

Original article

Interleukin (IL)-31 induces pro-inflammatory cytokines in human monocytes and macrophages following stimulation with staphylococcal exotoxins

Background: IL-31 is a cytokine expressed by T cells following activation with cytokines or staphylococcal exotoxins. A major function of IL-31 in atopic dermatitis (AD) is the induction of pruritus in the skin via the IL-31 receptor on sensory nerve cells. However, the regulation of the IL-31 receptor and pro-inflammatory functions of IL-31 in human monocytes and monocyte-derived cells are yet to be studied in detail.

Objective: To investigate the regulation and function of IL-31 receptors in resting and activated human monocytes, macrophages and dendritic cells.

Methods: Human monocytes, macrophages and dendritic cells were stimulated with staphylococcal exotoxins (SEB, α -toxin) or cytokines (IFN- γ , IL-13). IL-31RA expression and regulation were then investigated at both the mRNA and the protein level. Subsequently, functional effects of IL-31 stimulation on cytokine secretion were measured at the protein level.

Results: Staphylococcal exotoxins significantly up-regulated IL-31RA expression on monocytes and macrophages but not on dendritic cells at both the mRNA and the protein level. IL-31 enhanced the secretion of IL-1 β , IL-6 and IL-18 and up-regulated CD86 expression. In patients with AD, functional IL-31RA was also detected following stimulation of PBMC with IFN- γ . However, this was not observed in healthy individuals.

Conclusion: IL-31 induces pro-inflammatory effects in activated human monocytes and macrophages. This may have implications for cutaneous inflammation in eczema where an over-expression of IL-31 has been described previously. Moreover, our findings provide a new link between staphylococcal colonization and the worsening of inflammation via IL-31. Further therapeutic considerations may include IL-31 as a target in AD.

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Key words: atopic dermatitis; IL-31–IL-31R; macrophages; PBMC; human; SEB; *Staphylococcus enterotoxin B*; α -toxin.

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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). Skin-infiltrating T cells have been suggested to play a major role in chronic skin inflammation by the production and release of Th2 cytokines in the acute and Th1 cytokines in the chronic phase (3, 4). Although significant insight has been

obtained into the immunopathogenesis of AD, the molecular basis of pruritus and of the pathogenesis of flare-ups remain elusive (5). Pruritus is one hallmark of AD which has a negative impact on the quality of life (3). IL-31 has been shown to induce skin inflammation, pruritus and severe dermatitis in over-expressing transgenic mice (1). Further studies have demonstrated that IL-31 is associated with atopic dermatitis-induced skin inflammation and pruritus in humans (5, 6). Schulz et al. (7) found that a mutation in the IL-31 gene is associated with intrinsic AD.

The expression of IL-31RA and OSMR on human epidermal keratinocytes and the number of IL-31-expressing, skin-infiltrating Th2 and CD45RO⁺ (memory) cutaneous lymphocyte antigen (CLA)⁺ T cells is higher in atopic dermatitis patients than in healthy controls (6). Furthermore, IL-31–IL-31R interactions

Abbreviations: AD, atopic dermatitis; APC, Antigen-presenting cells; FACS, Fluorescence-activated cell sorting; HLA-DR, Human Leucocyte Antigen DR; IFN- γ , Interferon γ ; IL, interleukin; IL-31R, interleukin 31 Receptor; IL-31RA, interleukin receptor alpha; OSMR, oncostatin M receptor beta; PBMC, Peripheral blood mononuclear cells; *S. aureus*, *Staphylococcus aureus*; SEB, Staphylococcal enterotoxin B; Th2, T helper 2 cells.

negatively regulate type 2 inflammation in the lung (8). It has also been shown that measurement of IL-31 in serum or at the mRNA level in PBMCs may be a valuable parameter for the diagnosis of allergic asthma (9). Moreover, we recently provided evidence that serum IL-31 levels are significantly higher in patients with AD and correlated with disease activity in AD (10).

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), which has recently been demonstrated to activate the signal transducer and activator of transcription factor 3 (STAT-3), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and Akt signalling pathways in human alveolar epithelial cells transfected with IL-31RA (11, 12).

IL-31RA has homology to gp130-like type 1 cytokine receptors and is specific for IL-31 receptor signalling pathways (1).

There are putative target cells in the skin which are implicated in cutaneous inflammation (1, 5, 6, 13, 14). In this study, we investigated monocytes and macrophages that are known to accumulate in acutely and chronically inflamed skin in AD. Macrophages differentiate from blood-derived monocytes *in situ* (15). In addition to phagocytosis and secretion of lytic enzymes, macrophages are known to be an important source of pro-inflammatory cytokines such as IL-1 β , IL-6 and IL-18.

A recent study showed that human IL-31 is significantly up-regulated in pruritic (AD) but not in nonpruritic (psoriasis) forms of chronic skin inflammation. Furthermore, exposure to staphylococcal superantigen SEB rapidly induced IL-31 mRNA expression in AD both *in vivo* and *in vitro* (5). Here, we stimulated our cells with SEB or sublytic doses of a staphylococcal haemolysin (α -toxin). It has been shown previously that *S. aureus*-derived superantigens play an important role in the regulation of IL-31 production (5). α -Toxin is another exotoxin produced by *S. aureus* which may have an influence on eczema (16, 17). Isolation of α -toxin producing *S. aureus* from the skin of highly sensitized adult patients with severe atopic dermatitis was approximately 65 % after antimicrobial treatment (17).

Breuer et al. (16) showed that low α -toxin concentrations activate T lymphocytes and induce IFN- γ . Sublytic concentrations of α -toxin can induce a TH1 response and lead to chronification of eczema. Although the intracellular signalling pathways of IL-31 have recently been characterized (11), the function of IL-31 in human monocytes and monocyte-derived cells still remains unclear. In an attempt to further understand the role of IL-31 in pruritic skin inflammation, particularly within AD, we investigated the expression, regulation and function (i.e. regulation of cytokine secretion and membrane molecule expression) of the IL-31R in human PBMCs and macrophages following stimulation with staphylococcal components and cytokines.

Materials and methods

Preparation of cells

Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) density-gradient centrifugation from heparinized leucocyte-enriched buffy coats, which were obtained from the local blood bank from anonymous healthy donors. Total IgE levels as well as specific IgE against the common aeroallergens (sx-1: birch, elder, hazel, grass, rye, cat and dog epithelia, *Cladosporium* sp. and *Derma-tophaoides* sp.) were measured (CAP system; Pharmacia, Freiburg, Germany) and were below the normal limits of 100 and 0.35 kU/l, respectively. Moreover, PBMCs were isolated from informed consenting healthy donors and patients with AD as previously described. AD was determined by the diagnostic criteria of Hanifin & Rajka (18).

Approximately 1×10^8 PBMCs were plated in 80-cm² culture flasks (NuclonTM Δ ; Nunc GmbH & Co. AG, Wiesbaden, Germany) in Iscove medium supplemented with 5% v/v AB (serum from blood group AB), 1% w/v nonessential salts, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.5% w/v gentamycin (all from Biochrom AG, Berlin, Germany) for 1 h (37°C, 5% CO₂, humidified atmosphere). After 1 h of plastic adherence, nonadherent cells were removed by five vigorous washes with phosphate-buffered saline (PBS) and the remaining adherent cell fraction was cultured in RPMI 1640 medium supplemented with 5% v/v foetal calf serum, 12 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin ('macrophage medium') and granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.2 μ g/ml; Berlex Pharmaceutical Company, Montville, NJ, USA). In some indicated experiments, macrophages were differentiated by macrophage colony-stimulating factor (M-CSF) (ImmunoTools, Friesoythe, Germany) or by a long-term culture of monocytes. Adherent cells, thereafter called macrophages, were harvested on day 5. Before initiation of further experiments, cells were analysed by two-colour flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg, Germany) for contaminating CD3⁺ T cells, CD20⁺ B cells, CD56⁺ natural killer (NK) cells and CD16⁺ granulocytes, as described in the following paragraphs.

In order to generate monocyte-derived dendritic cells (moDC) and human inflammatory dendritic epidermal cells (IDEC), adherent cells were further cultured in 'macrophage medium' supplemented with IL-4 (10 ng/ml; R&D Systems, Minneapolis, MN, USA) and GM-CSF (10 ng/ml; Berlex Pharmaceutical Company, Montville, NJ, USA) or IL-4 (10 ng/ml; R&D Systems), GM-CSF (50 ng/ml; Berlex Pharmaceutical Company) and 50 μ M β -mercaptoethanol (Sigma-Aldrich, Munich, Germany), respectively, as described previously (19). T cells were generated from the peripheral blood. CD4⁺ T cells were enriched to >95% purity by negative selection with the MACS CD4⁺ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Stimulation of cells

Cells were left either unstimulated or were stimulated for various periods of time with highly purified staphylococcal enterotoxin B (SEB) (10, 1 μ g/ml, 100 or 10 ng/ml; Toxin Technology, FL, USA), lyophilized staphylococcal α -toxin (α -toxin) (100 ng/ml; Sigma-Aldrich, Germany), lipopolysaccharide (LPS) (100 ng/ml; Sigma-Aldrich, Germany), recombinant human IFN γ (10 ng/ml; ImmunoTools, Friesoythe, Germany), IL-13 (50 ng/ml; Promokine, Heidelberg, Germany) or recombinant human IL-31 (100 ng/ml; Peprotech, NJ, USA).

FACS analysis

PBMCs, macrophages, macrophages co-cultured with T cells, moDC and IDEC ($1-2 \times 10^5$ per well) were washed and resuspended in phosphate-buffered saline (PBS) containing 0.2% gelatine, 20 mM sodium azide and 10 µg/ml heat-aggravated human immunoglobulin G (IgG) (Sigma, Deisenhofen, Germany) to block FcR for 15 min. Subsequently, cells were incubated with specific surface markers which were directly conjugated fluorescently labelled monoclonal antibodies, on ice for 30 min: CD4-phycoerythrin (PE), CD8-fluorescein isothiocyanate (FITC), CD14-PE, CD11b-FITC, CD16-PE, CD20-FITC, CD40-PE, CD56-FITC, CD80-FITC, CD83-FITC, CD86-PE and CD64-FITC (all from Beckman Coulter, Krefeld, Germany), CD68 (Biolegend, San Diego, USA), CD206-FITC (BD Biosciences) and human leucocyte antigen (HLA)-DR-PE (BD Biosciences). Isotype control staining was performed with unlabelled (Beckman Coulter), FITC- and phycoerythrin-labelled (Sigma-Aldrich, Munich, Germany) mouse IgG1 antibodies and with FITC- and phycoerythrin-labelled mouse IgG2a and IgG2b antibodies (Sigma-Aldrich). CD14⁺, CD64⁺ and CD68⁺ macrophages had a purity of at least 86%. The cells were also stained indirectly with polyclonal biotinylated anti-human IL-31RA antibody (R&D Systems, Minneapolis, USA) and goat IgG biotin isotype control (Abcam, Cambridge, UK) which were indirectly conjugated with Streptavidin-Allophycocyanin (APC) (BD Biosciences, Heidelberg, Germany). Stained cells were washed three times, fixed in PBS with 1% paraformaldehyde and analysed by flow cytometry (FACScalibur, Becton Dickinson, Heidelberg, Germany).

Cell viability assay

Approximately 3×10^5 PBMCs and macrophages were prestimulated with SEB or α -toxin in a dose-dependent manner. Cell viability was assessed by flow cytometry by staining with 7-amino actinomycin D (7-AAD) (BD Biosciences, Heidelberg, Germany) after 10–15 min to determine the percentage of viable cells that were treated with SEB or α -toxin in a dose-dependent manner.

mRNA isolation, reverse transcription and quantitative RT-PCR

mRNA was isolated from 1×10^5 macrophages as well as PBMCs either unstimulated or stimulated for 2, 4, 6, 8 and 24 h with SEB (10, 1 µg/ml, 100 and 10 ng/ml) or α -toxin (100 ng/ml) and 24 h with IFN γ (100 ng/ml) using the High Pure mRNA Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany). Reverse transcription was performed using the First Strand cDNA Synthesis Kit (MBI Fermentas, St Leon-Rot, Germany). Real-time quantitative PCR was performed on a LightCycler (Roche Molecular Biochemicals) using SYBR Green with Quantitect primer assays for IL-31RA (QT00010794), OSMR (QT00020132) and glyceraldehyde-3-phosphate dehydrogenase GAPDH (QT01192646), according to manufacturer's instructions (Qiagen, Hilden, Germany). The following settings were used: an initial activation step of 15 min at 95°C with ramp 20°C/s was followed by three-step cycling (45 cycles): denaturation 15 s, 94°C; annealing 20 s, 55°C; extension 20 s, 72°C (all three with ramp 2°C/s). Melting curve analysis was performed from 60–90°C with ramp 20°C/s. The amount of IL-31RA and OSMR relative to the reference GAPDH was quantified using the Relative Quantification Software (Roche Molecular Biochemicals).

Cytokine assessment

Macrophages and PBMCs were prestimulated as indicated for 24 h to facilitate the up-regulation of IL-31RA and stimulated with

IL-31 for an additional 24 h in order to investigate the role of IL-31. Supernatants from macrophages were harvested after 48 h and analysed for IL-6, IL-1 β , IL-8, IL-10, MIP-1 α , MCP-1 α , TNF- α , IL-12p70 and IL-18 (Bender MedSystems, Vienna, Austria) using a flow cytometry assay and IL-23p19/p40 (Ready-Set-go, eBioscience, San Diego, CA, USA) by ELISA. Supernatants from PBMCs were harvested after 48 h and analysed for IL-6, IL-23p19/p40 (Ready-Set-go, eBioscience, San Diego, CA, USA) and IL-1 β (Duo Set, R&D Systems, MN, USA) using a commercially available enzyme-linked immunosorbent assay (ELISA) system following the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using the student's paired *t*-test. *P*-values below 0.05 were regarded as significant. *P* < 0.05 is depicted with * and *P* < 0.005 with **. The program GRAPHPAD PRISM version 3.02 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis.

Results

SEB and α -toxin up-regulate IL-31RA expression on human monocytes and macrophages

Viability of PBMCs and macrophages in response to SEB or α -toxin stimulation were measured by 7-AAD staining. Low concentrations of α -toxin (< 10 µg/ml) and SEB in a dose-dependent manner do not induce cell death in macrophages as well as PBMCs (data not shown).

IL-31RA expression was investigated at the mRNA level as determined by quantitative RT-PCR and at the protein level by flow cytometry. Up-regulation of IL-31RA was observed in a time- and dose-dependent manner following SEB stimulation or α -toxin stimulation at the mRNA level in PBMC (Fig. 1A, B) and macrophages (Fig. 1C, D). The maximal up-regulation of IL-31RA mRNA was found after 6–8 h in PBMC and after 24 h in macrophages. IFN- γ or combination of IFN- γ with LPS, which has been shown previously to up-regulate IL-31RA mRNA in human monocytes, (1) was used as a positive control and led to similar results as α -toxin (data not shown).

The findings with exotoxins could be confirmed at the protein level: FACS data revealed an up-regulation of IL-31RA expression on monocytes which were gated from PBMCs (Fig. 2A, C), macrophages (Fig. 2B) and on macrophages co-cultured with autologous T cells (data not shown) following stimulation with SEB and α -toxin for 24 h compared to unstimulated cells. In these experiments, low concentrations of SEB (10 ng/ml) could also up-regulate IL-31RA expression (data not shown). IFN γ and IL-13 did not lead to significant up-regulation of IL-31RA under these conditions. There was no difference in up-regulation of IL-31RA following staphylococcal exotoxins in macrophages differentiated under alternative conditions (using M-CSF or long-term cultures, see methods) at protein level (data not shown).

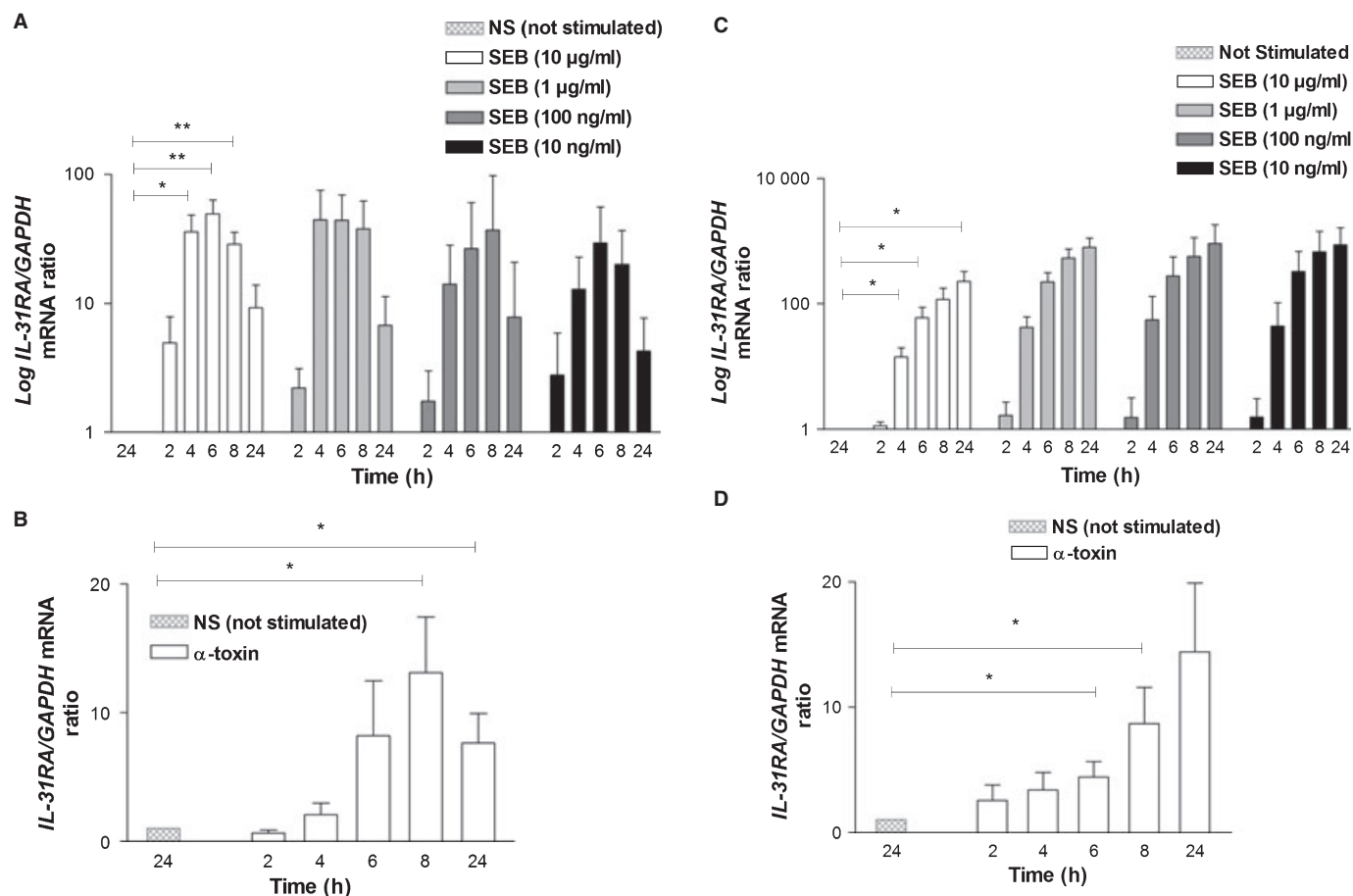


Figure 1. Expression and regulation of IL-31RA at the mRNA level. Approximately 1×10^5 PBMCs (A and B) or macrophages (C and D) were either unstimulated (medium control, NS) or stimulated for 2, 4, 6, 8 and 24 h with SEB (10, 1 µg/ml, 100 and 10 ng/ml) (A and C) or α-toxin (100 ng/ml) (B and D). qRT-PCR was employed to determine IL-31RA mRNA expression. Data are shown as mean IL-31RA/GAPDH ratio $\pm$ SEM of $n = 5-9$ (A and B) and $n = 5-11$ (C and D) independent experiments. * $P < 0.05$, ** $P < 0.005$.

IL-31RA was not detected on resting or stimulated mo-DC and IDEC by flow cytometry (data not shown). Based on our FACS results, up-regulation of IL-31RA was not related to the T cell cytokines IFN γ or IL-13 (Fig. 2A, B). Moreover, IL-31 itself did not have an effect on IL-31RA expression on monocytes or macrophages (data not shown). Surprisingly, OSMR mRNA or protein expression was not detected in monocytes or in macrophages. Because of the absence of OSMR in these cells, macrophages differentiated under alternative conditions (see methods) were studied; however, these cells were also negative for OSMR expression. Keratinocytes that were used as positive control cells express OSMR constitutively which was confirmed both on the mRNA and on the protein level in this study (data not shown).

IL-31 induces pro-inflammatory cytokines in human PBMCs and macrophages following up-regulation of IL-31RA with SEB or α-toxin. To study the functional role of IL-31 on IL-31RA-positive cells, PBMCs and macrophages were stimulated

for 24 h with IL-31. These were either unstimulated or prestimulated with staphylococcal exotoxins for 24 h to up-regulate IL-31RA expression. Subsequently, cell-culture supernatants were analysed for the secretion of pro-inflammatory cytokines by flow cytometry or ELISA.

Functional effects of IL-31 on the cytokine secretion in PBMCs. IL-31 significantly increased secretion of IL-6 from PBMCs following up-regulation of the IL-31 receptor with SEB in a dose-dependent manner or α-toxin (Fig. 3A, B). In addition, the combination of IL-31 and SEB was found to significantly up-regulate the secretion of IL-1 β in PBMCs (Fig. 3C). A similar trend for IL-1 β secretion was detected following up-regulation of the IL-31 receptor with α-toxin (Fig. 3D). IL-6 and IL-1 β secretion following IL-31 stimulation alone was below the level of detection.

Functional effects of IL-31 on cytokine secretion in macrophages. IL-31 significantly increased the secretion of IL-6 and IL-1 β from macrophages following

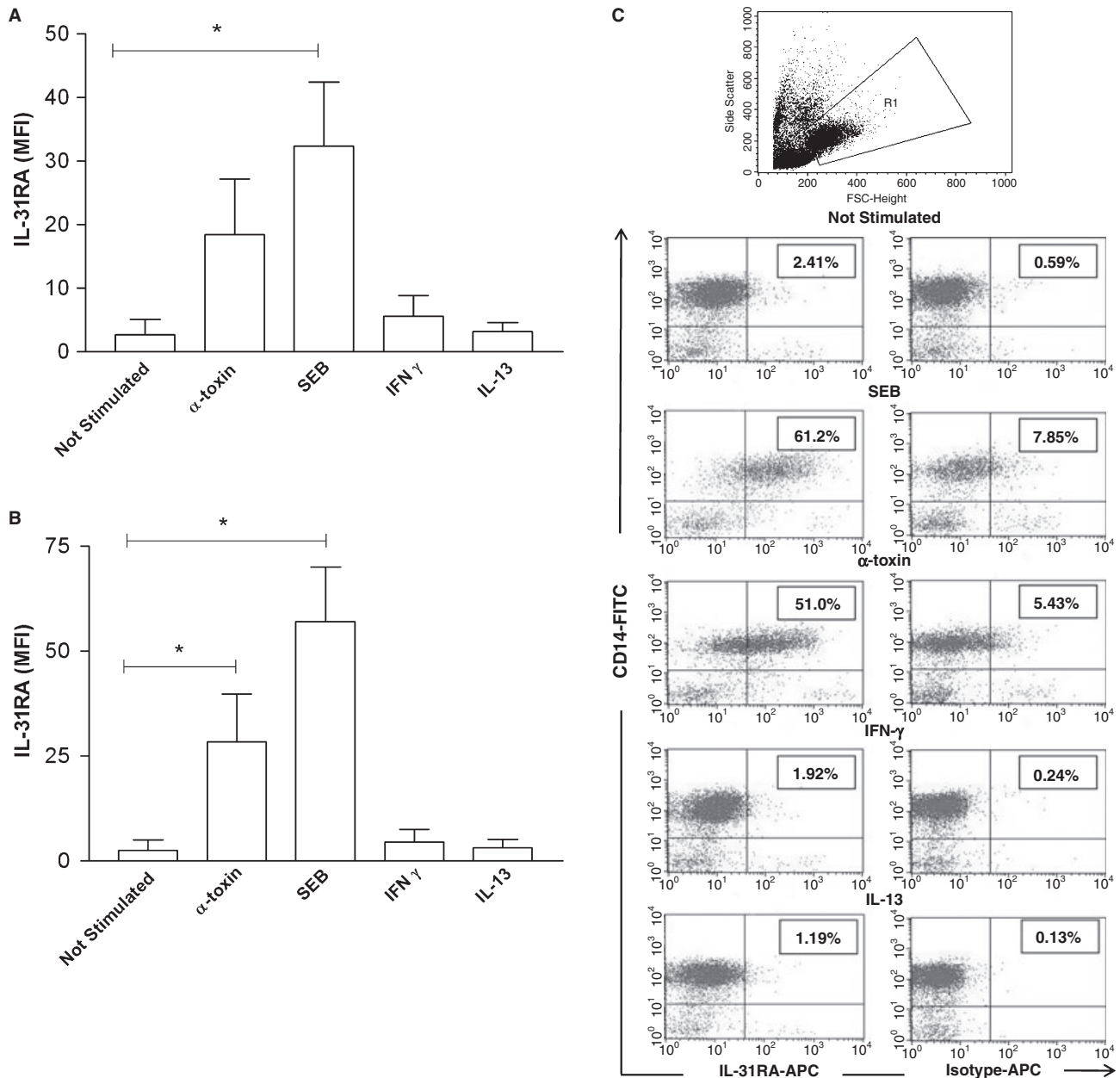


Figure 2. Expression and regulation of IL-31RA at the protein level. Regulation of IL-31RA on monocytes (A) and macrophages (B) following 24 h SEB (10 μ g/ml), α -toxin (100 ng/ml), IFN γ (10 ng/ml) or IL-13 (50 ng/ml) stimulation. Mean fluorescence intensity (MFI) of IL-31RA-positive cells $\pm$ SEM of $n = 8$ (A) and $n = 6$ (B) are shown. * $P < 0.05$. (C) Representative dot plots are shown from eight independent experiments with monocytes from different donors. Dot plots are gated physically on CD14 $^{+}$ cells. The values shown in dot plots correspond to percentage of positive IL-31RA cells.

up-regulation of the IL-31 receptor with SEB (Fig. 4A, B). Moreover, the combination of IL-31 and α -toxin significantly up-regulated the secretion of IL-6 and a similar trend was detected for IL-1 secretion (Fig. 4A, B). IL-31 also had minor but significant effects on the secretion of additional cytokines from macrophages: IL-18 was up-regulated following up-regulation of the IL-31 receptor with SEB or α -toxin (Fig. 4C) and IL-8 was down-regulated following up-regulation of the IL-31

receptor with α -toxin (Fig. 4D). We screened other cytokines and chemokines of interest (IL-10, IL-12p70, IL-23p19/p40, TNF- α , MCP1- α and MIP-1 α). These molecules were found not to be significantly regulated by IL-31 in SEB or α -toxin prestimulated cells (data not shown).

Functional effects of IL-31 on surface marker expression on macrophages. Following up-regulation of the IL-31

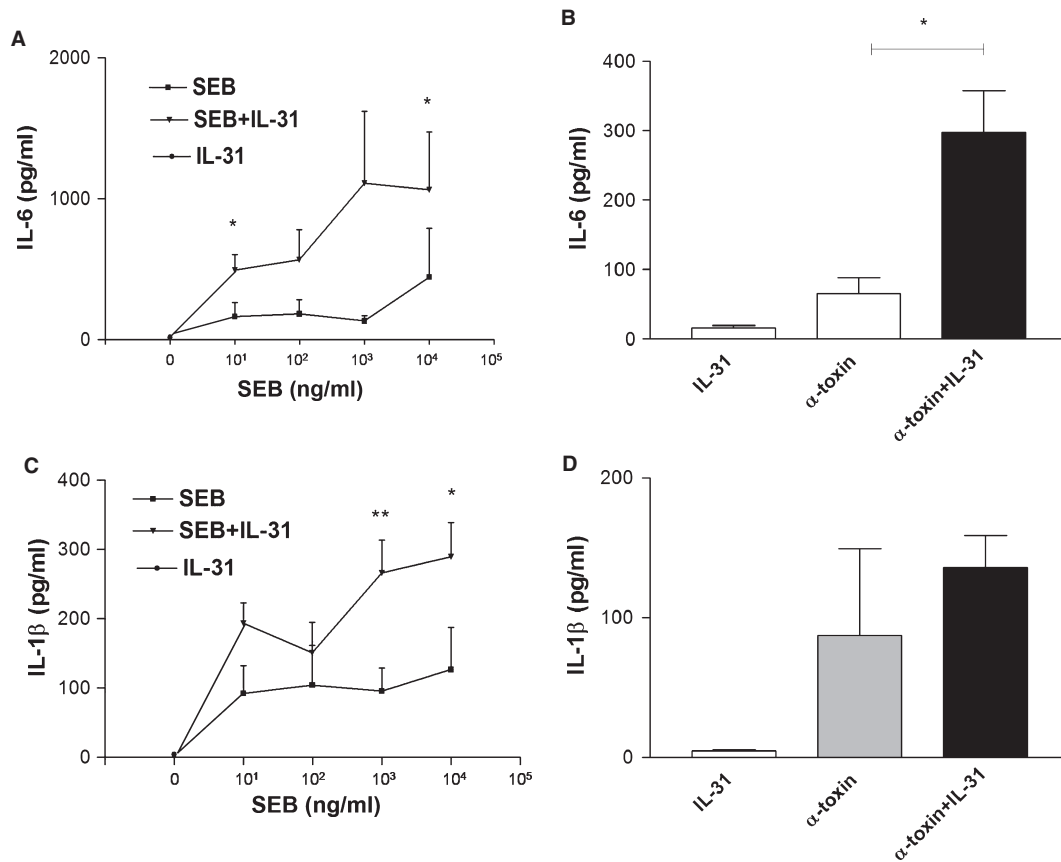


Figure 3. Functional effects of IL-31 following up-regulation of the IL-31R with SEB or α -toxin in PBMCs. Approximately 2×10^5 PBMCs were stimulated with SEB in a dose-dependent manner or α -toxin (100 ng/ml) with and without IL-31 (100 ng/ml) for 48 h. Cell-culture supernatants were measured for IL-6 secretion after up-regulation of the IL-31RA with SEB in a dose-dependent manner (A) or α -toxin (100 ng/ml) (B) as well as IL-1 β secretion following up-regulation of the IL-31RA with SEB in a dose-dependent manner (C) or α -toxin (100 ng/ml) (D), using an ELISA. The mean value $\pm$ SEM of $n = 5$ is shown. * $P < 0.05$, ** $P < 0.005$.

receptor with SEB or α -toxin on macrophages, IL-31 significantly up-regulated CD86 expression (Fig. 5) but not CD80 (data not shown). Moreover, HLA-DR measured by MFI increased significantly but only by approximately 20% following α -toxin/IL-31 stimulation (data not shown).

Comparison of IL-31RA and its function on monocytes in AD compared to healthy control individuals. Because IL-31 is of particular interest in atopic dermatitis, we compared the expression of IL-31RA and its function on monocytes in PBMC fractions from patients with AD with that of cells from healthy controls. IL-31RA expression was strongly induced on monocytes by staphylococcal exotoxins (α -toxin or SEB studied in different concentrations) in the same results between patients with AD and healthy controls (Fig. 6A). Moreover, monocytes from patients with AD pre-activated with staphylococcal exotoxins reacted to IL-31 with similar increases of cytokine production (IL-1 β , IL-6) as cells from control individuals (data not shown).

In contrast, IFN- γ led to significantly higher expression of IL-31RA on monocytes from patients with AD compared to control individuals (Fig. 6A). Furthermore, our data indicate that IL-31 induced greater levels of IL-1 β secretion in PBMCs from patients with AD compared to healthy controls following up-regulation of the IL-31RA with IFN γ (Fig. 6B).

Discussion

Up-regulation of the IL-31 receptor following stimulation with SEB and α -toxin

IL-31 is a newly characterized T cell-derived cytokine, which is involved in AD. A major function that has been attributed to IL-31 is the induction of pruritus in the skin via IL-31 receptors on sensory nerve cells (1). In humans, IL-31 has been detected in the skin mainly in acute eczema (20).

However, the regulation of the IL-31 receptor and the functions of IL-31 in human monocytes and monocyte-derived cells following stimulation with staphylococcal

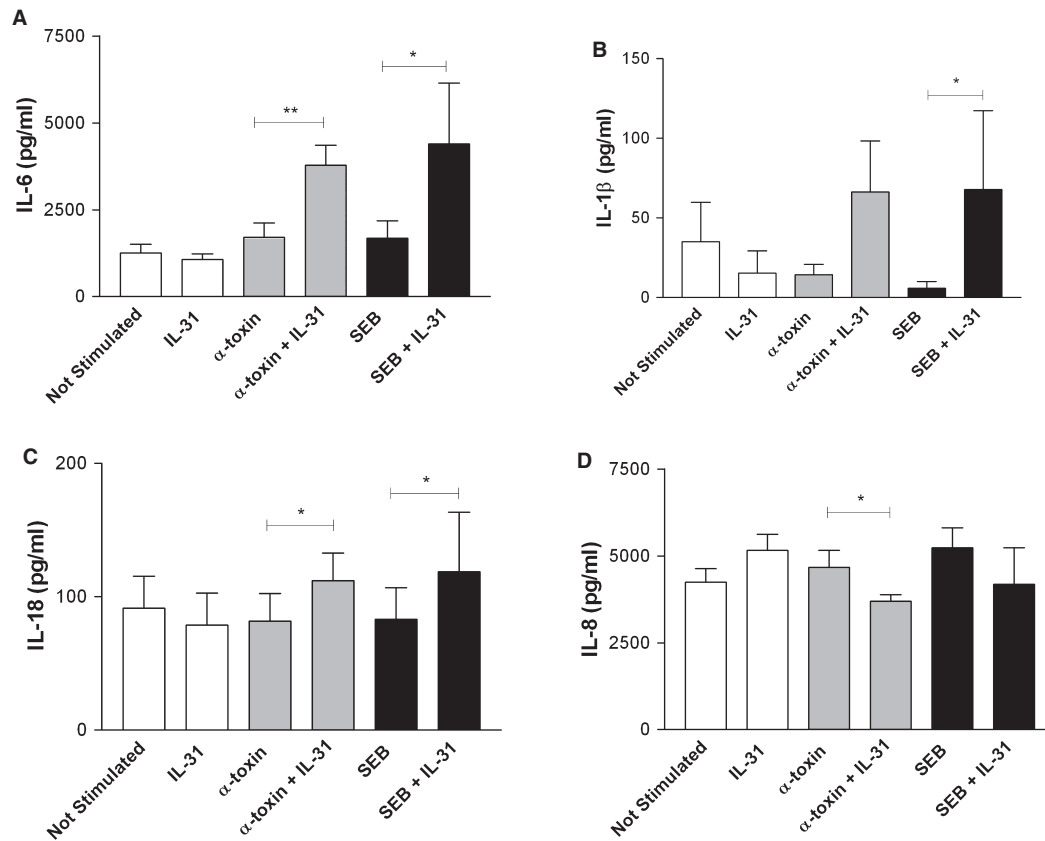


Figure 4. Functional effects of IL-31 following up-regulation of the IL-31R with SEB and α -toxin in macrophages. Approximately 2×10^5 macrophages were left either unstimulated or stimulated with SEB (10 μ g/ml) or α -toxin (100 ng/ml) with and without IL-31 (100 ng/ml) for 48 h. Cell-culture supernatants were measured for (A) IL-6, (B) IL-1 β , (C) IL-18 and (D) IL-8 secretion, using a flow cytometry assay. The mean value $\pm$ SEM of $n = 6-7$ is shown. * $P < 0.05$, ** $P < 0.005$.

components have not been studied in detail. The involvement of IL-31 in AD offers the potential of serving as a useful tool for investigating the basic immunological role of this cytokine in human macrophages and monocytes, target cells of IL-31.

We show that mRNA IL-31RA expression was not detectable in unstimulated monocytes (resp. PBMC) and macrophages while significant up-regulation of IL-31RA was observed when these cells were stimulated with the staphylococcal exotoxins SEB or α -toxin. In this context, Dillon et al. (1) demonstrated that human IL-31RA mRNA, although undetectable in fresh peripheral blood monocytes, was up-regulated substantially in monocytes cultured with IFN- γ . Jawa et al. (21) demonstrated that in pulmonary macrophages, IFN γ played the prominent role in up-regulating IL-31RA mRNA expression, whereas TGF- β was ineffective. Here, we could not confirm a significant up-regulation of IL-31RA by IFN- γ on monocytes or macrophages from healthy individuals. However, IFN γ up-regulated the receptor on cells from patients with AD.

Moreover, we show for the first time an up-regulation of IL-31RA on human macrophages and PBMCs

following SEB and α -toxin stimulation. SEB stimulates both T cells and antigen-presenting cells (APC) by binding with high affinity to the major histocompatibility complex (MHC) class II molecules on APC and subsequently interact with specific V β regions of the T-cell antigen receptors. Low α -toxin concentrations can activate cells, including mononuclear cells, by inducing ion influxes as a result of their pore forming ability (16). Both findings are important because of the enhanced susceptibility of patients with AD to infections with *S. aureus*. More than 90% of skin lesions are colonized with *S. aureus* in atopic dermatitis (2, 22, 23) and the number of bacteria on the skin positively correlates with the severity of skin lesions (24). As many as 65% of *S. aureus* strains isolated from atopic skin lesions produce exotoxins with superantigenic properties, which may penetrate the skin barrier and contribute to the exacerbation and maintenance of atopic skin inflammation (23). Furthermore, approximately 33% of *S. aureus* strains isolated from atopic skin lesions produce α -toxin (16). α -Toxin-producing *S. aureus* were found more often than expected on the skin of patients treated with antiseptic and anti-inflammatory substances (17).

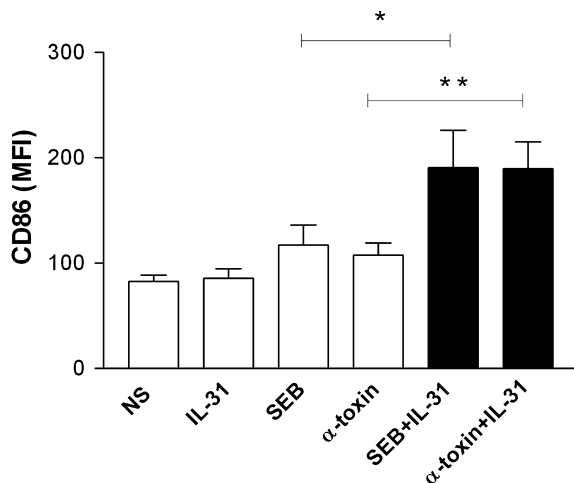


Figure 5. Functional effect of IL-31 on surface marker expression on macrophages. Regulation of CD86 is shown on macrophages. Approximately 3×10^5 macrophages were stimulated with SEB (10 μ g/ml) or α -toxin (100 ng/ml) with or without IL-31 (100 ng/ml) for 48 h. Mean fluorescence intensity (MFI) of CD86-positive cells $\pm$ SEM of $n = 5$ is shown. * $P < 0.05$, ** $P < 0.005$.

As shown previously, IL-31 transcripts were rapidly induced following topical exposure of the nonlesional skin of patients with atopic dermatitis to the staphylococcal superantigen SEB *in vivo* (5). Moreover, IL-31 was rapidly and selectively up-regulated in PBMCs treated with staphylococcal superantigens (SEB and TSST-1) *in vitro*, whereas other trigger factors of atopic dermatitis such as TH1-type or TH2-type cytokines or viruses did not induce its expression (5). Our data confirm the involvement of staphylococcal components in regulation of IL-31. However, we could not confirm previous data on inducible OSMR mRNA expression in monocytes (1).

Heise et al. (25) demonstrated that the expression of IL-31RA mRNA was greatly reduced in terminally differentiated keratinocytes while the expression of OSMR was not significantly altered during the late phase of keratinocyte differentiation. The discrepancy between IL-31RA and OSMR expression suggests an independent regulation of these two parts of the IL-31 receptor.

Based on the role of IL-31 in AD, we investigated the expression and regulation of IL-31RA in PBMCs from patients with AD compared to healthy controls. Our data suggest that SEB and α -toxin can up-regulate the IL-31RA in patients with AD as well as in normal control individuals. Interestingly, IFN γ up-regulated the receptor in patients with AD significantly higher compared to healthy controls.

There are a limited number of investigations regarding the effects of IL-31 on inflammatory mechanism. One study showed that IL-31 could significantly increase gene and protein expression of epidermal growth factor

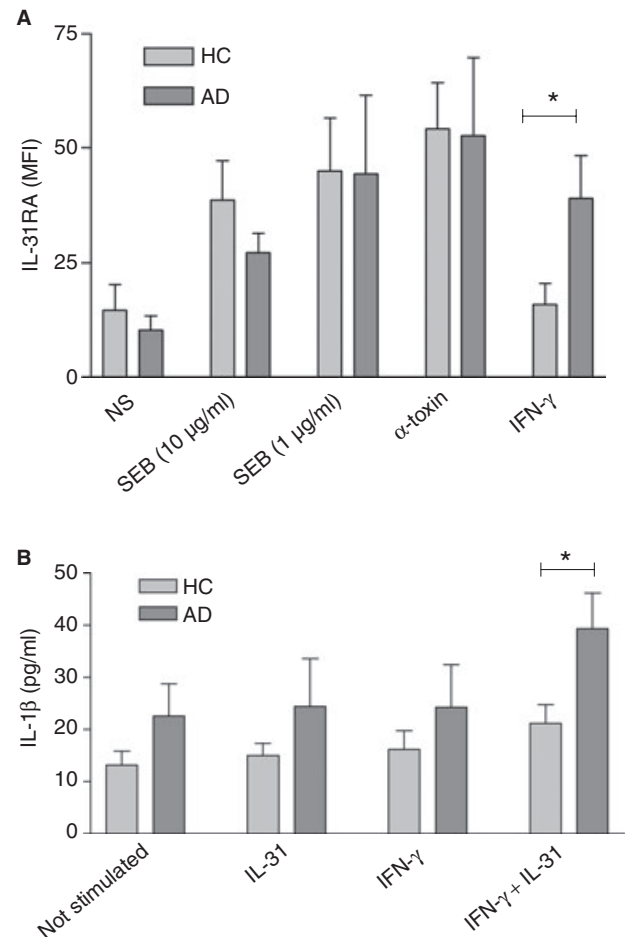


Figure 6. IL-31RA expression and regulation at the protein level on monocytes from patients with AD compared to healthy controls (HC). Approximately 3×10^5 PBMCs were either unstimulated or stimulated for 24 h with (A) SEB (10, 1 μ g/ml), α -toxin (100 ng/ml) or IFN γ (10 ng/ml), (B) Functional effect of IL-31 following up-regulation of the IL-31R with indicated stimulation in PBMCs from patients with AD compared to healthy controls. Approximately 2×10^5 PBMCs were left unstimulated or were stimulated with IFN γ (10 ng/ml) with and without IL-31 (100 ng/ml) for 48 h. Cell-culture supernatants were measured for IL-1 β secretion, using an ELISA. The mean value $\pm$ SEM of $n = 6$ –7 patients with AD and $n = 6$ healthy controls (HC) is shown. * $P < 0.05$.

(EGF), vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1/CCL2) in human bronchial epithelial cells (BEAS-2B cells) both in a time and dose-dependent manner. The combination of IL-31 with either IL-4 or IL-13 further enhanced VEGF and CCL2 production. Moreover, IL-31 augmented the release of EGF, VEGF, CCL2, IL-6 and IL-8 in co-cultures of BEAS-2B cells and eosinophils (26). Another study demonstrated that LPS and the pro-inflammatory cytokines TNF- α , IL1 β , IFN- γ and sodium butyrate stimulation increased IL-31, IL-31RA and

OSMR mRNA expression, while IL31 itself enhanced IL-8 expression in intestinal epithelial cells (IEC) (13).

Our investigations suggest new functional effects of IL-31 in human macrophages and monocytes: The combination of IL-31 with either SEB or α -toxin may significantly increase expression of IL-6. This cytokine has previously been shown to play an important role as a pro-inflammatory cytokine in AD which is up-regulated in the skin of humans and in animal models of atopic dermatitis (27). Macrophages and PBMCs treated with SEB and IL-31 secreted higher levels of IL-1 β compared to SEB stimulated cells. IL-1 β is another important pro-inflammatory cytokine which has been studied in many disease models but only in a few studies of AD thus far (28, 29). Our data also revealed that IL-31 could enhance IL-1 β production following up-regulation of IL-31RA with SEB and α -toxin in both PBMCs from patients with AD and normal controls. Interestingly, IL-31 induced more IL-1 β in PBMCs from patients with AD compared to healthy controls after up-regulation of the IL-31RA with IFN γ . This was probably related to the effect of IFN γ on IL-31RA expression in AD. Pro-inflammatory cytokines such as IL-1 and IL-6 play an important role in many physiological processes including the regulation of immune and inflammatory responses. A variety of studies have shown that these pro-inflammatory cytokines contribute to the pathophysiology of a range of diseases, when they are expressed at high concentrations (30). In concordance with this, Yagi et al. (31) showed that IL-31 stimulates the secretion of IL-6 (besides other mediators) from human colonic subepithelial myofibroblasts (SEMFs).

Furthermore, our results indicate that IL-31 moderately up-regulates the secretion of IL-18 in stimulated macrophages. IL-18 is a cytokine that plays an important role in the TH1 cell response, primarily through its ability to induce IFN- γ production, especially in combination with IL-12. The possible involvement of IL-18 in AD and bronchial asthma has previously been discussed (32, 33).

Our data also suggest the role of IL31 in modulating macrophages function by up-regulation of HLA-DR and CD86 expression following up-regulation of IL-31 receptor with SEB or α -toxin.

CD80 (B7.1) and CD86 (B7.2) play a critical role in the initiation of T-cell responses. They are expressed by antigen-presenting cells, such as dendritic cells, macrophages and B cells, but can also be up-regulated on other cell types such as endothelial cells by pro-inflammatory stimuli. The interaction of CD80 or CD86 with CD28, promotes T-cell responses by decreasing apoptosis and enhancing the expansion of antigen-specific T cells. CD80 and CD86 have also been shown to regulate Th1/Th2 differentiation and are critical for antibody production and isotype switching (34). It has been shown that CD80 promotes Th1 and CD86 Th2 responses (35, 36). Our data revealed the role of IL-31 in regulation of Th2 differentiation by up-regulation of CD86 expression on macrophages following staphylococcal exotoxin stimulation and enhancing their antigen-presenting capacity (APC) to T lymphocytes by up-regulation of HLA-DR expression following α -toxin stimulation.

Based on our findings, the functional effects of IL-31 are only observed following the up-regulation of the receptor with staphylococcal components, which is of great clinical interest because of the high levels of *S. aureus* colonization in patients with AD.

In conclusion, our findings identify new functional roles of IL-31, a cytokine that is over-expressed in eczematous skin diseases such as atopic dermatitis. This indicates that IL-31 not only plays a role in the induction of pruritus but may also enhance inflammatory reactions in the skin.

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