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INVESTIGATIVE REPORT

IL-31 Expression by Inflammatory Cells is Preferentially Elevated in Atopic Dermatitis

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Interleukin-31 (IL-31) is a recently discovered cytokine expressed in many human tissues, and predominantly by activated CD4+ T cells. IL-31 signals through a heterodimeric receptor consisting of IL-31 receptor alpha (IL-31RA) and oncostatin M receptor beta (OSMR). Earlier studies have shown involvement of IL-31 and its receptor components IL-31RA and OSMR in atopic dermatitis, pruritus and Th2-weighted inflammation at the mRNA level. The aim of this study was to investigate IL-31 protein expression in skin of such conditions. Immunohistochemical staining for IL-31, IL-31RA and OSMR was performed in formalin-fixed paraffin-embedded biopsy specimens. IL-31 expression was increased in the inflammatory infiltrates from skin biopsies taken from subjects with atopic dermatitis, compared with controls (p≤0.05). IL-31, IL-31RA and OSMR protein immunoreactivity was not increased in biopsies from subjects with other Th2-weighted and pruritic skin diseases. Our results confirm, at the protein level, the relationship between IL-31 expression and atopic dermatitis. Our results do not support a general relationship between expression of IL-31/IL-31RA and pruritic or Th2-mediated diseases. Key words: atopic dermatitis; IL-31; pruritus; Th2-inflammation.

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Interleukin-31 (IL-31) is a recently discovered four-helix bundle cytokine that is expressed in low abundance in many human tissues (1) and at relatively high levels by activated CD4+ T cells, in particular those cells skewed toward a Th2-phenotype. Furthermore, expression of IL-31 has been shown in human cultured mast cells in myeloproliferative disorders (2). It has been shown that staphylococcal enterotoxin B (SEB) upregulates IL-31 in peripheral blood mononuclear cells (3). Induction of IL-31 mRNA expression has been shown by stimulation of histamine receptor 4 in combination with SEB (4). IL-31 signals through a heterodimeric receptor consisting of IL-31 receptor alpha (IL-31RA) and oncostatin M receptor beta (OSMR). Through the receptor complex, IL-31 induces the activation of various signaling molecules, including Jak-STAT and PI-3 kinase signaling pathways (1, 5, 6). The receptor components IL-31RA and OSMR are co-expressed on keratinocytes and activated monocytes (1). Besides the skin, other tissues, such as testis, thymus, trachea, intestinal epithelial cells and dorsal root ganglia, show mRNA co-expression for the receptor components (1, 3, 7, 8).

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is associated with intense pruritus. The mechanisms underlying dermatitis and pruritus are complex and not fully understood (9, 10), but there is emerging evidence that IL-31 may play a role. Transgenic mice that overexpress IL-31 develop severe pruritus and skin lesions similar to AD (1). In an AD-like murine model (NC/Nga mice), high IL-31 mRNA expression is associated with scratching behavior (11), while an anti-IL-31 antibody reduces scratching behavior (12). IL-31 mRNA expression is increased in biopsies of human AD compared with normal skin (13), and correlates with IL-4 and IL-13 mRNA expression. In humans, IL-31 serum levels are higher in AD compared with non-atopic healthy controls, and correlate with disease activity (14). An IL-31 gene haplotype may be linked to genetic susceptibility to non-atopic eczema in humans (15). Taken together, these data suggest that IL-31 and its receptor components IL-31RA and OSMR are involved in AD, and may be functionally related to pruritus and Th2-weighted inflammation. The relationship between IL-31 and Th1-weighted inflammation is less clear. IL-31 mRNA expression was not increased in psoriasis (16), but was upregulated in inflammatory bowel disease mucosa (8). It was reported that the Th1 cytokines tumor necrosis factor (TNF)-α and interferon (IFN)-γ up regulate IL-31, IL-31RA and OSMR mRNA expression in human cell lines (8).

Most investigations of IL-31 and its receptor components have been done at the mRNA level. On the protein level, only IL-31RA expression has been studied (13). The objective of this study was to collect immunohistochemistry data on IL-31, IL-31RA and OSMR protein expression in different skin diseases and to explore a correlation with pruritus or with Th2-weighted inflammation.

MATERIALS AND METHODS

Samples

Following ethics committee approval, appropriate formalin-fixed paraffin-embedded tissue samples were selected from
the archive of the dermatohistopathological laboratory of the Department of Dermatology. Samples were diagnostic biopsies taken before treatment. The histology of the different skin diseases studied showed the typical histologic features overall and the typical distribution of inflammatory infiltrate for the particular disease. Only samples with typical histological findings were included in the study.

We identified at least 5 appropriate samples of each skin disease from our archives (except for pruritus sine materia \((n = 2)\); Sézary syndrome \((n = 4)\)).

We investigated samples of AD, alopecia areata, pruritus sine materia, mycosis fungoides (MF) and Sézary syndrome (SS), and classified them into groups with or without pruritus, with Th1- or Th2-weighted inflammation or without inflammation (Table I). Such a classification inherently carries problems by grouping together heterogeneous disease conditions \((17–30)\). For AD, biopsies of fresh lesions from patients with the extrinsic type of AD were chosen. Since AD is characterized by an initial phase predominated by Th2 cytokines, we classified AD as Th2-weighted disease \((17)\). Itch is commonly reported in psoriasis, while the association of itch with a particular clinical presentation of psoriasis is less well defined \((19–21)\). As samples for psoriasis, we used biopsies from non-itching chronic plaque type psoriasis \((psoriasis vulgaris)\) and itching inverse type psoriasis \((psoriasis inversa)\). Prurigo nodularis can be associated with atopy \((23)\) and can be initiated by the scratching in AD \((prurigo-type atopic dermatitis)\). For our investigations, we selected only samples of patients without history of AD. Data on the Th1- or Th2-weighted inflammation in alopecia areata vary \((24–28)\). In our study, we used samples of localized alopecia areata, which probably reflects a Th2-weighted inflammation \((25)\). MF and SS are Th2-weighted cutaneous T-cell lymphomas \((29)\). Whereas MF in the patch and plaque stage often presents without pruritus, SS presents with the hallmark of severe pruritus \((30)\). The clinical history of our patients with MF was without pruritus, the patients with SS had severe pruritus.

As a control for non-itching skin with little or no inflammation, we used perilesional skin collected from safety margin tumor resections with no detectable residual tumor. Samples were collected from patients with no history of itching and/or known inflammatory skin disease.

### Immunohistochemistry

Immunohistochemistry was performed for IL-31, IL-31RA, OSMR, CD45RO and CD68 with the following antibodies: monoclonal IgG1 mouse antibody specifically binding human IL-31 \((MAB28241\) clone 308202; R&D Systems Europe Ltd., Abingdon, UK), polyclonal rabbit antibody specifically binding human IL-31RA \((Lot \#\) E6292; ZymoGenetics Inc., Seattle, USA), polyclonal IgG rabbit antibody specifically binding human OSMR \((10982-1-AP;\) ProteinTech, Chicago, USA), monoclonal IgG2a mouse antibody specifically binding human CD45RO \((M0742,\) clone UCHL1; Dako, Glostrup, Denmark), monoclonal IgG1 mouse antibody specifically binding human CD68 \((M0814,\) clone KP1; Dako, Glostrup, Denmark). Adjacent sections of tissue 3–5 \(\mu\)m thickness were used for immunohistochemistry. For antigen retrieval, treatment conditions for deparaffinized sections were adapted for each antibody. M0742: no antigen retrieval necessary. M0814: incubation with proteinase K solution \((Dako,\) Glostrup, Denmark) 10 min at room temperature. MAB28241: heating in a pressure cooker for 15 min in 1 mM EDTA – buffer concentrate \((Biocyt GmbH,\) Luckenwalde, Germany). E6292: heating in a pressure cooker for 15 min in citrate buffer \((Target Retrieval Solution pH 9;\) Dako, Glostrup, Denmark). 10982-1-AP: heating in a pressure cooker for 15 min in citrate buffer \((Target Retrieval Solution pH 6;\) Dako, Glostrup, Denmark). The primary antibody was then incubated at room temperature \((M0742\) dilution 1:20 for 30 min, M0814 dilution 1:200 for 30 min, MAB282421 dilution 1:6 for 30 min, E6292 dilution 1:1000 for 60 min, 10982-1-AP dilution 1:50 for 60 min). Secondary staining was performed using the alkaline phosphatase anti-alkaline phosphatase method \((31)\). Slides were counterstained with hemalaun solution. Fibroblasts, as well as other cells of the subcutaneous tissue, served as internal negative controls; appropriate external positive controls were used as recommended by the manufacturer.

### RESULTS

#### IL-31 immunoreactivity by inflammatory cells predominates in atopic dermatitis

Immunoreactivity for IL-31 was present in 41 out of all 47 samples \((87\%)\) in cells of the dermal infiltrate in a predominantly cytoplasmatic pattern \((Fig. 1)\), in contrast to the epidermal cells where reliable immunoreactivity of cells was not found. Cytokine staining in general is difficult, often leading to rather high background staining. In our experiments, background staining was seen especially in stainings with the IL-31 antibody, despite optimized antigen retrieval, blocking, incubation and concentration of the antibody. The inflammatory infiltrate in samples of atopic dermatitis showed the highest number of positively stained cells

### Table I. Schematic classification of skin conditions examined according to their inflammatory profile and pruritus

<table>
<thead>
<tr>
<th>No inflammation</th>
<th>Th1-weighted inflammation</th>
<th>Th2-weighted inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pruritus</td>
<td>Perilesional skin ((n = 5))</td>
<td>Psoriasis vulgaris ((n = 5))</td>
</tr>
<tr>
<td>Pruritus</td>
<td>Pruritus sine materia ((n = 2))</td>
<td>Prurigo nodularis ((n = 5))</td>
</tr>
<tr>
<td></td>
<td>Notalgia paresthetica ((n = 5))</td>
<td>Psoriasis inversa ((n = 6))</td>
</tr>
</tbody>
</table>
for IL-31. ANOVA with post-hoc Bonferroni’s multiple comparison test showed increased expression of IL-31 in the infiltrate of atopic dermatitis compared with perilesional skin ($p<0.05$; 95% confidence interval (CI): 0.12–24.43), psoriasis ($p<0.01$; 95% CI: 2.62–23.35), alopecia areata ($p<0.05$; 95% CI: 1.18–25.48), MF ($p<0.01$; 95% CI: 3.93–28.23), prurigo nodularis ($p<0.01$; 95% CI: 4.22–28.53), notalgia paresthetica ($p<0.01$; 95% CI: 5.19–29.5) and SS ($p<0.001$; 95% CI: 6.13–31.91) (Fig. 2a). For pruritus sine materia, no differences were found compared with AD ($p>0.05$; 95% CI: –4.39–27.77) (Fig. 2a).

Comparisons with all examined itching skin diseases showed increased IL-31 immunoreactivity only for AD (AD vs. pruritus sine materia $p>0.05$; vs. psoriasis inversa $p<0.01$, 95% CI: 4.5–28.25; vs. prurigo nodularis $p<0.01$, 95% CI: 5.47–29.22; vs. Sézary syndrome $p<0.01$, 95% CI: 6.43–31.62). Similar results were found when comparing different Th2-weighted skin diseases (AD vs. alopecia areata $p<0.01$, 95% CI: 2.81–23.84; vs. MF $p<0.01$, 95% CI: 5.65–26.59; vs. SS $p<0.001$, 95% CI 7.87–30.17).

IL-31RA and oncostatin M receptor beta immunoreactivity occurs in the dermal infiltrate of all samples

Immunoreactivity stainings for IL-31RA and OSMR yielded a predominantly cytoplasmatic staining pattern. Comparisons by ANOVA showed no difference in the expression of IL-31RA ($p=0.13$) and OSMR ($p=0.72$) in the dermal infiltrate of investigated skin diseases (Figs 2 b and c).

Antigen retrieval and incubation conditions for the primary antibody (duration, temperature, concentration) were permuted. Epidermal staining for IL-31RA and OSMR yielded a moderate homogenous immunoreactivity of keratinocytes in all samples analyzed. Staining intensity, however, did not vary much between disease conditions. A grading of immunoreactivity was thus not performed (data not shown).

Similar CD68 and CD45RO immunoreactivity in the dermal infiltrate of the different skin diseases

Except for Sézary syndrome, which is characterized by a distinct increase of cells in the dermal infiltrate, statistical analyses showed no differences for the total cell number in the dermal infiltrate of different skin diseases (Figs S1a, S2a, S3a (available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1191)). Immunohistochemical stainings for CD68 and CD45RO as markers for macrophages/dendritic cells and activated T lymphocytes, respectively, showed positive results in all diseases. Statistical analyses by ANOVA showed no differences in the expression rate of these markers in different skin diseases (Figs S1b, c, S2b, c, S3b, c (available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1191)).

**DISCUSSION**

Earlier studies suggest that IL-31 and its receptor components IL-31RA and OSMR are involved in AD (1, 3, 4, 13, 14, 16), other itching dermatoses (3, 16) and Th2-weighted inflammation (1, 4, 16).

Our investigations showed increased immunoreactivity of IL-31 in infiltrating cells in AD compared with other skin diseases. This supports earlier findings based on mRNA expression and supports a postulated role of IL-31 in AD (3, 13, 16). In the epidermis, moderate homogenous immunoreactivity could be detected in all samples for receptor components IL-31RA and OSMR. This emphasizes a constitutive expression of the receptor components in keratinocytes, as has been shown in earlier investigations (1, 13). In infiltrating cells in the dermis, we found a mean of $<5\%$ immunoreactivity for IL-31RA and OSMR in all skin diseases. The immunoreactivity in the dermal infiltrate mainly reflects the
presence of macrophages, which have been shown to express the receptor components (1, 13). For IL-31RA, four splice variants have been described (1). With the antibody used, splice variants cannot be distinguished. Even though we found only low numbers of cells with positive immunoreactivity for the receptor components in the dermal infiltrate, it is important to mention that different receptor splice variants might be associated with differential function in different skin diseases.

Samples from subjects with AD were distinguished by increased IL-31 immunoreactivity compared with samples from other Th2-weighted skin diseases. Acute AD shows an increased number of Th2 cytokines compared with uninvolved or normal skin, while in a later and more chronic stage of AD, Th1-weighted inflammation dominates (17). Dillon et al. (1) reported RNA expression of IL-31 in both Th2 and Th1 T cells, though higher levels of IL-31 mRNA were observed in Th2 cells. Our results suggest that IL-31 is not so much involved in Th2-weighted-inflammation in general, but may rather play a role unique to AD. Investigations in mice, using an anti-IL-31-antibody, showed an initial reduction of scratching behavior waning over time (12). The authors explained their finding as possibly related to a transition from an acute Th2 dominant phase to a chronic state where Th1 inflammation plays a more dominant role, and suggested that IL-31 may be more important during the Th2-weighted stage of AD. Serial investigations on the same patient at different stages of disease may help to further characterize the impact of IL-31 in AD evolution.

There are many open questions in the pathogenesis of pruritus. Pruritus-modulators such as nerve growth factor and semaphorin, receptors such as µ opioid or Mrgpr receptors and pathways vary among pruritic diseases and are targets of current investigations (10, 32, 33). Pruritus-modulators activate unmyelinated afferent neurons that originate as free nerve endings in the epidermis (34). IL-31 has been suggested to be a key player in the development of pruritus (1, 2, 13). It is unknown whether IL-31 acts directly via its receptor on sensory nerve endings or indirectly, for example via keratinocytes. IL-31RA and OSMR were found in afferent cutaneous nerve fibers/dorsal root ganglia (3, 7, 35); thus, IL-31 may be a link between the immune and the neural system.

Our investigations for expression of neurons in the epidermis did not show differences in itchy dermatoses (immunohistochemical stainings for PGP9.5, data not shown). Increased peripheral sensory neurons have been reported in AD and prurigo nodularis. We believe that our biopsies were taken from earlier and subtle lesions, which, at this point in time, already display changes for IL31 expression, while not yet having had time to induce nerve sprouting visibly. Unlike our findings, Sonkoly et al. (3) have reported increased IL-31 mRNA expression in prurigo nodularis as well in patients with a history of atopy. The difference may be explained by technique, the small number of individuals studied, the stage of disease, or disease heterogeneity. For this study, we chose samples with typical clinical and histological findings while excluding an additional history of AD (prurigo type AD). Furthermore, we analyzed protein rather than mRNA expression.

Our findings that samples from subjects with psoriasis showed no increased IL-31 immunoreactivity correspond with earlier findings based on mRNA expression (3, 16). In the group of psoriasis samples, we separately analyzed samples of psoriasis inversa, a variant of psoriasis that is often accompanied by itching. This subgroup did not show increased IL-31 immunoreactivity, supporting our other findings that increased IL-31 expression is not a general feature of itching skin disease.

We found no differences in the expression rate of CD68 and CD45RO in different skin diseases and samples from perilesional skin. We interpret these findings, first of all, as a result of having examined samples of very early inflammatory cell infiltrates, because these clinically earlier lesions are probably the ones that will be biopsied most for diagnostic purposes. Skin samples from perilesional skin are not the same as normal skin, and inflammatory cells can be found, first of all because of ongoing wound repair resulting from the primary tumor resection.

Our study is limited by its retrospective nature and a limited number of samples for each skin condition. However, the scope of diagnoses covered allows a broader overview of IL-31 involvement in itching and Th2-weighted diseases than has been reported previously. To conclude, our data point to a role of IL-31 preferential in AD distinct from other Th2-weighted or other itching skin diseases. Follow-up studies will be based on fresh tissue in order to allow a wider range of antigens to be detected and to isolate cells for flow cytometry analysis.

Fig. 2. Percentage of dermal cells immunoreactive for (a) interleukin (IL)-31, (b) IL-31RA and (c) oncostatin M receptor beta (OSMR) in different skin diseases overall. Asterisks indicate differences between atopic dermatitis and the other skin diseases (*p<0.05,**p<0.01, ***p<0.001). Error bars indicate SEM.
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IN THIS ISSUE…

IL-31 Expression by Inflammatory Cells is Preferentially Elevated in Atopic Dermatitis

In 2004, interleukin-31 (IL-31) was described as a short-chain 4-helix bundle cytokine that is expressed by activated CD4^+ T cells, preferentially by T cells skewed toward a T_{H2} phenotype (1). Recently it has been demonstrated that human mast cells (MC) are also a source of IL-31 (2). Moreover, monocytes, macrophages, immature and especially mature monocyte-derived dendritic cells produce IL-31 in response to ultraviolet (UV) irradiation and hydrogen peroxide (H_2O_2) treatment (3). Low IL-31 mRNA expression levels have been detected in tissues from testis, bone marrow, skeletal muscle, kidney, colon, thymus, small intestine and trachea (1). In normal human epidermal keratinocytes and dermal fibroblasts-enhanced IL31 mRNA expression is measured upon H_2O_2 stimulation (3). In the skin of NC/Nga mice with scratching behaviour, an animal model of atopic dermatitis (AD), expression of IL-31 mRNA was significantly higher than that in NC/Nga mice without scratching behaviour. Together, these findings suggest that IL-31 is associated with T_{H2}-driven pruritic skin diseases, and that IL-31 may participate in the cause for itch sensation (4, 5).

IL-31 signals through a heteromeric receptor complex composed of the IL-31 receptor alpha (IL-31RA) and the oncostatin M receptor beta (OSMR) subunits. Engagement of the receptor complex results in activation of Janus kinase (JAK) tyrosine kinases and subsequently of different signalling molecules, including different signal transducers and activators of transcription (STAT) factors, as well as the MAPK and PI3K signalling pathways (Fig. 1). These pathways are activated in various cell types, including lung epithelial or malignant melanoma cells (6–8).

Expression of IL-31Rα and OSMRβ mRNA can be induced in activated monocytes, while their expression is constitutive in skin, testis and thymus, arguing that these organs are probably responsive to IL-31 (1, 9, 10). IL-31RA and OSMRβ are also found co-expressed in a subset of neurons of murine and human dorsal root ganglia (9, 11), which represents the site where the soma of cutaneous sensory neurons are located, while their sensory fibres protrude directly into the skin. These sensory neurons might be implicated in the sensing...
of itch and are possibly stimulated by IL-31. Recent studies demonstrated variations of IL-31RA expression in normal human epidermal keratinocytes that are dependent on the status of cellular differentiation and influenced by pro-inflammatory cytokines such as interferon-gamma (8).

While enhanced IL-31 mRNA expression has previously been detected in skin samples of inflammatory skin diseases such as AD, allergic contact dermatitis or prurigo nodularis (9, 12, 13), hardly anything is known about the protein expression of this cytokine in lesional skin from patients with inflammatory skin diseases. This question has now been addressed in an article in this issue of *Acta Dermato-Venereologica* by Nobbe and colleagues (p. 24–28) (14).

In their study they performed immunohistochemical staining for IL-31, IL-31RA and OSMR in formalin-fixed paraffin-embedded biopsy specimens of T1/2- and T12-weighted, pruritic and non-pruritic skin diseases. The authors demonstrate an enhanced IL-31 expression in biopsies of fresh lesions from patients with the extrinsic type of AD compared with controls. In 87% of samples from patients with AD cytoplasmic immunoreactivity for IL-31 was present in cells infiltrating the dermis, in contrast to epidermal cells, in which no significant staining could be detected (14). Comparisons of immunoreactivity in the dermal infiltrate of the examined itching skin diseases (AD, pruritus sine materia, psoriasis inverse, prurigo nodularis, Sézary syndrome, notalgia paraesthetica) revealed increased IL-31 immunoreactivity only in AD. Similar results were obtained by comparing different T12-weighted diseases (AD, localized alopecia areata, mycosis fungoides, Sézary syndrome, natalgia paraesthetica) showing increased IL-31 immunoreactivity only in samples from subjects with AD.

These studies confirm, at the protein level, the relationship between IL-31 expression and AD that was previously identified at the mRNA level. However, the presented data do not support a general relationship between IL-31 protein expression and pruritic or T12-mediated diseases (14). Therefore IL-31 might play a role in the pathogenesis of AD and presents a putative therapeutic target, especially in the acute phase of this disease.

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*Acta Derm Venereol* 92

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