IL-31 regulates differentiation and filaggrin expression in human organotypic skin models

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Background: Atopic dermatitis (AD) is an inflammatory skin disease affecting 10% to 20% of children and 1% to 3% of adults in industrialized countries. Enhanced expression of IL-31 is detected in skin samples of patients with AD, but its physiological relevance is not known.

Objective: We sought to determine the role of IL-31 in skin differentiation.

Methods: We used human 3-dimensional organotypic skin models with either primary keratinocytes or HaCaT keratinocytes with inducible IL-31 receptor α to evaluate the effect of IL-31. The consequences were studied by using histology, the expression of markers analyzed by immunofluoresence and quantitative RT-PCR, and gene expression arrays.

Results: We observed that IL-31 interferes with keratinocyte differentiation. Gene expression analysis revealed a limited set of genes deregulated in response to IL-31, including *IL20* and *IL24*. In HaCaT keratinocytes with inducible IL-31 receptor α, IL-31 inhibited proliferation upon induction of IL-31 receptor α by inducing cell cycle arrest. As in primary cells, IL-31–treated HaCaT cells elicited a differentiation defect in organotypic skin models, associated with reduced epidermal thickness, disturbed epidermal constitution, altered alignment of the stratum basale, and poor development of the stratum granulosum. The differentiation defect was associated with a profound repression of terminal differentiation markers, including filaggrin, an essential factor for skin barrier formation, and a reduced lipid envelope. The highly induced proinflammatory cytokines IL-20 and IL-24 were responsible for part of the effect on *FLG* expression and thus for terminal differentiation.

Conclusion: Our study suggests that IL-31 is an important regulator of keratinocyte differentiation and demonstrates a link between the presence of IL-31 in skin, as found in patients with AD, and filaggrin expression. (J Allergy Clin Immunol 2012;129:426-33.)

Key words: *Atopic dermatitis, differentiation, filaggrin, HaCaT, IL-20, IL-24, IL-31, keratinocyte, organotypic skin model, proliferation*

Atopic dermatitis (AD) is a frequent chronic inflammatory skin disease with a number of different cytokines being involved.[1,2] Studies from different laboratories reported enhanced expression of the gene that encodes the cytokine IL-31 in skin lesions of patients with AD.[3] Moreover, IL-31 serum levels are enhanced in patients with AD and correlate with the severity of the phenotype.[4,5] Despite these findings, the functional consequences of IL-31 expression in the pathogenesis of AD remain largely undefined. Originally, IL-31 was described as synthesized by T helper cells, in particular T_H2.[6] More recent studies identified additional sources of IL-31 expression, including monocytes, dendritic cells, and mast cells (MCs).[7-9] Thus, several different cell types that potentially infiltrate the skin or are resident skin cells have the capacity to produce IL-31.[3]

IL-31 signals through the IL-31 receptor α (IL-31RA)/oncostatin M receptor β (OSMRβ) heterodimeric complex, which activates the Janus kinase (JAK)-signal transducer and activator of transcription (STAT), mitogen-activated protein kinase, and phosphoinositide-3-kinase (PI3K) signaling pathways.[10,11] The IL-31 receptor has a rather broad expression spectrum. Keratinocytes of the human epidermis express significant amounts of IL-31RA and OSMRβ. Differentiation leads to a downregulation of IL-31RA, whereas IFN-γ treatment induces IL-31RA expression in not only proliferating but also differentiated keratinocytes in tissue culture.[11] Moreover, keratinocytes of patients with AD possess higher IL-31RA expression and may therefore be more responsive to IL-31.[3] These and other studies have shown that human skin keratinocytes can respond to IL-31; however, the consequences are largely unknown.

One of the proteins important for the terminal differentiation of keratinocytes and for skin barrier function is filaggrin, which is extensively processed to participate in the formation of the stratum corneum.[12,13] Recently, loss-of-function mutations in *FLG*, the gene encoding filaggrin, have been identified as the cause of the common skin condition ichthyosis vulgaris, which is characterized by dry scaly skin.[14,15] These mutations, which are found in up to 10% of the general population, represent a strong genetic predisposing factor for AD, asthma, and allergies.

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Abbreviations used
3D: Three-dimensional
AD: Atopic dermatitis
IL-31RA: IL-31 receptor α
JAK: Janus kinase
MCs: Mast cells
NHEKs: Normal human epidermal keratinocytes
OSMRβ: Oncostatin M receptor β
PI3K: Phosphoinositide-3-kinase
STAT: Signal transducer and activator of transcription

FLG is located within the epidermal differentiation complex on chromosome 1q21.3, a gene cluster expressed late in epidermal differentiation.[14] Loss of filaggrin leads to a poorly formed stratum corneum (ichthyosis), resulting in skin prone to water loss (xerosis) and to enhanced percutaneous transfer of allergens. *FLG* null mutations are found in a large proportion of patients with AD.[14,16,17] Heterozygotes with an *FLG* 2282del4 mutation are observed not only in European American patients with AD (8.8%) but also in normal healthy subjects (3.7%). Interestingly, the acute lesional skin of patients with AD having this mutation exhibited lower levels of filaggrin expression as compared with the uninvolved skin in the same patients.[18] This suggests that additional factors contribute to filaggrin expression and the AD phenotype.

In this study, we addressed the putative role of IL-31 in skin development and in the pathogenesis of AD and observed that this cytokine interfered with the proliferation and differentiation of keratinocytes in a 3-dimensional (3D) organotypic model system, at least in part by activating the expression of IL-20 and IL-24. Furthermore, IL-31 repressed the expression of several differentiation markers, including filaggrin. Thus, the expression of IL-31 is critical for proper skin differentiation, and our findings suggest that increased expression of this cytokine is at least in part responsible for the AD phenotype.

METHODS

Primary cell culture and skin equivalents

Keratinocytes and fibroblasts were prepared from sterile human skin samples and hair follicles (approved by the ethic committee of the RWTH Medical School), cultivated, and skin equivalents prepared (see additional information in the Methods section of this article's Online Repository at www.jacionline.org).

HaCaT cells with inducible IL-31RA

HaCaT cells were obtained from N. Fusenig (DKFZ, Heidelberg, Germany).[19] IL-31RA was expressed stably in HaCaT cells using a tet-inducible lentiviral expression vector (see additional information in the Methods section of this article's Online Repository). All methods are described in detail in the Methods section of this article's Online Repository.

RESULTS

IL-31 interferes with keratinocyte differentiation

To understand the role of IL-31 for keratinocyte proliferation and differentiation, recombinant human IL-31 (rhIL-31) was applied to organotypic human 3D models generated from primary normal human epidermal keratinocytes (NHEKs) and primary dermal fibroblasts. IL-31 treatment resulted in a disturbed epidermal differentiation, characterized by a thin epidermal layer with hypogranulosis and an abolished alignment of keratinocytes in the stratum basale (Fig 1, *A*). The proliferation of keratinocytes was impaired as measured by the reduced staining for Ki67. In addition, the expression of epidermal differentiation markers, including filaggrin and cytokeratin 10, was reduced in the upper epidermal layer. Moreover, hair follicle keratinocytes of an atopic patient showed a similar phenotype in response to IL-31 (see Fig E1 in this article's Online Repository at www.jacionline.org). Of note, none of the donors of the primary cells carried either the p.R501X mutation or the c.2282del4 *FLG* mutation. These histological findings were confirmed by measuring the *FLG* and *KRT10* mRNA levels of the 3D models. *FLG* and *KRT10* expression was considerably delayed and reduced during differentiation in response to IL-31 (Fig 1, *B* and *C*). The expression of involucrin was also reduced (Fig 1, *A*), but this was not apparent at the mRNA level (data not shown). Moreover, the expression of β4-integrin and cytokeratin 14 was disorganized (Fig 1, *A*), suggesting that IL-31 has broad effects on keratinocyte differentiation.

To address the molecular consequences of IL-31, NHEKs were stimulated for 1 hour with IL-31 and differences in gene expression measured. A rather limited set of genes was responsive (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). The most highly regulated gene was *SOCS3*, a direct target of the JAK-STAT3 pathway activated by the IL-31RA/OSMRβ receptor complex.[10] In addition, the expression of several cytokines and transcriptional regulators was enhanced. Of particular interest to us were the genes that encode IL-20 and IL-24. These 2 genes are located in the *IL10* gene cluster containing 2 further cytokine genes, *IL10* and *IL19*, flanked by additional genes (summarized in Fig E2, *B*).

We observed that *IL20* and *IL24* were robustly activated within 1 hour of IL-31 application (Fig 1, *D*). In contrast, the neighboring genes were either not expressed (ie, *IL10* and *IL19*) or their expression was not affected by IL-31 treatment (ie, *MAPKAPK2*). Similarly, the expression of *IL31RA* and *OSMR* was not regulated by IL-31. These findings validate the IL-31–dependent regulation of *IL20* and *IL24* and suggest that these 2 cytokines are involved in the downstream effects of IL-31. Indeed, both IL-20 and IL-24 have been suggested to affect keratinocyte differentiation, although some of the reports are controversial.[20]

IL-31 induces *IL20* and *IL24* expression in HaCaT keratinocytes expressing IL-31RA

Primary keratinocytes often show donor-dependent variability, which we also noted by studying the response to IL-31 in many different batches of primary NHEKs. Therefore, we established a donor-independent reproducible cellular system for subsequent studies. HaCaT cells are spontaneously immortalized primary keratinocytes that are still competent to differentiate in organotypic skin models.[19] HaCaT keratinocytes constitutively express the OSMRβ (Fig E3, *A*) but not the IL-31RA chain and are therefore unresponsive to IL-31. The expression of IL-31RA in HaCaT cells can be induced by IFN-γ (see Fig E3, *A* and *B*, in this article's Online Repository at www.jacionline.org),[11] which enables the cells to respond to IL-31 and to induce target genes, including *IL20* and *IL24* (Fig E3, *C*). But IFN-γ controls many cellular processes and induces cell cycle arrest, making it difficult to investigate IL-31–specific functions during differentiation. To overcome

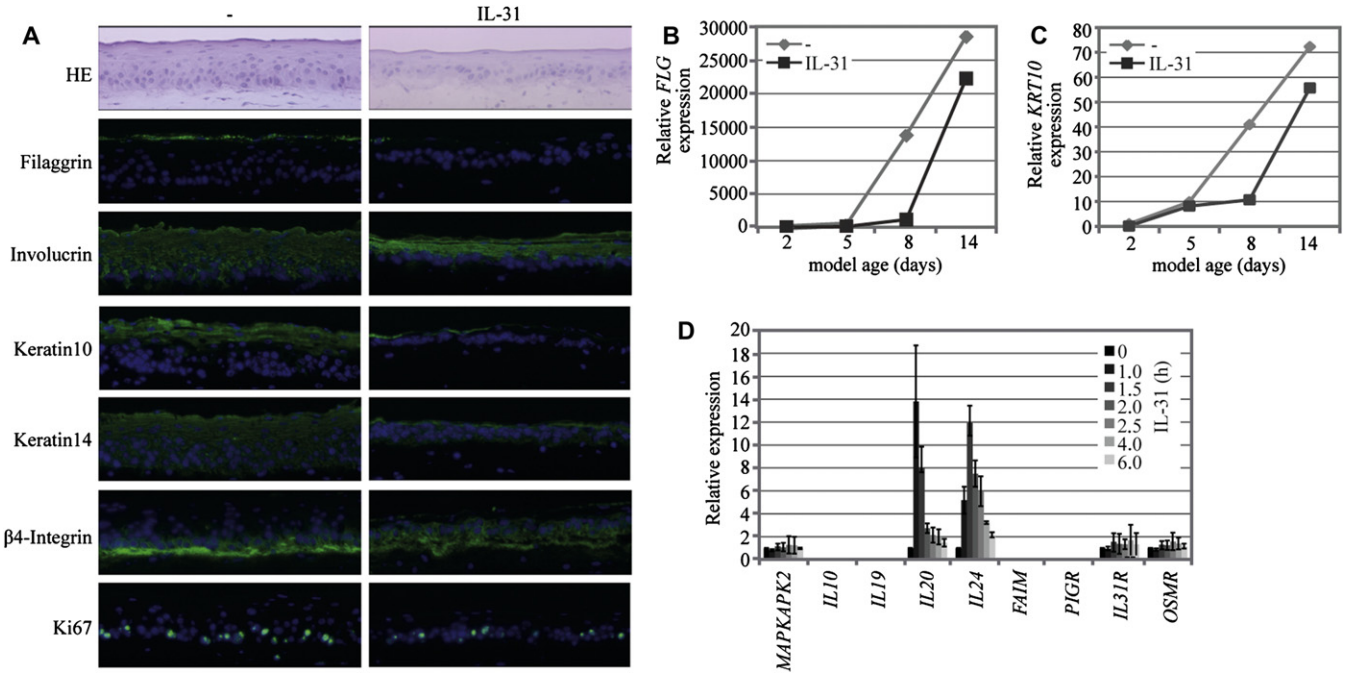

FIG 1. IL-31 disturbs differentiation of NHEKs in organotypic skin equivalents. **A,** 3D organotypic skin equivalents were stimulated with or without rhIL-31 (100 ng/mL; every second day), and histological sections were stained for the indicated markers at day 8 (1 of 6 models). **B** and **C,** Organotypic skin equivalents were harvested at the days indicated, and *FLG* (*Fig 1, B*) and *KRT10* (*Fig 1, C*) expression was determined by using quantitative RT-PCR. Relative expression is displayed (compared with nonstimulated day 2, normalized to *HPRT*). One representative experiment of 3 is shown. **D,** NHEKs from 3 different donors were stimulated with rhIL-31 (100 ng/mL). The indicated genes were analyzed by using quantitative RT-PCR. *HE*, Hematoxylin-eosin.

this limitation, we generated stable cell clones expressing IL-31RA under a tetracycline regulatable promoter. These HaCaT-IL31RA clones expressed *IL31RA* mRNA (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org) and protein (Fig E4, *B*) on addition of doxycycline. IL31-RA was expressed at the cell surface (Fig 2, *A*) and was functional because stimulation with IL-31 resulted in the phosphorylation of STAT3 (Fig 2, *B*), an immediate downstream target of receptor-bound JAK kinases. Also, the HaCaT-IL31RA clones responded with induction of *IL20* and *IL24* mRNA expression (Fig E4, *C*). Clones K12 and K14 were selected for the following experiments. In these 2 clones, the induction of the *IL20* and *IL24* genes was observed only when IL-31RA was induced and the cells were stimulated by IL-31 (Fig 2, *C*). The specificity of induction of these 2 genes was addressed by using kinase inhibitors. The inhibition of JAK kinases completely blocked the expression of *IL20* and *IL24*, consistent with a key role of these kinases in IL-31RA/OSMRβ–dependent signal transduction (Fig 2, *D*). In addition, the p38 and extracellular signal-regulated kinases but neither c-Jun N-terminal kinases nor PI3K kinases were critical to induce the 2 cytokine genes (Fig 2, *D*). Thus, HaCaT cells stably expressing an inducible IL-31RA subunit and NHEK cells respond comparably to IL-31 signaling when *IL20* and *IL24* expression is measured, requiring the activation of distinct signaling pathways.

Next, we addressed how the HaCaT clones reacted to IL-31 stimulation. The combination of the induction of IL-31RA and stimulation with IL-31 resulted in reduced proliferation in clone 14 cells (Fig 3, *A*). Fluorescence-activated cell sorting analyses revealed that within 48 hours the number of cells in S/G2 was significantly reduced with a parallel increase in G1 cells (Fig 3, *B*). Comparable results were obtained with clone 12 cells (data not shown). Since we did not observe any signs of apoptosis by AnnexinV staining (Fig E4, *D*), by evaluating the cells morphologically, and by measuring sub-G1 cells (data not shown), the proliferation effect is most likely due to reduced cell cycle progression, consistent with the reduced Ki67 staining observed in the 3D NHEK model (Fig 1, *A*).

IL-31 interferes with differentiation of HaCaT-IL31RA cells

The consequences of IL-31 signaling on HaCaT cells were measured in organotypic 3D skin reconstructs. Representative micrographs depict the effect of continuous IL-31 treatment (at day 7 and day 14) on 3D reconstructs of a HaCaT-IL31RA cell clone (see Fig E5, *A* and *C*, in this article's Online Repository at www.jacionline.org). Similar to primary cells, IL31-treament significantly disturbed epidermal differentiation. This was specific for IL-31 signaling, because neither doxycycline nor IL-31 alone resulted in changes in the differentiation compared with the control. The epidermal layer was thinner and disordered. Moreover, the expression of epidermal differentiation markers, including filaggrin, involucrin, and cytokeratin 10, was reduced in the upper epidermal layer (Fig 4, *A*, and Fig E5, *B*). For a semiquantitative estimation of IL-31–induced disordered differentiation, Hematoxylin-eosin–stained slides from 3 independent experiments were examined blinded by a dermatopathologist.[21] The epidermal thickness of each reconstruct was measured (Fig 4, *B*), and the

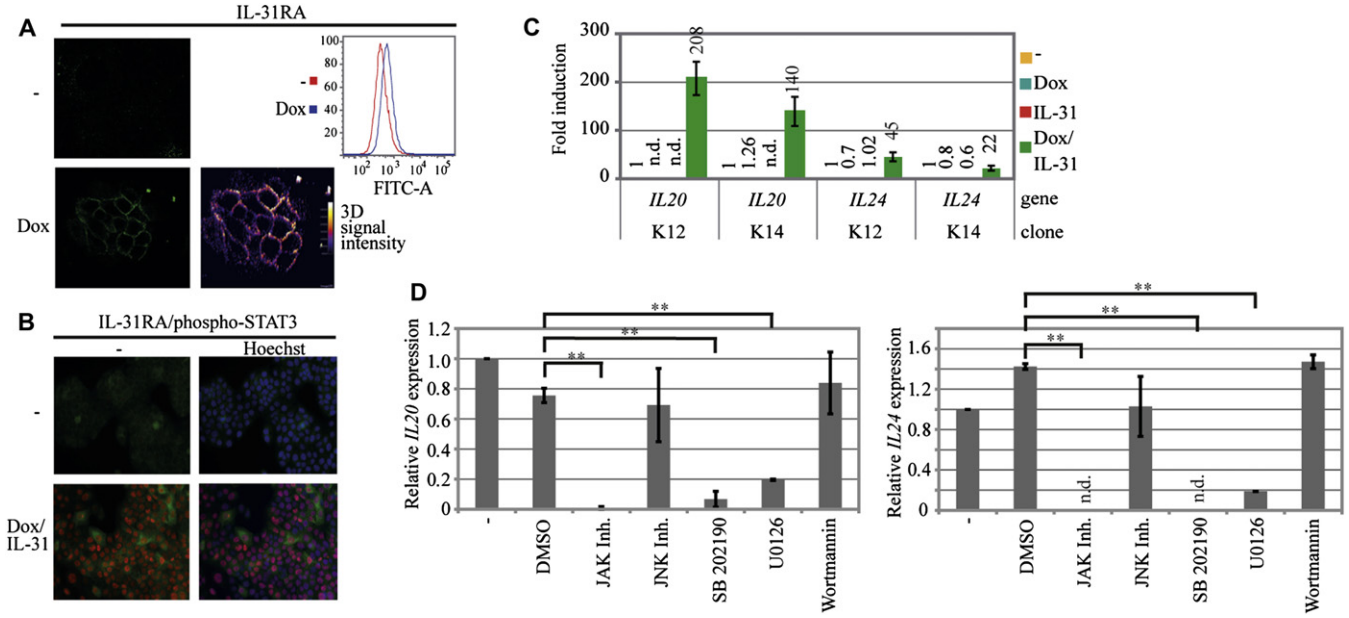

FIG 2. IL-31 induces *IL20* and *IL24* in HaCaT-IL31RA cells. **A,** IL-31RA protein expression was evaluated in clone 14 cells treated with or without dox (1 μg/mL) for 16 hours and visualized by using indirect confocal immunofluoresence microscopy *(panels on the left)* and fluorescence-activated cell sorting *(panel on the right)*. **B,** Clone 14 cells were treated with or without dox (1 μg/mL, 16 hours), stimulated with rhIL-31 for 45 minutes, fixed, and subsequently stained for IL-31RA *(green)* and phospho-STAT3 *(red)*. The DNA was stained with Hoechst 33258. **C,** The expression of *IL20* and *IL24* was analyzed by using quantitative RT-PCR in 2 individual clones in response to dox (16 hours), rhIL-31 (1 hour), or cotreatment. **D,** The cells were treated as in panel C. In addition, the indicated kinase inhibitors (JAK inhibitor I [100 nM]; c-Jun N-terminal kinase inhibitor II [20 μM]; SB202190, selective for p38 MAP kinases [20 μM]; U0126, selective for extracellular signal-regulated kinases [20 μM]; Wortmannin, selective for PI3K kinases [500 nM]) were added 1 hour prior to stimulation with IL-31. The expression of *IL20 (left panel)* and *IL24 (right panel)* was analyzed by using quantitative RT-PCR. Mean values and standard deviations of 3 independent experiments are displayed. $^{**}P < .01$. *dox*, Doxycycline; *n.d.*, not detectable.

specimens were scored according to the epidermal constitution, alignment of the stratum basale, and development of the stratum granulosum on a scale from 0 (completely disturbed) to 3 (normally differentiated, ie, equivalent to healthy human skin) as described previously (Fig 4, *C*).[21] IL-31 signaling resulted in a significantly disturbed differentiation according to all 3 criteria.

Most striking was the reduced expression of both proliferation (Ki67) and differentiation (filaggrin, involucrin, cytokeratin 10) markers, which are strong indications for inappropriate differentiation. Prominent was the effect on filaggrin expression, as also seen in the primary cell model (Fig 1, *A*). The *FLG* and *KRT10* expression was strongly reduced when the 3D model was analyzed on permanent IL-31 treatment at day 7 (Fig 4, *D*). The expression of β4-integrin was also disorganized and no longer confined to the basal layer (Fig 4, *A*). In IL-31 pulse experiments, 2 hours were not sufficient to observe a phenotype in the 3D model (data not shown). But an 8- and 24-hour treatment resulted in a partial phenotype (Fig E5, *D*), suggesting that an initial, relatively short IL-31 stimulus is sufficient to deregulate late differentiation processes. *FLG* was a particular sensitive marker because we observed a reduction in expression already after an IL-31 pulse of 2 hours, while *KRT10* expression was less sensitive (Fig 4, *D*).

In addition to the altered expression of protein markers, disturbed differentiation was measurable by analyzing the lipid envelope. The overall lipid content was reduced on IL-31 treatment (see Fig E6, *A*, in this article's Online Repository at www.jacionline.org). The mRNA expression of enzymes involved in the metabolism of ceramids (spingomyelinase and sphingomyelin synthase), of cholesterol (steroid sulfatase), and of free fatty acids (phospholipase A2) was measured.[22] IL-31 signaling did not affect the expression of these genes in HaCaT-IL31RA organotypic skin models (Fig E6, *B*). Nevertheless, the expression of ceramids in the cornified envelope was reduced in response to IL-31 (Fig E6, *C*), suggesting that IL-31 signaling affects lipid envelope metabolism, possibly at the posttranslational level. Together, these findings support the hypothesis that IL-31 induces a series of molecular events that culminate in defective differentiation.

IL-20 and IL-24 mediate part of the IL-31 effect on keratinocyte differentiation

Candidates for mediating this IL-31 effect are IL-20 and IL-24, which are induced rapidly in response to IL-31. In addition, an enhanced expression of both genes was measured upon prolonged stimulation of HaCaT-IL31RA cells with IL-31 (Fig 5, *A*). The 3 receptor subunits that form 2 heterodimeric receptors, that is, IL-20Rα/IL-20Rβ and IL-22Rα/IL-20Rβ, which are both recognized by IL-20 and IL-24, are expressed at the cell surface (Fig 5, *B*). Therefore, we tested whether IL-20 and IL-24 affected keratinocyte differentiation. While IL-20 and IL-24 alone gave variable and small effects (data not shown), the combination of the 2 cytokines resulted in reduced differentiation with decreased *FLG* and, to a lesser extent, *KRT10* expression (Fig 5, *C* and *D*). Similar effects were seen in HaCaT-IL31RA clone 12 cells. Moreover,

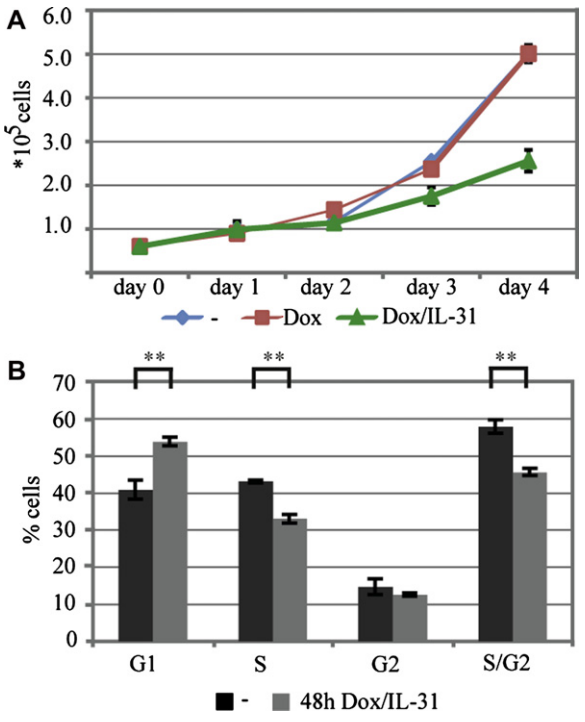

FIG 3. IL-31 inhibits proliferation in HaCaT-IL31RA cells. **A,** The proliferation rate of HaCaT-IL31RA clone 14 cells was measured by counting cells incubated in the presence or absence of dox (1 μg/mL) and IL-31 (100 ng/mL). The data are of 3 biological replicates with standard deviation. **B,** Cell cycle distribution of HaCaT-IL31RA clone 14 cells was determined in response to dox/IL-31 treatment after 48 hours. The percentage of cells in G1, S, and G2 phases was determined by using fluorescence-activated cell sorting. Displayed are mean values with standard deviation of 3 biological replicates. $^{**}P < .01$. *dox,* Doxycycline.

reduced expression of *FLG* and *KRT10* was measured in 3D equivalents of primary NHEK (Fig 5, *D*). IL-20/IL-24 treatment was clearly less efficient than IL-31 treatment, suggesting that additional IL-31–regulated genes are involved in controlling keratinocyte proliferation and differentiation.

DISCUSSION

AD is characterized by the deregulation of a number of cytokines that are postulated to participate in disease progression.[1,2] One of the T_H2 effector cytokines implicated in AD is IL-31, in addition to a number of other cytokines including IL-4 and IL-13. IL-31 is also produced by monocytes, dendritic cells, and MCs.[3] UV and reactive oxygen species enhance the expression of IL-31 in at least some of these cells that have the capacity to infiltrate human skin or are resident skin cells. MCs play a prominent role in allergic disorders.[23,24] Antimicrobial peptides β-defensins and cathelicidin LL-37 stimulate IL-31 expression and secretion in MCs.[8] Moreover, $CD4^+$ T cells, especially T_H2 cells, express the histamine H_4 receptor, which is upregulated on IL-4 treatment. Stimulation with a histamine agonist results in the activation of *IL31* mRNA expression in these T cells.[25] A possible scenario is that the reduced skin barrier formation observed in patients with AD allows a more readily activation of monocytes, T cells, and MCs during infection and by allergens, subsequently stimulating IL-31 production. For example, the superantigen staphylococcal enterotoxin B of *S aureus*, which colonizes frequently AD lesions, is a strong activator of IL-31 production in immune cells.[26] From these observations, an important role of IL-31 in AD pathology has been postulated. However, IL-31–responsive cells and the physiological consequences in these cells are less well described. Keratinocytes express the IL-31RA and OSMRβ receptor subunit; the expression of the former can be further stimulated with proinflammatory cytokines including IFN-γ,[11] suggesting that keratinocytes are responsive to IL-31. Another relevant cell type found in lesions of patients with AD are eosinophils, which express the IL-31 receptor.[27] IL-31 stimulates the secretion of a number of different proinflammatory cytokines and chemokines by eosinophiles. Together, these findings suggest that IL-31 has broad proinflammatory effects in skin and thus IL-31 is a valid candidate to contribute to the development of AD pathology.

Another important aspect of AD is the development of pruritus. An enhanced expression of the specific IL-31RA was discovered in cells of the human and murine dorsal root ganglia and in murine primary afferent fibers of the spinal cord and dermis that are proposed to be involved in the sensation of itch.[26,28] In an AD-like murine model (NC/Nga mice), high IL-31 mRNA expression correlates with itch-associated scratching behavior.[29] Moreover, IL-31 overexpression in mice results in the development of severe pruritus with skin lesions characterized by hyperkeratosis, acanthosis, inflammatory cell infiltration, and an increase in MCs.[6] However, in humans with AD, a direct correlation of itch sensation and IL-31 levels has not been demonstrated. Nevertheless, it is worth noting that a correlation of pruritus has been found with IL-31 serum levels and the number of MCs in patients suffering from Philadelphia chromosome-negative myeloproliferative neoplasms.[7] These MCs also released significantly larger amounts of pruritogenic factors, providing support for a role of IL-31 in pruritus development in humans.

Finally, it is worth noting that genetic predispositions associated with the *IL31* locus appear to influence the pathogenesis of AD. Three different haplotypes of single nucleotide polymorphisms in the *IL31* gene and promoter were identified.[30] One of these is significantly associated with non-IgE–mediated AD. Monocytes of healthy individuals of this risk haplotype responded with a stronger induction of *IL31* expression in response to CD3/CD28-mediated stimulation compared with the other haplotypes. This study provides additional evidence for a determining role of IL-31 in AD.

We observed that IL-31 inhibited proliferation and deregulated differentiation in both primary NHEK and HaCaT cells conditionally expressing the IL-31RA subunit (Figs 1, 3, 4, E1, E4, and E5). The effect of IL-31 on the capacity to proliferate is consistent with observations that this cytokine interferes with the proliferation of lung epithelial cells and of the colon carcinoma cell line HCT116.[31,32] In lung epithelial cells, IL-31 represses different cell cycle regulators, including cyclin B1 and cyclin-dependent kinase 1 (CDK1), and stimulates the expression of $p27^{KIP1}$, a negative regulator of cyclin/CDK complexes. These findings were somewhat unexpected since IL-31 stimulates different pathways, that is, the RAS/extracellular signal-regulated kinase pathway, the PI3K/AKT pathway, and the JAK/STAT pathway, all of which are rather proproliferative.[6,10,33-35] However, we note that IL-31 did not have a cytostatic effect on several cell lines tested (data not shown). Thus, at present it is not clear how IL-31 antagonizes proliferation and whether this effect is cell type specific.

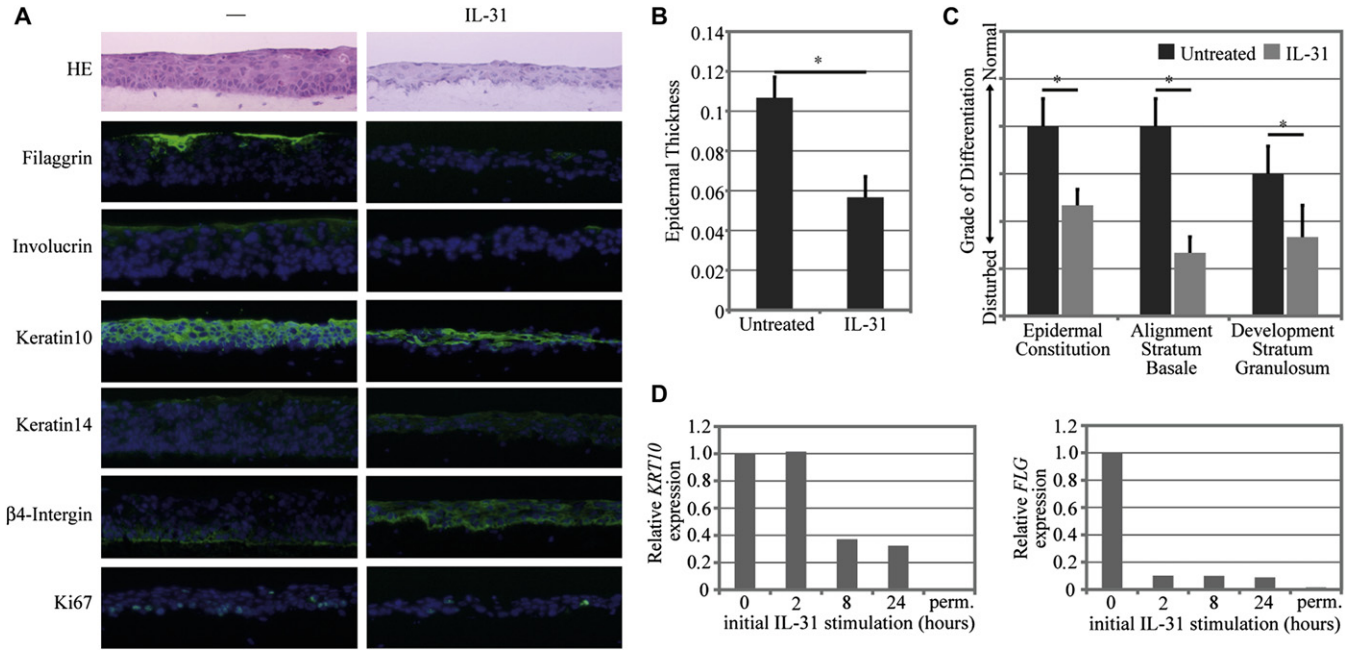

FIG 4. IL-31 disturbs the differentiation of HaCaT-IL31RA cells in organotypic 3D cocultures. **A,** Clone 14 cells organotypic skin equivalents were grown in the presence or absence of doxycycline and rhIL-31 (100 ng/mL). Histology (hematoxylin-eosin [HE]) and protein expression was analyzed at day 7 (identical to Fig E5, *A*). **B,** Epidermal thickness of HE-stained sections was measured. **P* < .05. **C,** Epidermal constitution, the alignment of the stratum basale, and the development of the stratum granulosum of HE-stained sections were measured. **P* < .05 (Mann-Whitney U test). **D,** The expression of *KRT10* and *FLG* mRNA was determined from biopsies of the 3D recontructs shown in panel A and Fig E5. A representative experiment performed in duplicates is shown.

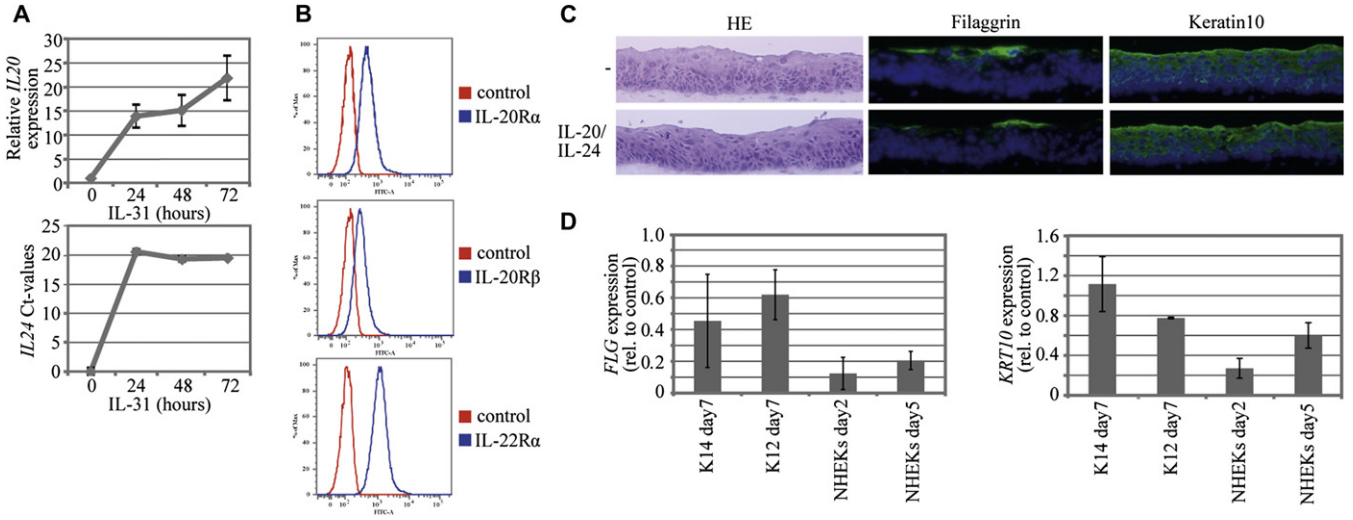

FIG 5. IL-20 and IL-24 impair differentiation of HaCaT-IL31RA cells in organotypic 3D cocultures. **A,** HaCaT-IL31RA clone 14 cells were stimulated with doxycycline/IL-31 (1 μg/mL/100 ng/mL) and *IL20* and *IL24* mRNA measured by quantitative RT-PCR. **B,** Fluorescence-activated cell sorting analysis of fixed cells incubated with antibodies specific for IL-20Rα, IL-20Rβ, and IL-22Rα and secondary antibodies coupled to Alexa-Fluor 488. **C,** Clone 14 organotypic skin equivalents in the presence or absence of IL-20 (20 ng/mL) and IL-24 (20 ng/mL), harvested at day 7 and stained as indicated. **D,** Total RNA was isolated from the organotypic skin equivalents (panel C) and from comparable samples of clone 12 and of 3D equivalents of primary NHEKs that were grown in the presence or absence of IL-20 (20 ng/mL) and IL-24 (20 ng/mL). The expression of *FLG* and *KRT10* was analyzed by using quantitative RT-PCR. *HE*, Hematoxylin-eosin.

Our findings suggest that the expression of filaggrin is a relevant downstream target of IL-31. As pointed earlier, loss-of-function mutations in *FLG* cause ichthyosis vulgaris and are correlated with AD, eczema, and asthma.[14,15,36,37] Filaggrin is essential for the formation of the stratum corneum and for skin barrier function.[12,13] Considering that *FLG* mutations are

frequent in the general Western population, the enhanced expression of IL-31 with the herein-described consequence on filaggrin suggests that IL-31 has the capacity to aggravate AD pathology and to further weaken skin barrier function. The IL-31 pulse experiments that we performed suggest that *FLG* is not a direct target of IL-31–induced signaling pathways (Figs 1, 5, and E5). Although the *FLG* promoter has not been studied in great detail, the AP1 family of transcriptional regulators, POU-domain transcription factors, and p63 have been implicated in controlling *FLG* expression.[38-40] Moreover, AP1 factors have been demonstrated to regulate different aspects of skin differentiation.[41] While IL-20 and IL-24, as direct targets of IL-31 (Figs 1, 2, and E2), contribute to the IL-31 differentiation phenotype (Fig 5), our array data indicate that different AP1 family members, that is, *FOS* and *JUNB*, as well as genes that encode components of mitogen-activated protein kinase signaling pathways are also targets of IL-31. This suggests that these genes will also contribute to altered differentiation of keratinocytes and disregulated filaggrin expression, which will require more detailed studies in the future. In summary, our study reveals a significant effect of IL-31 on the terminal differentiation of the human epidermis. These novel findings uncover a clear link between IL-31 expression, as found in patients with AD, and keratinocyte differentiation including filaggrin expression.

We thank N. Fusenig for providing HaCaT cells.

Key messages

- **IL-31 interferes with keratinocyte proliferation.**
- **IL-31 inhibits keratinocyte differentiation.**
- **The expression of filaggrin, a protein essential for skin barrier function, is reduced by IL-31.**
- **A cooperative effect of IL-31 and mutations in the gene encoding filaggrin on the AD phenotype is proposed.**

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METHODS

Primary cell culture and skin equivalents

Keratinocytes and fibroblasts were prepared from sterile human skin samples and cultivated under regular cell culture conditions. Hair follicle keratinocytes were prepared as described previously.[E1] Organotypic skin equivalents were constructed as described previously, cultured over a period of up to 14 days at the air-liquid interphase,[E2] and treated with the different cytokines as indicated in the figure legends. The reconstructs were harvested at the days indicated, cut into pieces, and either fixed according to a standardized protocol for routine histology or embedded in Tissue Tec (O.C.T.) compound (Sakura Finetek, Zoeterwoude, The Netherlands) for cryosectioning. Parts of the cultures were stored in RNA later (Ambion/Applied Biosystems, Darmstadt, Germany) for RNA isolation.

Analysis of gene expression using Exon Expression Arrays

For gene expression analysis, NHEKs were stimulated with recombinant IL-31 (100 ng/mL, PeproTech, Hamburg, Germany) for 1 hour. mRNA was then purified and analyzed on the GeneChip Human Exon 1.0 ST array as reported previously.[E3]

HaCaT cells with inducible IL-31RA

HaCaT cells were obtained from N. Fusenig (DKFZ, Heidelberg, Germany) and cultured in DMEM with 10% FCS.[E4] The *IL31RA* cDNA (nucleotide accession number: ENST00000297015) was obtained from ImaGenes (Berlin, Germany) in an open reading frame Gateway entry vector (pENTR™221) and fully sequenced. The cDNA was subcloned to add an hemagglutinin (HA)-tag and finally cloned into the tet-inducible lentiviral expression vector pSLIK-Neo,[E5] to obtain pSLIK-IL31RA-HA. HEK293T cells were transiently transfected with pSLIK-IL31RA-HA, together with the lentiviral packaging and helper plasmids pLP1, pLP2, and pLP/VSVG (Stratagene, La Jolla, Calif). The supernatants containing lentiviral particles were harvested 48 hours later, sterile filtered, diluted 1:2 with fresh medium, and supplemented with 8 μg/mL polybrene. This mixture was incubated with 50% confluent HaCaT cells for 24 hours. For selection, the cells were cultivated in medium containing 800 μg/mL G418. Seventy-two hours later, G418-resistant cells were washed, highly diluted in fresh media containing G418, and seeded onto 10-cm plates. Single-cell colonies were transferred into 48-well plates, expanded, and tested for *IL31RA* expression upon doxycycline treatment (1 μg/mL). Positive clones were further cultured in DMEM with 10% FCS containing 800 μg/mL of G418.

For 3D organotypic cultures, primary dermal fibroblasts were isolated, cultured, and seeded into collagen gels at a density of 5×10^5 mL^{-1}. These dermal equivalents were covered with DMEM containing 10% FCS. The following day, HaCaT-IL31RA cells (8×10^5 cm^{-2}) were plated onto the fibroblast-collagen matrix. The next day doxycycline (1 μg/mL) was added to induce IL-31RA expression. After 2 days of culturing, the skin equivalents were lifted to the air-liquid interphase. The treatment with IL-31 was as described above. Once lifted to the air-liquid interphase, interleukins were added as described in the figure legends. Untreated models were maintained as negative controls. For the pulse experiments, IL-31 (100 ng/mL) was added to the culture for 2, 8, and 24 hours. Models were carefully washed and further cultured with medium at the air-liquid interphase.

RNA preparation, reverse transcription, and quantitative RT-PCR

The RNAeasy Mini Kit (Qiagen, Hilden, Germany) was used for total RNA extraction, according to the manufacturer's instruction, and residual genomic DNA was removed by DNaseI (Qiagen) digestion. A total of 1 μg of RNA was reverse transcribed into cDNA by using the QuantiTect reverse transcription kit (Qiagen) and analyzed by quantitative real-time PCR by using the Corbett RotorGene system (Qiagen). The real-time PCR reactions were performed with the SensiMix SYBR Kit (Bioline, Luckenwalde, Germany) or the Universal Mastermix (Diagenode, Liège, Belgium). All used primer pairs were QuantiTect primer assays (Qiagen) except the primer pairs for hypoxanthine guanine phosphoribosyl transferase (*HPRT*) (forward: 5′-TGACACTGG CAAAACAATGCA-3′; reverse: 5′-GGTCCTTTTCACCAGCAAGCT-3′) and *OSMRβ* (forward: 5′-GTGTGGGTGCTTCTCCTGCTTC-3′; reverse: 5′-TCTGTGCTAATGACTGTGCTTG-3′). All measurements were performed in duplicate. The relative quantification was calculated by using the comparative cycle threshold method and normalized to *HPRT*. Kinase inhibitors were purchased from Calbiochem/Merck (Darmstadt, Germany): c-Jun N-terminal kinase Inhibitor I (420099); **c**-Jun N-terminal kinase Inhibitor II (420119); SB202190 (559388); U0126 (662005); Wortmannin (681675).

In case of organotypic 3D models, the tissues were mechanically disrupted and homogenized by using a tissue lyzer (Qiagen). Total RNA was extracted with Nucleo Spin RNA II (Macherey-Nagel, Düren, Germany) according to the protocol of the manufacturer. Purified RNA was reverse transcribed by utilizing High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, Calif). TaqMan experiments were carried out on an ABI PRISM 7300 sequence detection system (Applied Biosystems) using Assay-on-Demand gene expression products (Applied Biosystems) for *FLG* (Hs00418578_m1) and *KRT10* (Hs00166289_m1) according to the manufacturer's recommendations. An Assay-on-Demand product for *HPRT* rRNA (Hs99999909_m1) was used as an internal reference to normalize the target transcripts. For *FLG, KRT10,* and *HPRT*, all measurements were performed in triplicate in separate reaction wells.

Statistical significance was evaluated by using the 2-sided Student *t* test for all the experiments as indicated in the figure legends.

Western blotting

Cell lysis and western blotting were performed as described elsewhere.[E6,E7] Primary antibodies: IL-31RA (BAF2769, R&D Systems, Wiesbaden, Germany); α-tubulin (T-5168, Sigma-Aldrich, St Louis, Mo). The detection was performed by using horseradish peroxidase–labeled secondary antibodies with either "chemiluminescence ECL kit" (Pierce/Thermo Scientific, Rockford, Ill) or "SuperSignal West Femto Maximum Sensitivity Substrate" (Pierce).

Light microscopy and immunofluorescence

Cells were seeded at a density of 1.5×10^5 cells on a cover slip in a 12-well plate and incubated for 24 hours. Afterward the cells were stimulated with doxycycline for 16 hours. The cells were fixed with 3.7% paraformaldehyde for 20 minutes at room temperature and washed/blocked with PBS/1% BSA. The IL-31RA surface expression was analyzed by using an IL-31RA–specific primary antibody (BAF2769, R&D Systems) and a fluorescently labeled secondary antibody. For analysis of the induction of STAT3 phosphorylation after IL-31 stimulation, the cells were preincubated with doxycycline for 16 hours and stimulated with IL-31 for 45 minutes prior to fixation with 3.7% paraformaldehyde. The cells were permeabilized by incubation with 100% ice-cold methanol at −20°C for 20 minutes. STAT3 phosphorylation (Tyr705) was stained by using a specific primary antibody (9131S, Cell Signaling, Danvers, Mass) and an appropriate fluorescently labeled secondary antibody. Genomic DNA was stained by incubation with 0.2 μg/mL Hoechst 33258 for 5 minutes at room temperature.

For light microscopy and immunofluorescence of the 3D-skin models, 4-μm cryosections were processed as described previously.[E2] The following antibodies specific for the indicated proteins were used: β4-integrin (1:200, Millipore, MAB2059 [Temecula, Calif]); keratin 14 (1:200, Chemicon, CBL 197 [Temecula]); involucrin (1:2000, Sigma-Aldrich, I-9018); Ki67 and keratin 10 (M7240 at 1:100 and M7002 at 1:500, respectively, Dako [Glostrup, Denmark]); filaggrin (1:100, Santa Cruz, sc-66192 [Santa Cruz, Calif]); ceramid (1:25, antibodies-online ABIN 480714 [Aachen, Germany]). DNA was stained with DAPI (Applichem, Darmstadt, Germany). Nile red staining was performed on 4-μm cryosections with 12.5 μg/mL of Nile red (Invitrogen, N1142; Darmstadt, Germany) for 20 minutes followed by PBS washing steps and DAPI-DNA staining.

Flow cytometry and cell proliferation

Cultured HaCaT or HaCaT-IL31RA cells were washed with PBS/EDTA and dissociated by addition of trypsin/EDTA. To inactivate the trypsin, culture

medium containing 10% FCS was added. The cells were washed with PBS. For analysis of the surface expression of IL-31RA, IL-20Rα, IL-20Rβ, and IL-22Rα, the cells were fixed in 3.7% paraformaldehyde for 20 minutes, washed and blocked with PBS/1% BSA, and incubated with receptor-specific antibodies (IL-31RA: BAF2769, R&D Systems; IL-20Rα: AF1176, R&D Systems; IL-20Rβ: sc-47058, Santa Cruz; IL-22Rα, MAB2770, R&D Systems) at room temperature for 30 minutes. The cell surface fluorescence intensity was measured by adding fluorescently labeled secondary antibodies. For analysis of the cell cycle, the cells were fixed in 100% ice-cold methanol at −20°C for 20 minutes and washed 3 times with PBS/1% BSA. RNA was removed by adding 20 μg/mL of RNaseA at room temperature for 5 minutes. The genomic DNA was stained by adding a dilution of propidium-iodide in PBS at room temperature for at least 20 minutes (1:20 from 1 mg/mL of PI stock solution; Sigma-Aldrich). For Annexin V staining, the Annexin V-eFluor 450 Apoptosis detection kit (88-8006-74, eBioscience, Frankfurt, Germany) was used according to the manufacturer's recommendations. The fluorescence intensity in all experiments was measured by using the FacsCanto flow cytometry system (BD, Franklin Lakes, NJ).

To evaluate cell proliferation, HaCaT-IL31RA cells were seeded at low density (0.5×10^5 cells/6 wells) and immediately stimulated with doxycycline and IL-31. The cells were washed and restimulated daily. The total cell number was determined daily by dissociation of the cells in trypsin/EDTA and measuring the total cell number per well by using the CASY cell counting system (Innovatis/Roche, Basel, Switzerland).

FLG mutation analysis in normal human keratinocytes

The analysis of *FLG* mutations was performed as described with modifications detailed below.[E8,E9] We used the QIAamp DNA Mini Kit (Qiagen) for DNA extraction from NHEKs. With specific primer pairs we amplified the coding regions of the *FLG* gene harboring the 2 most frequent germline mutations p.R501X and c.2282del4. In detail, the primers used were FIL501F-CACGGAAAGGCTGGGCTGA and FIL501R-ACCTGAGTGTCCAGAC CTATT for mutation p.R501X and FIL2282F-GGGAGGACTCAGAC TGTTT and FIL2282R-AATAGGTCTGGACACTCAGGT for mutation c.2282del4. PCR reactions contained ~50 ng of DNA, 8.5 μM of each forward and reverse primers, and 15 μL of Platinum PCR Supermix (Life Technologies Europe BV, Bleiswijk, The Netherlands) in a total volume of 25 μL. PCR conditions for mutation p.R501X were initial denaturation at 95°C for 7 minutes; 35 cycles of denaturation at 95°C for 5 seconds, annealing at 58°C for 15 seconds, and elongation at 72°C for 30 seconds, followed by a final elongation step at 72°C for 10 minutes. For mutation c.2282del4, PCR conditions were initial denaturation at 95°C for 7 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 45 seconds, and elongation at 72°C for 60 seconds, followed by a final elongation step at 72°C for 10 minutes. PCR products were directly sequenced by using an ABI 3103 DNA Analyzer (Applied Biosystems, Foster City, Calif). Sequence analysis was performed by visual inspection.

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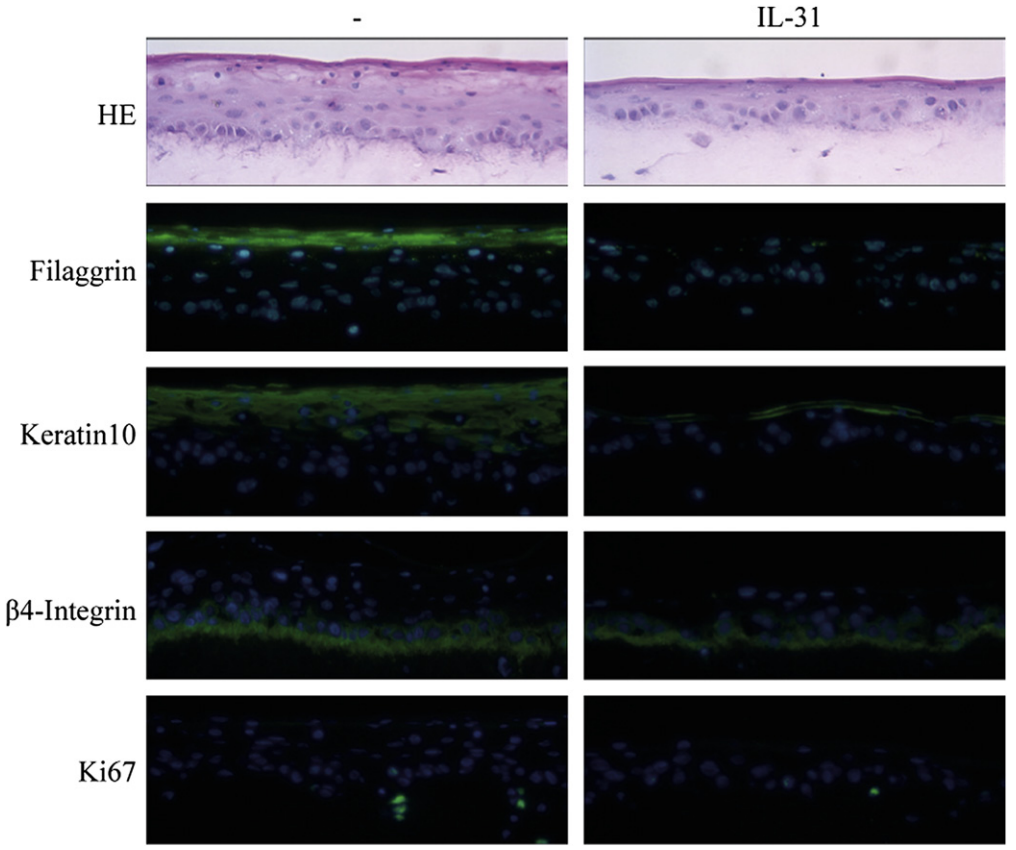

FIG E1. IL-31 disturbs differentiation of hair follicle keratinocytes of a patient with AD. 3D organotypic skin equivalents of hair follicle keratinocytes obtained from an atopic patient with elevated total IgE serum levels, allergic rhinitis, and moderate AD were stimulated with or without rhIL-31 (100 ng/mL; every second day), harvested at day 5, and histological sections stained for the indicated proteins. *HE*, Hematoxylin-eosin.

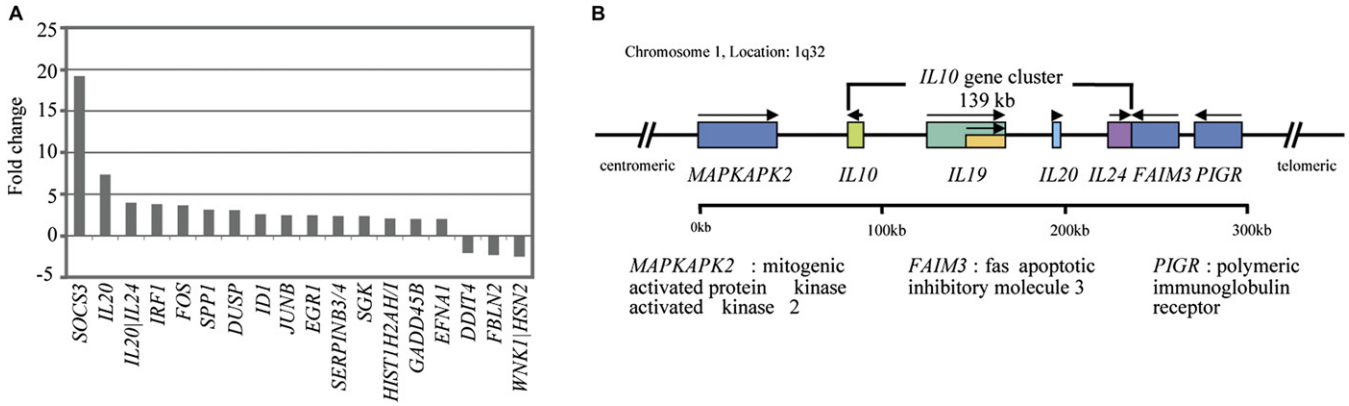

FIG E2. IL-31 regulated genes. **A,** NHEKs were stimulated with rhIL-31 for 1 hour and mRNA was isolated and analyzed on Affymetrix Exon arrays. Displayed are genes that are regulated more than 2-fold compared with a nonstimulated control in 2 independent experiments. **B,** Schematic representation of the *IL10* gene cluster located on chromosome 1 that spans a region of about 140 kb. The *IL19* gene is transcribed from 2 different promoters.

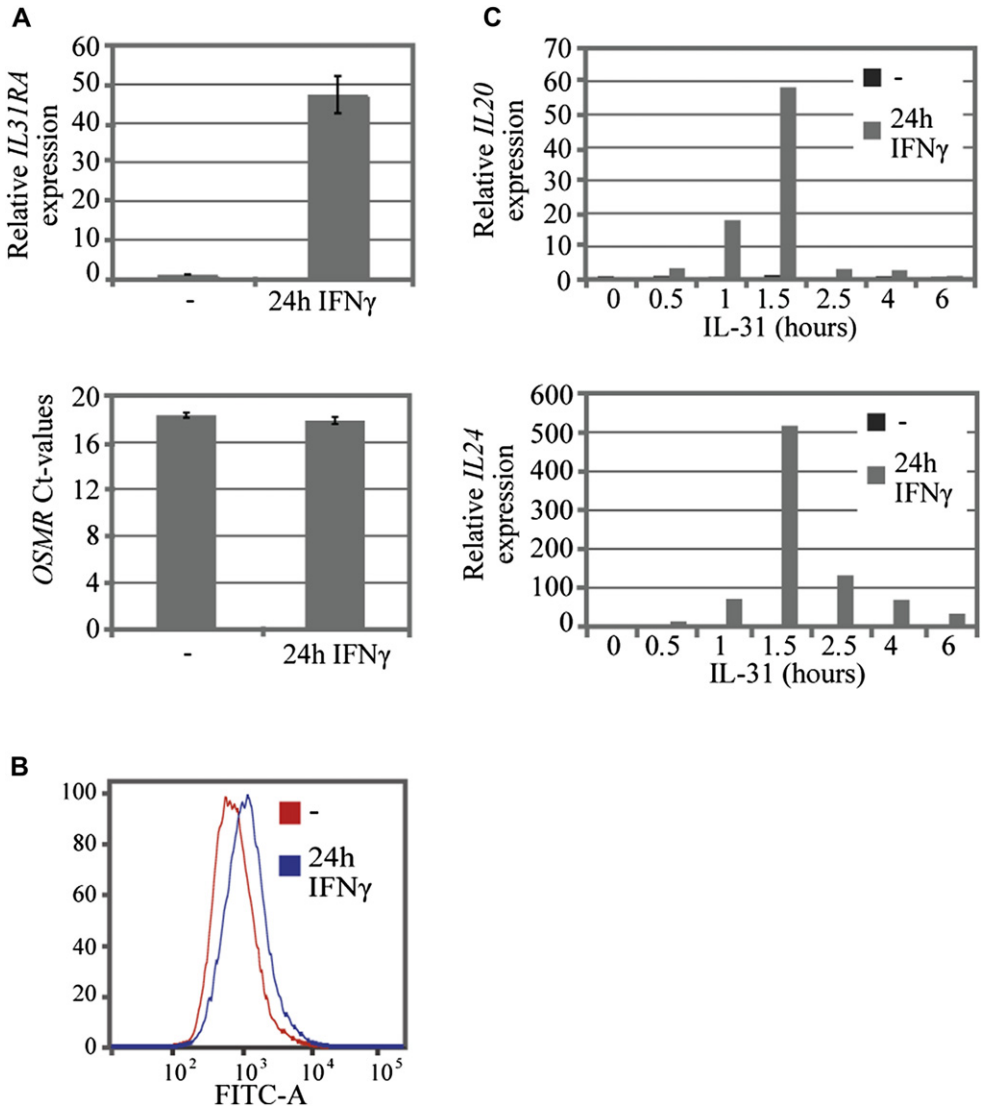

FIG E3. HaCaT cells express IL-31RA in response to IFN-γ and subsequently respond to IL-31. **A,** HaCaT cells were stimulated with IFN-γ (20 ng/mL) for 24 hours. Then, *IL31RA* and *OSMRβ* expression was analyzed by using quantitative RT-PCR. **B,** The expression of IL-31RA at the cell surface was evaluated in cells treated as described in panel A using FACS. **C,** HaCaT cells, either pretreated or not with IFN-γ for 24 hours, were stimulated with rhIL-31 (100 ng/mL). The expression of *IL20* and *IL24* was analyzed by using quantitative RT-PCR.

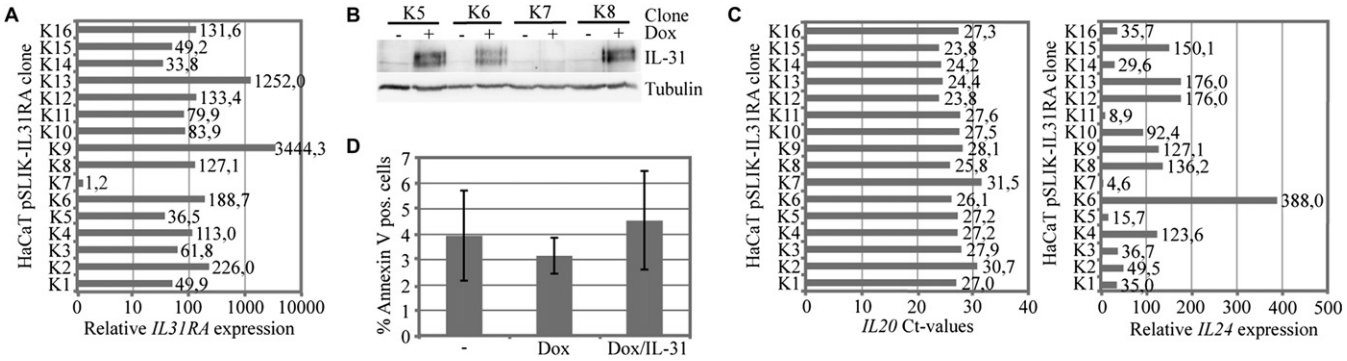

FIG E4. Characterization of HaCaT cell clones carrying an inducible *IL31RA* transgene. **A,** HaCaT cells were infected with pSLIK-IL31RA and individual clones selected with G418. The clones were stimulated with or without doxycycline (1 μg/mL) for 16 hours and the expression of *IL31RA* mRNA was analyzed by using quantitative RT-PCR. **B,** IL-31RA protein expression was analyzed in lysates of cells treated with or without doxycycline (1 μg/mL, 16 hours) by western blotting. Tubulin served as loading control. **C,** The expression of *IL20* and *IL24* was analyzed by using quantitative RT-PCR in response to 16 hour dox/1 hour IL-31 stimulation. The *IL20* expression in individual clones is displayed as CT values because the basal expression values for *IL20* in the different clones were either not detectable or very low. The *IL24* expression is shown as relative expression compared with cells without dox and IL-31 treatment. *HPRT* expression was used for normalization. **D,** HaCaT-IL31RA cells were stimulated with or without dox (1 μg/mL) and IL-31 (100 ng/mL) as indicated. The cells were then stained for AnnexinV and analyzed by using fluorescence-activated cell sorting. Displayed are mean values with standard deviation of 3 experiments. The differences were not statistically significant. *dox*, Doxycycline.

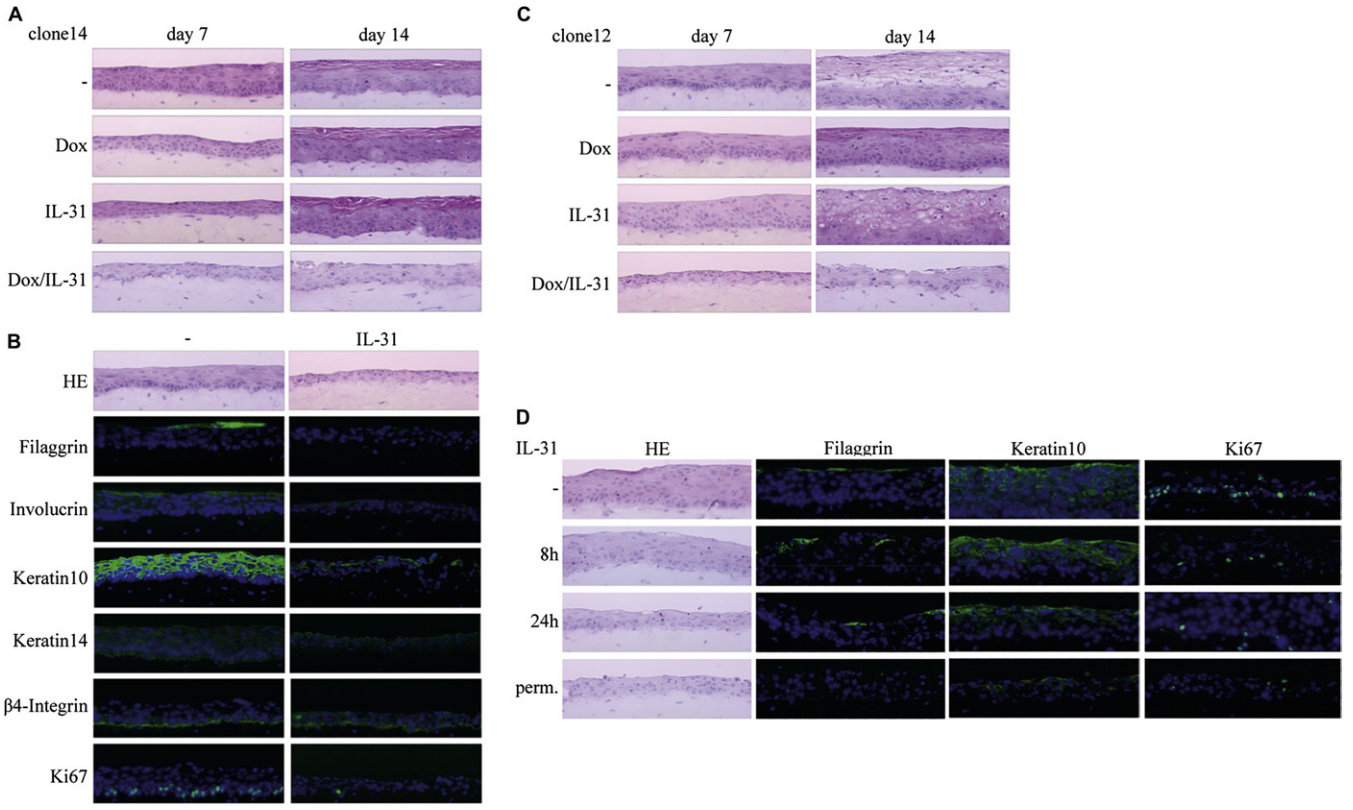

FIG E5. IL-31 interferes with HaCaT-IL31RA cell differentiation. **A** and **C**, Clone 14 (*Fig E5, A*) and clone 12 (*Fig E5, C*) cells and primary dermal fibroblasts were grown as organotypic skin equivalents in the presence or absence of dox and/or rhIL-31 (100 ng/mL). Dox and IL-31 were added every second day. The skin equivalents were harvested after 7 and 14 days and histological sections were stained with hematoxylin-eosin (HE). **B,** Clone 12 cells and primary dermal fibroblasts were grown as organotypic skin equivalents in the presence or absence of dox and rhIL-31 (100 ng/mL). Dox and IL-31 were added every second day. The skin equivalents were harvested after 7 days and analyzed histologically (HE) or stained for the indicated marker proteins. These results are from the same experiment as shown in panel C. **D,** Clone 14 cells and primary dermal fibroblasts were grown as organotypic skin equivalents in the presence or absence of dox. rhIL-31 (100 ng/mL) was added for the indicated times, and the skin equivalents were harvested after 7 days and processed as described in panels A to C. *dox*, Doxycycline.

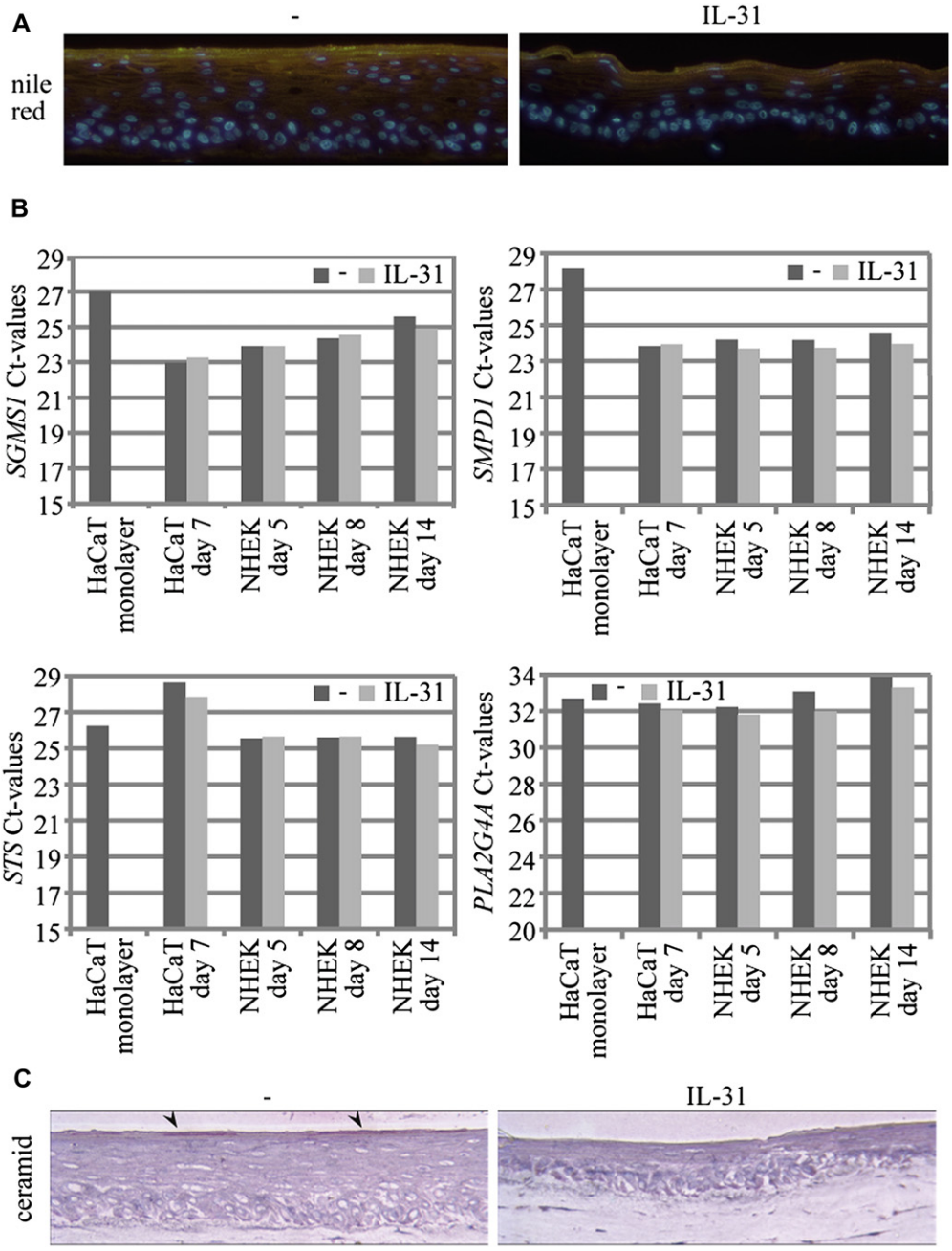

FIG E6. IL-31 reduces lipid envelope formation. **A,** 3D organotypic skin equivalents were stimulated with or without rhIL-31 (100 ng/mL; every second day) and histological sections were stained with Nile red. **B,** Organotypic skin equivalents from HaCaT-IL31RA cells and NHEKs were harvested at day 7 (HaCaT) or days 5, 8, and 14 (NHEKs) and the expression of spingomyelinase (SMPD1), sphingomyelin synthase (SGMS1), steroid sulfatase (STS), and phospholipase A2 (PLA2G4A) was measured by using quantitative RT-PCR. **C,** Sections of 3D organotypic skin equivalents (as in panel A) were stained for ceramid.