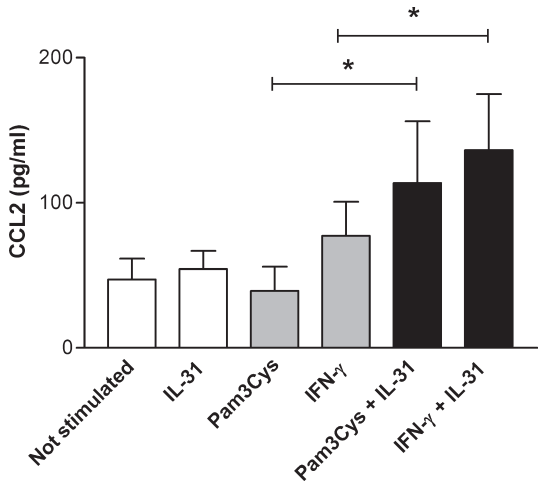

Figure 3 Effect of IL-31 on CCL2 secretion following up-regulation of the IL-31R with Pam3Cys or IFN-γ in HPKs at the protein level. The cells were left either unstimulated or were prestimulated with Pam3Cys (10 μg/ml) or IFN-γ (10 ng/ml) with and without IL-31 (100 ng/ml) for 48 h. The cell culture supernatants were measured for CCL2 secretion, using an ELISA. The mean value + SEM of $n = 5$ experiments is shown. $^*P < 0.05$.

Comparison of IL-31RA expression and regulation as well as its functions on hair-derived HPKs from AD patients compared with healthy control individuals

Next, we investigated the expression and regulation as well as functional effects of IL-31 on important cytokines and chemokines for AD in keratinocytes from AD patients compared with healthy controls.

Pam3Cys up-regulated IL-31RA expression in keratinocytes both from AD and healthy controls. However, the up-regulation of IL-31RA upon Pam3Cys stimulation was lower in keratinocytes from AD compared with healthy controls (Fig. 4). Moreover, IL-31 induced lower levels of CCL2 secretion in keratinocytes from AD patients compared with healthy controls following up-regulation of the IL-31RA with Pam3Cys (Fig. 5).

Interferon-γ did not regulate IL-31RA expression (Fig. 4) nor IL-31 function (data not shown) in keratinocytes from both patients with AD and healthy controls. As shown for HPKs from healthy controls, CCL20, MMP-9, IL-1β, IL-6

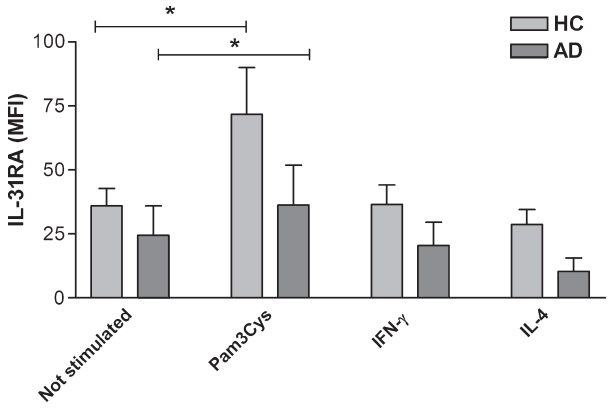

Figure 4 IL-31RA expression and regulation on HPKs from patients with atopic dermatitis (AD) compared with healthy controls (HC) at the protein level. Hair-derived HPKs were either left unstimulated or stimulated for 24 h with Pam3Cys (10 μg/ml), IFN-γ (10 ng/ml) or IL-4 (20 ng/ml). Mean fluorescence intensity of IL-31RA positive cells + SEM of seven AD patients and 10 HC is shown. $*P < 0.05$ compared with medium control.

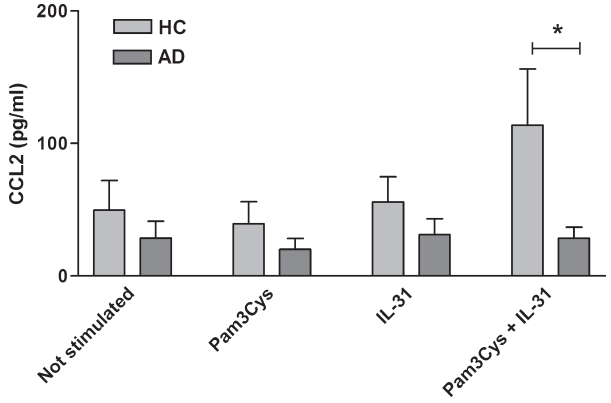

Figure 5 Effect of IL-31 on CCL2 secretion following up-regulation of the IL-31R in HPKs from atopic dermatitis (AD) patients compared with healthy controls (HC). Hair derived HPKs were left unstimulated or were prestimulated with Pam3Cys (10 μg/ml) with and without IL-31 (100 ng/ml) for 48 h. The cell culture supernatants were measured for CCL2 secretion, using an ELISA. The mean value + SEM of 11 AD patients and nine HC is shown. $*P < 0.05$.

and IL-8 were also not regulated by IL-31 in Pam3Cys or IFN-γ prestimulated keratinocytes from AD patients (data not shown).

Impaired TLR-2 expression in keratinocytes from inflamed AD skin compared with healthy controls

To clarify the altered function of IL-31 and an impaired CCL2 production by keratinocytes from AD patients following treatment with Pam3Cys and IL-31, we investigated TLR-2 expression in inflamed AD skin. Immunohistochemical stainings from punch biopsies were performed to address a possible deficient TLR-2 expression on keratinocytes from AD.

Immunohistological staining of TLR-2 in keratinocytes from inflamed skin showed a decreased TLR-2 expression in AD patients compared with healthy skin (Fig. 6).

Discussion

Interleukin-31 is a T-cell-derived cytokine, which is involved in AD (6). Recently, an enhanced expression of IL-31RA in keratinocytes has been described for AD (28), however, the regulation and function of IL-31R in HPKs still remains unclear.

As staphylococcal components have been previously shown to play an important role for IL-31 regulation (8, 20), we investigated staphylococcal LTA and PGN as well as the synthetic TLR-2 ligand Pam3Cys in this study for their impact on IL-31 regulation. We demonstrate for the first time the up-regulation of IL-31RA following stimulation with Pam3Cys in HPKs both at the mRNA and the protein level. Pam3Cys also significantly up-regulated the second chain of the IL-31R, namely OSMR, in HPKs. Pam3Cys (triacylated lipoprotein) acts via TLR-2/TLR-1 heterodimers (19). Therefore, our data indicate that in addition to staphylococcal exotoxins (20), staphylococcal lipoproteins which are mainly TLR-2 ligands could regulate the IL-31R.

Dillon et al. demonstrated that human IL-31RA mRNA, although undetectable in fresh peripheral blood monocytes, was up-regulated substantially in monocytes cultured with IFN-γ (6). Moreover, Jawa et al. showed that IFN-γ played the prominent role in up-regulating IL-31RA mRNA expression in pulmonary macrophages (29). Here, we showed that IFN-γ also significantly up-regulates both chains of the IL-31R (IL-31RA, OSMR) in HPKs. This finding points to an interaction between Th-1 cells and keratinocytes in the skin.

In our study, both Pam3Cys and IFN-γ were effective in the up-regulation of IL-31RA and OSMR in keratinocytes.

Based on the signal transduction mechanisms of IL-31, it has been shown that engagement of the receptor complex results in activation of Jak-1, and to a minor extent of Jak-2, as well as STAT-1, STAT-3, STAT-5, ERK, MAPK and PI3K signalling pathways in glioblastoma and melanoma tumour cells, human intestinal epithelial cells and human lung epithelial cells (11, 30, 31). We confirmed that IL-31 activates STAT-3 in keratinocytes which is augmented following the up-regulation of IL-31R with Pam3Cys or IFN-γ, whereas we failed to detect an involvement of IL-31 in STAT-1, STAT-5 and ERK phosphorylation. This points to distinct signalling pathways in different cell types, differences between tumour cell lines and primary cells.

Subsequently, IL-31 signalling in keratinocytes led to the induction of CCL2 following up-regulation of its receptor with Pam3Cys or IFN-γ. CCL2 has been previously shown to be STAT-3 dependent as well. One study substantiated the role of the STAT-1/3 pathway in CCL2 synthesis by using shRNA targeting STAT-1 and STAT-3 which in turn reduced OSM-induced CCL2 expression in Osteoblastic cells (32).

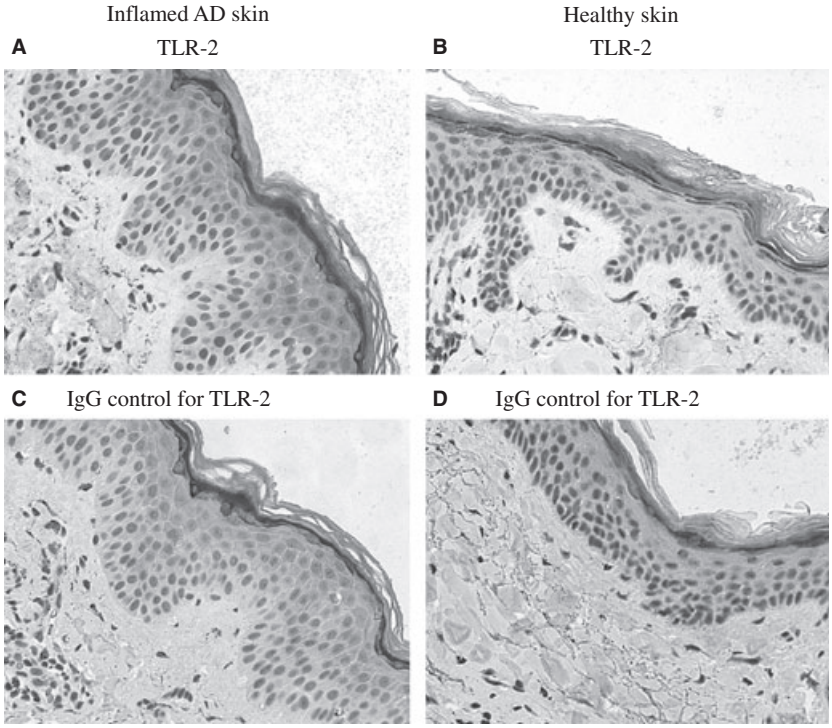

Figure 6 Impaired TLR-2 expression in keratinocytes from inflamed skin of patients with atopic dermatitis (AD) compared with healthy controls. Immunohistological stainings show TLR-2 expression in keratinocytes from inflamed epidermal skin of (A) AD patients compared with (B) healthy controls and (C and D) the isotype controls for TLR-2. One representative analysis of two AD patients and two healthy controls is depicted. Original magnification ×400.

There are a limited number of investigations regarding the effects of IL-31 on inflammatory mechanisms. We recently showed that IL-31 induces pro-inflammatory effects in activated human monocytes and macrophages following up-regulation of the receptor with staphylococcal exotoxins (20). One study demonstrated that IL-31 increases the expression of pro-inflammatory cytokines such as IL-8 in human intestinal epithelial cells (11). Another study revealed that IL-31 could significantly increase gene and protein expression of EGF, VEGF and CCL2 in human bronchial epithelial cells (BEAS-2B cells). Moreover, IL-31 augmented the release of EGF, VEGF, CCL2, IL-6 and IL-8 in co-cultures of BEAS-2B cells and eosinophils (12). Our data confirmed effects of IL-31 for CCL2 and VEGF secretion after up-regulation of the IL-31R with Pam3Cys or IFN-γ in keratinocytes. In contrast to the literature (11, 12, 27, 31), we could not find direct effects of IL-31 in keratinocytes both at the mRNA and the protein level on CCL2, VEGF, EGF, IL-1β, IL-6 and IL-8 production. In concordance with this, a recent study showed the release of pro-inflammatory cytokines, namely IL-1β, IL-6, CXCL1, CXCL8, CCL2 and CCL18 from eosinophils following IL-31 stimulation and such induction was further enhanced upon co-culture of eosinophils and keratinocytes. However, no direct effects of IL-31 in keratinocytes were observed (33).

Our findings show that only stimulation with IL-31 after up-regulation of the IL-31 receptor with either the TLR-2 ligand Pam3Cys or the Th1 cytokine IFN-γ could significantly enhance CCL2 production in keratinocytes via the phosphorylation of STAT-3. This suggests a new functional effect of IL-31 in keratinocytes. This is of clinical interest as keratinocytes are known to play a crucial role in local inflammatory responses by producing a broad panel of pro-inflammatory cytokines and chemokines (34). Dysregulated production of these pro-inflammatory mediators in general and CCL2 in particular are thought to be contributing factors to the development of AD (35).

Pam3Cys strongly up-regulated IL-31RA expression in keratinocytes from healthy controls and moderately enhanced IL-31RA expression in keratinocytes from AD patients.

In contrast to the experiments performed using foreskin-derived keratinocytes, IL-31RA in hair-derived keratinocytes was only up-regulated by Pam3Cys but not by IFN-γ stimulation. This discrepancy might be attributed to the different phases of differentiation in these cells as it has been shown previously that IL-31RA expression depends on the differentiation state of keratinocytes (36). Heise et al. showed that IFN-γ stimulation of normal epidermal human keratinocytes (NHEK) in early phases of keratinocyte differentiation led to an up-regulation of IL-31RA expression. However, IFN-γ treatment of NHEK had no effect on IL-31RA expression in the late phases of differentiation, (36). As we failed to induce an up-regulation of IL-31RA upon IFN-γ stimulation, keratinocytes from inflamed skin might be more responsive to

IL-31 in the early stages of differentiation what should be addressed more in detail in further studies.

Moreover, IL-31 induced lower levels of CCL2 secretion in keratinocytes from AD patients compared with healthy controls following up-regulation of the IL-31RA with Pam3Cys. CCL2 is known as a potent chemotactic factor for monocytes and activated Th1 lymphocytes (37). It might be anticipated that the minor effect of IL-31 regarding CCL2 production in keratinocytes derived from AD might not strongly favour the Th1 phenotype compared with healthy controls, thus it might switch to Th2 responses and support Th2 cytokines such as IL-31 itself. In turn, activated skin-infiltrating T cells may become new sources of IL-31, thereby amplifying atopic skin inflammation and pruritus as a positive feedback loop. Furthermore, there is emerging evidence which supports a general impairment of TLR-2 expression and TLR-2-mediated pro-inflammatory cytokines in monocytes from AD patients (26, 38). In this context, we recently showed that macrophages from patients with AD expressed significantly less TLR-2, whereas the expression pattern of TLR-1 and TLR-6 were not altered. In addition, macrophages from patients with AD had a reduced capacity to produce pro-inflammatory cytokines and chemokines upon TLR-2 stimulation (IL-1β, IL-6, CXCL8) which might be an explanation for the enhanced susceptibility to skin infections with *S.aureus* (26). In this study, we also showed a decreased TLR-2 expression in inflamed AD skin compared with healthy skin. Therefore, moderate up-regulation of IL-31RA upon stimulation with the TLR-2 ligand Pam3Cys in keratinocytes from AD compared with healthy individuals could be attributed to an impaired TLR-2 expression on keratinocytes from AD patients. Consecutively, the impaired CCL2 production by keratinocytes from AD patients upon treatment with Pam3Cys and IL-31 might be explained by an impaired TLR-2 expression on these cells.

Our data also show a dysbalanced chemokine production following the combination of IL-31 and TLR-2 ligand stimulation in AD and support the emerging concept that AD patients have a dysbalance in innate and acquired immunity.

In conclusion, our findings identify a new functional role of IL-31 in keratinocytes and provide a new link between TLR-2 ligands and IL-31 which might be dysregulated in AD. This may have implications for cutaneous inflammation in eczema where skin colonization with *S. aureus* and dysregulation of TLR-2 have been described.

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Supporting Information

Additional Supporting Information may be found in the online version of this article found at: http://www.wileyonlinelibrary.com

Data S1. Materials and methods.
Figure S1. Expression and regulation of IL-31RA in HPKs at the mRNA level. HPKs were left either unstimulated (medium control, not stimulated) or were stimulated with Pam3Cys (10 μg/ml) or IFN-γ (10 ng/ml), respectively, for 4, 8 and 24 h. qRT-PCR was employed to determine IL-31RA mRNA expression. Data are shown as mean IL-31RA/GAPDH ratio + SEM of $n = 7$ independent experiments. $*P < 0.05$ compared with medium control.
Figure S2. Expression and regulation of IL-31R on HPKs at the protein level. Regulation of IL-31RA and OSMR on HPKs following 24 h Pam3Cys (10 μg/ml), IFN-γ (10 ng/ml) or IL-4 (20 ng/ml) stimulation. Histograms of representative stainings along with isotype controls are shown. NS, not stimulated.
Figure S3. STAT-3 activation in hair derived HPKs. Phospho-STAT (pSTAT)-3 and total STAT-3 levels were determined by means of Western blot analysis 15–60 min after IL-31 stimulation. Approximately 5×10^5 HPKs derived from hair follicles were left either unstimulated or were prestimulated with Pam3Cys (10 μg/ml) or IFN-γ (10 ng/ml) for 24 h to up-regulate the IL-31 receptor and with and without IL-31 (100 ng/ml) for 15 or 60 min. Cellular extracts were normalized on the basis of protein determination. One representative experiment of three independent experiments from different donors is shown.

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