

Interleukin (IL)-31 activates signal transducer and activator of transcription (STAT)-1, STAT-5 and extracellular signal-regulated kinase 1/2 and down-regulates IL-12p40 production in activated human macrophages

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Keywords

atopic dermatitis; human macrophages; interleukin-12; interleukin-31; signalling pathways.

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Abstract

Background: Interleukin-31 is a cytokine expressed by activated T cells. A major function of IL-31 in atopic dermatitis (AD) is the induction of pruritus in the skin. We recently showed that IL-31 induces pro-inflammatory cytokines following staphylococcal exotoxins' stimulation in human macrophages. However, signalling pathways of IL-31 in activated human macrophages still remain unclear. The aim of the study was to investigate the signalling pathways of IL-31 receptor as well as functional effects of IL-31 in activated macrophages.

Methods: Human macrophages were prestimulated with staphylococcal exotoxins (SEB, α -toxin) to up-regulate the IL-31 receptor with and without IL-31. Phospho-signal transducer and activator of transcription (pSTAT) 1/3/5, phospho-extracellular signal-regulated kinase (ERK 1/2), β -actin as well as p21/WAF/Cip1 levels were determined by means of Western blot analysis. Interleukin-12p40, IL-12p70 and IL-23 secretions were assessed by using an enzyme-linked immunosorbent assay.

Results: Interleukin-31 strongly activated STAT-1 and 5 but not STAT-3 in human macrophages after up-regulation of IL-31 receptor with staphylococcal exotoxins. p21/WAF/Cip1 expression was induced by IL-31 in activated human macrophages. Furthermore, IL-31 down-regulated IL-12p40 secretion via ERK 1/2 phosphorylation in human macrophages following up-regulation of IL-31 receptor with staphylococcal exotoxins.

Conclusions: The T helper (Th) 2 cytokine IL-31 induces pro-inflammatory effects in activated human macrophages via STAT-1 and 5 phosphorylation. Interleukin-31-induced ERK 1/2 activation contributes to the underlying mechanism of Th1 cytokine IL-12 suppression in macrophages. This mechanism may be relevant in Th2 inflammatory responses and may help to develop therapeutic strategies in IL-31-associated diseases such as AD.

Abbreviations

AD, atopic dermatitis; APC, antigen-presenting cells; CDK, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; HLA-DR, human leukocyte antigen DR; IFN- γ , interferon gamma; IL, interleukin; IL-31RA, interleukin receptor alpha; JNK, c-Jun N-terminal kinases; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; NF κ B, nuclear factor kappa B; NK, natural killer cells; OSMR, oncostatin M receptor beta; PBMC, peripheral blood mononuclear cells; PI3K, phosphatidylinositol-3-kinases; *S. aureus*, *Staphylococcus aureus*; SEB, Staphylococcal enterotoxin B; STAT, signal transducer and activator of transcription; Th, T helper cells.

Interleukin (IL)-31 has been identified as a four-helix bundle cytokine that is produced by activated T cells, particularly by T helper 2 cells, and is involved in promoting skin disorders and regulating other allergic diseases such as asthma (1, 2).

Interleukin-31 plays an important role in atopic dermatitis (AD) and has been shown to induce skin inflammation, pruritus and severe dermatitis in IL-31-over-expressing transgenic mice (1, 2). Interleukin-31 is significantly up-regulated in pruritic (e.g., AD) but not in nonpruritic (e.g., psoriasis) forms of chronic skin inflammation (2).

Recently, we provided evidence that serum IL-31 levels were significantly higher in patients with AD and correlated with disease activity in AD (3).

Interleukin-31 is closely related to the IL-6-type cytokines oncostatin M (OSM), leukaemia inhibitory factor and cardiotrophin-1 and signals through a heterodimeric receptor complex composed of the IL-31 receptor alpha (IL-31RA) and the oncostatin M receptor (OSMR) (1, 4). Target cells for IL-31 expressing IL-31RA are activated macrophages, epithelial cells including keratinocytes as well as eosinophils in the skin (1, 5, 6).

Activation of the receptor complex resulted in recruitment of Janus kinase (JAK) 1, JAK 2, signal transducer and activator of transcription (STAT)-1, STAT-3, STAT-5 and phosphatidylinositol 3-kinase (PI3K) signalling pathways in glioblastoma and melanoma tumour cells, and lung epithelial cells (4, 5, 7). Interleukin-31 mediates extracellular signal-regulated kinase (ERK)-1/2, Akt, STAT-1 and STAT-3 activation in intestinal epithelial cells (IEC) (8). Recently, we showed that staphylococcal exotoxins significantly up-regulated IL-31RA expression in macrophages. Moreover, IL-31 induces pro-inflammatory cytokines following staphylococcal exotoxins' stimulation in human macrophages (9). However, signalling pathways of IL-31 in activated human macrophages still remain unclear.

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 *Dermatophagoides* sp.) were measured (CAP system; Pharmacia, Freiburg, Germany) and were below the normal limits of 100 and 0.35 kU/l, respectively.

Approximately 1×10^8 PBMCs were plated in 80 cm² culture flasks (Nuclon™Δ; Nunc GmbH & Co. AG, Wiesbaden, Germany) in Iscove's medium supplemented with 5% v/v AB serum (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) (10 ng/ml; Berlex Pharmaceutical Company, Montville, NJ, USA). Adherent cells, thereafter called macrophages, were harvested on day 5. Subsequently, adherent cells were washed five times with PBS to remove GM-CSF in the culture medium. Before initiation of further experiments, cells were analysed by two-colour flow cytometry (FACScalibur, Becton Dickinson, Heidelberg, Germany) for contaminating CD3⁺ T cells, CD20⁺ B cells, CD56⁺ natural killer (NK) cells and CD16⁺ granulocytes, as described below.

Stimulation of cells

Cells were left either unstimulated or stimulated with highly purified staphylococcal enterotoxin B (SEB) (10 µg/ml, 1 µg/ml, 100 ng/ml or 10 ng/ml; Toxin Technology Inc., Sarasota, FL, USA), lyophilized staphylococcal α-toxin (α-toxin) (100 ng/ml; Sigma-Aldrich, Munich, Germany) or recombinant human IL-31 (100 ng/ml; Peprotech, Rocky Hill, NJ, USA). In indicated experiments, cells were pre-incubated for 30 min with the MEK1/2-specific inhibitor U0126 (10 µM; New England Biolabs, Frankfurt, Germany), PD98059 (60 µM; Calbiochem, Schwalbach, Germany) and SB202474 (10 µM; Calbiochem) (a negative control for PD98059).

Fluorescence-activated cell sorting analysis

Macrophages ($1-2 \times 10^5$ per well) were washed and resuspended in 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 with fluorescent-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, CA, USA), CD206-FITC (BD Biosciences, Heidelberg, Germany) and human leukocyte antigen (HLA)-DR-PE (BD Biosciences). Isotype control staining was performed with unlabelled (Beckman Coulter), FITC- and PE-labelled (Sigma-Aldrich, Munich, Germany) mouse IgG1 antibodies and with FITC- and PE-labelled mouse IgG2a and IgG2b antibodies (Sigma-Aldrich). CD14⁺, CD64⁺ and CD68⁺ macrophages had a purity of at least 86%.

Cells were also stained indirectly with PE-conjugated goat polyclonal anti-human IL-31RA (10 µg/ml; R&D Systems, Minneapolis, MN, USA) and goat IgG isotype control (R&D Systems). Stained cells were washed three times, fixed in PBS with 1% paraformaldehyde and analysed by flow cytometry (FACScalibur).

Cell viability assay

Approximately 3×10^5 PBMCs and macrophages were pre-stimulated with SEB or α -toxin in a dose-dependent manner. Cell viability was assessed by flow cytometry by staining with 7-aminoactinomycin D (7-AAD) (BD Biosciences) after 10–15 min to determine the percentage of viable cells that were treated with SEB or α -toxin in a dose-dependent manner. Viability of cells with $\leq 10 \mu\text{g/ml}$ SEB stimulation or with $<1 \mu\text{g/ml}$ α -toxin stimulation was not affected in macrophages (data not shown).

mRNA isolation, reverse transcription and quantitative RT-PCR

mRNA was isolated from 1×10^5 macrophages either unstimulated or stimulated with SEB ($10 \mu\text{g/ml}$ or α -toxin (100 ng/ml) for 24 h to up-regulate IL-31 receptor with and without IL-31 (100 ng/ml) for 6, 16 and 24 h 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-12p35 (QT00000357), IL-12p40 (QT 00000364) and glyceraldehyde-3-phosphate dehydrogenase GAPDH (QT011926 46), 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 to 90°C with ramp 20°C/s . The amount of IL-12p35 and IL-12p40 relative to the reference GAPDH was quantified using the Relative Quantification Software (Roche Molecular Biochemicals).

Western blot

A total of 10^6 macrophages were prestimulated with SEB ($10 \mu\text{g/ml}$) or α -toxin (100 ng/ml) for 24 h and stimulated with or without IL-31 (100 ng/ml) for 15 or 60 min, respectively.

In indicated experiments, cells were stimulated with LPS, which was derived from *Escherichia coli* serotype 055:B5 (500 ng/ml ; Sigma-Aldrich, Deisenhofen, Germany) or IL-27 (25 ng/ml ; R&D Systems) for 60 min as a positive control. Cell extracts were subjected to Western blot analysis as described previously (10).

Cells were homogenized in M-Per Mammalian Extraction Reagent (Thermo Fisher Scientific, Rockford, IL, USA) containing protease and phosphatase inhibitor (complete mini and PhosSTOP, Roche Molecular Biochemicals) After centrifugation ($3000 g$ for 10 min at 4°C), the protein concentration in the supernatants was determined with a protein assay (Bio-Rad, Munich, Germany). A total of $250 \mu\text{g}$ protein per sample was separated on a 4–20% SDS-polyacrylamide gel

(Precise Protein Gels, Thermo Fisher Scientific) under reducing condition using SDS-PAGE running conditions (Tris-HEPES-SDS Running Buffer, Thermo Fisher Scientific). The semidry system was used to transfer proteins to a nitrocellulose membrane (30 min at 18 V and room temperature). Membranes were incubated in blocking buffer composed of Tris-buffered saline (TBS) (Bio-Rad); 0.1% Tween and 5% nonfat milk powder (2 h at room temperature; Roth GmbH, Karlsruhe, Germany). First antibody incubation was followed with TBS/Tween 0.1% containing 5% BSA (overnight at room temperature, Sigma-Aldrich). Mouse p21/WAF/Cip1 (1 : 1000), rabbit anti-phospho-STAT-1 (Tyr701) (1 : 1000), rabbit anti-phospho-STAT-3 (Tyr705) (1 : 1000), rabbit anti-phospho-STAT-5 (Tyr694) (1 : 1000), rabbit anti-phospho-ERK1/2 (p44/42) (Thr202/Tyr204) (1 : 1000), rabbit anti-phospho-p38 mitogen-activated protein kinases (MAPK) (Thr180/Tyr182) (1 : 1000), rabbit anti-phospho-SAPK/ c-Jun N-terminal kinases (JNK) (Thr183/Tyr185) (1 : 1000), rabbit anti-phospho-Akt (Th308) (1 : 1000) and rabbit anti-phospho-nuclear factor kappa B (NF κ B) p65 (Ser536) (1 : 1000) mAbs as well as rabbit anti-STAT-1 (Tyr701) (1 : 1000), rabbit anti-STAT-3 (Tyr705) (1 : 1000), rabbit anti-STAT-5 (Tyr694) (1 : 1000), rabbit anti-ERK1/2 (p44/42) (Thr202/Tyr204) (1 : 1000), rabbit anti-p38 MAPK (Thr180/Tyr182) (1 : 1000), rabbit anti-SAPK/JNK (Thr183/Tyr185) (1 : 1000), rabbit anti-Akt (Th308) (1 : 1000) and rabbit anti-NF κ B p65 (Ser536) (1 : 1000) mAbs were used to detect p21/WAF/Cip1 expression, phosphorylated and total STAT-1, STAT-3, STAT-5, ERK1/2, MAPK p38, SAPK/JNK, Akt and NF κ B, respectively. β -actin mouse mAb (1 : 1000) was used as a reference protein, followed by a horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1 : 2000). All antibodies were purchased from Cell Signaling Technology, Danvers, MA, USA. Second antibody incubation was performed in blocking buffer (see above) for 1 h at room temperature. Immunoreactivity was detected using a chemiluminescence kit according to the instructions of the manufacturer (Roche Applied Science, Mannheim, Germany).

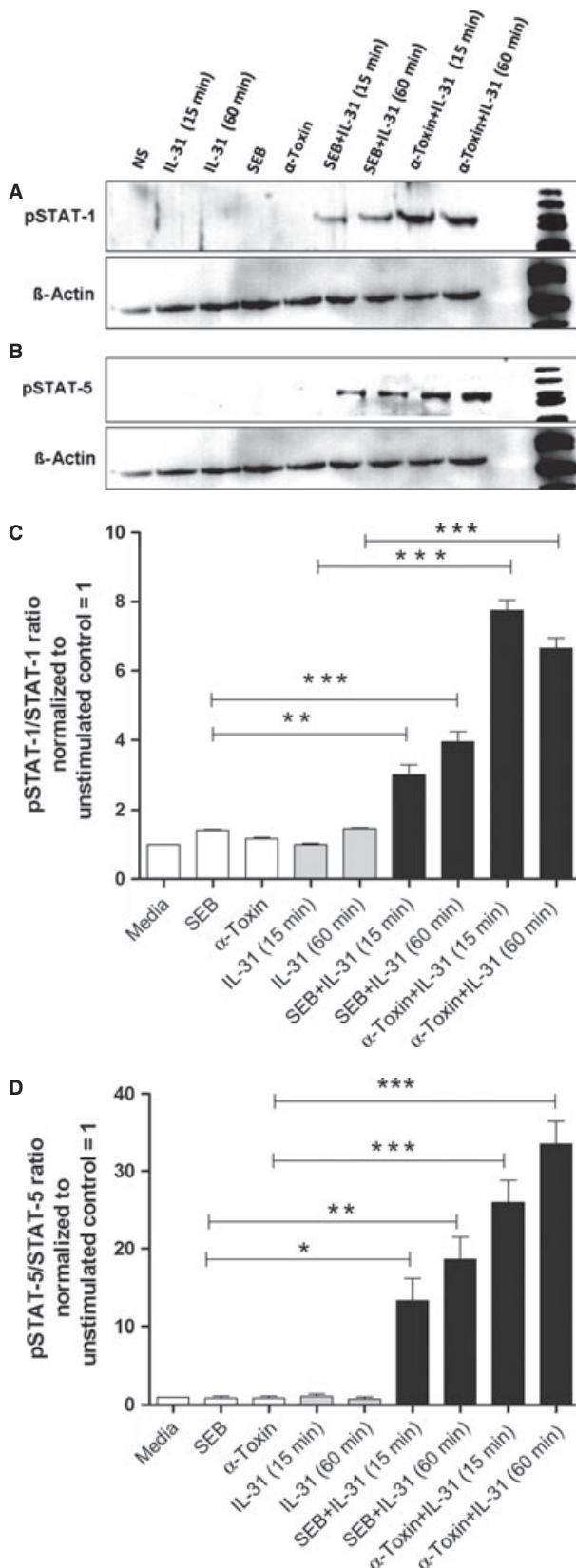
Detected bands were quantified by using ImageJ image analysis software (National Institutes of Health) and analysed after background subtraction.

Cytokine assessment by ELISA

Cell-free culture supernatants were harvested and analysed for IL-12p40, IL-12p70 (each Duo Set; R&D Systems) or IL-23p19/p40 (Ready-Setgo; eBioscience, San Diego, CA, 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 *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) and the software Sigma stat for Windows (Systat Software, San Jose, CA, USA) were used for statistical analysis.

Results

STAT signalling pathways of IL-31 in activated human macrophages

We investigated possible signalling pathways of IL-31 including STATs, MAPK family including ERK1/2, p38, JNK, PI3K-Akt as well as NFκB signalling pathways in human macrophages by means of Western blot.

To up-regulate the IL-31 receptor, cells were prestimulated with SEB or α-toxin for 24 h. As shown in Fig. 1, IL-31 activates STAT-1 (Fig. 1A) and STAT-5 (Fig. 1B) in human macrophages following up-regulation of its receptor with staphylococcal exotoxins. However, there was no difference in STAT-3 phosphorylation upon IL-31 stimulation, neither in resting nor in activated macrophages (Fig. S1A). Positive and negative controls for STAT-1, 3 and 5 were used according to manufacturer's instruction and worked as expected (data not shown).

p21/WAF/Cip1 expression is induced by IL-31 in activated human macrophages

As we could not show the involvement of STAT-3 activation by IL-31 in activated human macrophages despite its involvement in some other cells (5, 6, 8), we investigated the role of IL-31 in the induction of the cell cycle inhibitor p21, which is activated through STAT-1 and STAT-5 but not STAT-3. STAT-1 and STAT-5 have been implicated in the induction of p21 by interferon gamma (IFN-γ) and thrombopoietin, respectively (11, 12). To investigate whether STAT activation by IL-31 also leads to p21/WAF/Cip1 induction, we analysed p21/WAF/Cip1 expression by Western blot in human macrophages following up-regulation of the IL-31 receptor with staphylococcal exotoxins. Interleukin-31 induced p21/WAF/Cip1 expression in human macrophages following staphylococcal exotoxins' stimulation (Fig. 2).

Figure 1 Signal transducer and activator of transcription (STAT)-1 (A) and STAT-5 (B) activation in human macrophages following up-regulation of the receptor with staphylococcal exotoxins. Phospho-STAT (pSTAT) 1, phospho-STAT (pSTAT) 5, total STAT-1, STAT-3 and β-actin levels were determined by means of Western blot analysis 15–60 min after interleukin (IL)-31 stimulation. Approximately 10^6 macrophages were left either unstimulated or prestimulated with SEB (10 μg/ml) or α-toxin (100 ng/ml) for 24 h to up-regulate the IL-31 receptor and with and without IL-31 (100 ng/ml) for 15 or 60 min. Cellular extracts were normalized on the basis of protein determination. One representative experiment of three independent experiments from different donors is shown. The graphs represent the phosphorylated STAT-1 (C) or STAT-5 (D) to total STAT-1 or STAT-5 ratio obtained by densitometric analysis of each bands using Quantity ImageJ Software. The mean value + SD of $n = 3$ independent experiments is shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

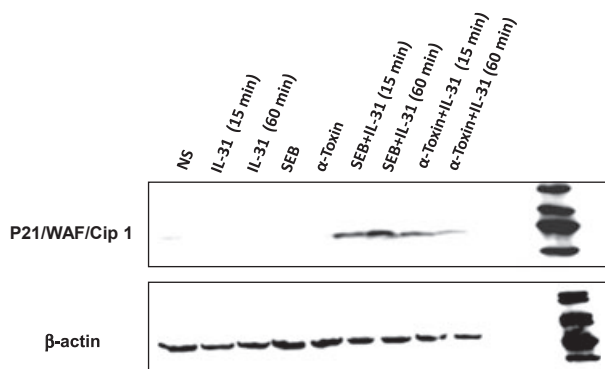


Figure 2 p21/WAF/Cip1 induction by interleukin (IL)-31 in human macrophages following up-regulation of the receptor with staphylococcal exotoxins. p21/WAF/Cip1 expression was determined by means of Western blot analysis 15–60 min after IL-31 stimulation. Approximately 10^6 macrophages were left either unstimulated or prestimulated with SEB (10 μ g/ml) or α -toxin (100 ng/ml) for 24 h to up-regulate the IL-31 receptor and with and without IL-31 (100 ng/ml) for 15 or 60 min. Cellular extracts were normalized on the basis of protein determination. One representative experiment of three independent experiments from different donors is shown.

MAPK signalling pathway of IL-31 in activated human macrophages

Previous studies showed that downstream signalling of IL-31 results in the phosphorylation of the p38 MAPK, ERK1/2 signalling pathways (5–8, 13). These findings were obtained by studying modified and/or over-expressed receptor complexes. More recently, these findings were substantiated in several reports demonstrating that IL-31 activates these pathways in different primary cells and cell lines through the endogenous receptor complex (14).

Thus, we investigated a possible MAPK signalling pathway involvement such as ERK1/2, p38 and JNK by IL-31 in activated human macrophages.

Our data indicate that IL-31 activates ERK1/2 in human macrophages following up-regulation of its receptor with staphylococcal exotoxins (Fig 3). Positive and negative controls for ERK1/2 were used according to manufacturer's instruction and worked as expected (data not shown).

However, in our experiments, IL-31 did not activate p38 as well as JNK phosphorylation in human macrophages following up-regulation of its receptor with staphylococcal exotoxins (Fig. S1 B,C).

In addition, we investigated the involvement of PI3K-Akt as well as NF κ B signalling pathways by IL-31. Our data show that IL-31 did not activate Akt as well as NF κ B phosphorylation in activated human macrophage (Fig. S1 D,E).

Involvement of the MAPK/ERK pathway in suppression of IL-12p40 by IL-31 at the mRNA level

As we have previously shown that IL-12 production is negatively regulated by CD40-activated ERK (15), we assessed

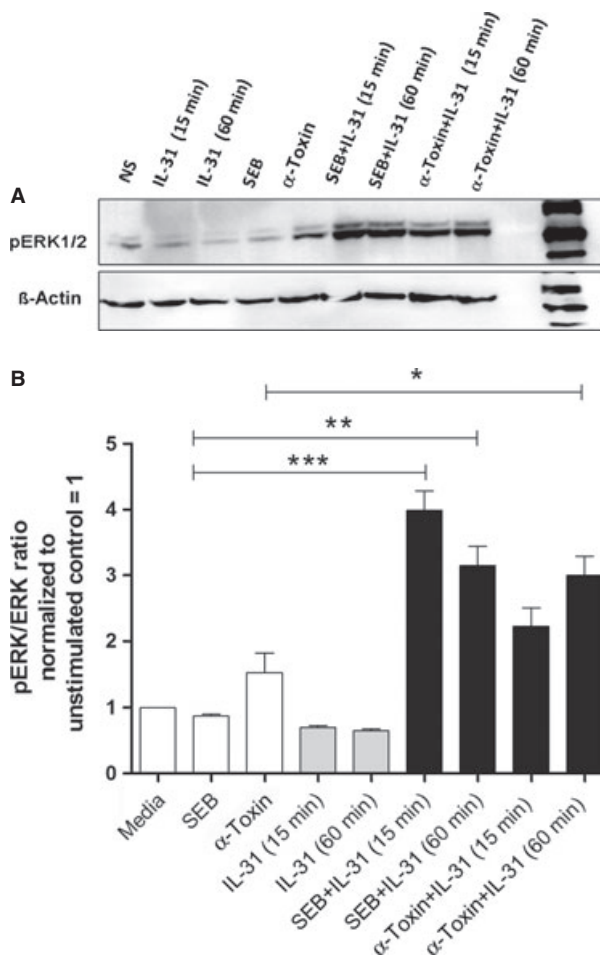


Figure 3 Extracellular signal-regulated kinase (ERK) 1/2 activation in human macrophages following up-regulation of the receptor with staphylococcal exotoxins. (A) Phospho-ERK (pERK) 1/2 and β -actin levels were determined by means of Western blot analysis 15–60 min after interleukin (IL)-31 stimulation. Approximately 10^6 macrophages were left either unstimulated or prestimulated with staphylococcal exotoxins (10 μ g/ml) or α -toxin (100 ng/ml) for 24 h to up-regulate the IL-31 receptor and with and without IL-31 (100 ng/ml) for 15 min or 60 min. Cellular extracts were normalized on the basis of protein determination. One representative experiment of 3 independent experiments from different donors is shown. (B) The graph represents the phosphorylated ERK1/2 to total ERK1/2 ratio obtained by densitometric analysis of each bands using Quantity ImageJ Software. The mean value $\pm$ SD of $n = 3$ independent experiments is shown. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

IL-12 expression to investigate the role of IL-31 in modulation of IL-12 production via the MAPK/ERK signalling pathway.

Using qRT-PCR, analysis of IL-12p35 and IL-12p40 expression was performed. Interleukin-31 suppressed IL-12p40 following up-regulation of the IL-31 receptor with staphylococcal exotoxins in human macrophages at the mRNA level (Fig. 4A,B). However, IL-12p35 was found not to be significantly regulated by IL-31 (Fig. S2A).

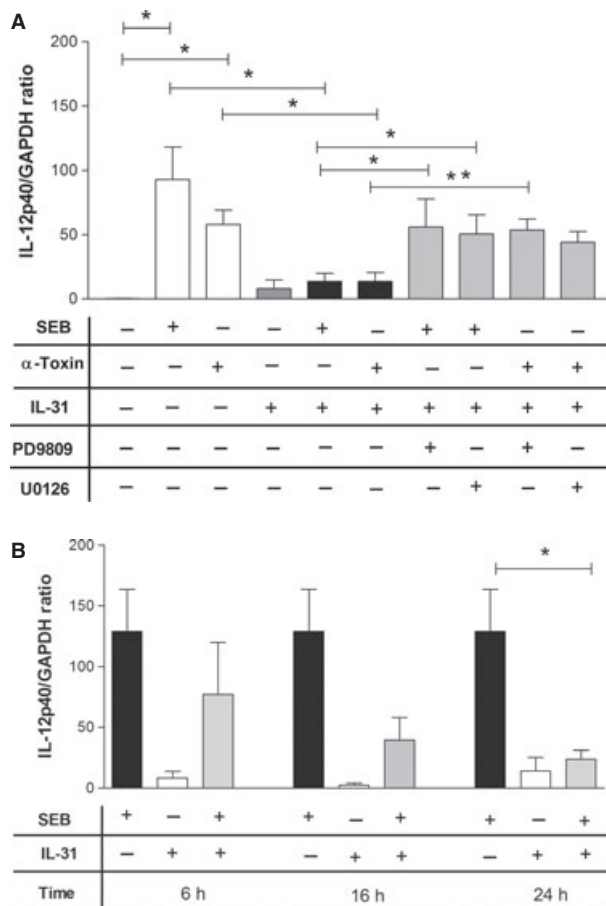


Figure 4 Involvement of the p44/42 MAPK pathway in interleukin (IL)-12p40 suppression by IL-31 following up-regulation of the IL-31R with staphylococcal exotoxins (SEB) or α -toxin in human macrophages at the mRNA level. Approximately 1×10^5 macrophages were either unstimulated (medium control, NS) or stimulated with SEB (10 μ g/ml) or α -toxin (100 ng/ml) for 24 h to up-regulate IL-31RA and subsequently with and without IL-31 (100 ng/ml) for 24 h (A) or 6, 16 and 24 h (B). In indicated experiments (A), 30 min prior to IL-31 stimulation, cells were pre-incubated with the MEK1/2-specific inhibitor U0126 (10 μ M) or PD98056 (60 μ M). qRT-PCR was employed to determine IL-12p40 mRNA expression. Data are shown as mean IL-12p40/GAPDH ratio $\pm$ SEM of $n = 6$ independent experiments. * $P < 0.05$; ** $P < 0.01$.

To analyse whether IL-31-induced down-regulation of IL-12p40 mRNA is controlled by ERK signalling, we performed experiments in the presence of specific inhibitors of the MAPK pathway. The MEK inhibitor PD98059, which primarily inhibits MEK1/2 activation by blockade of the access of activating enzymes (16), or U0126, which is able to inhibit the activated, phosphorylated form of MEK1/2 (17), can be used to inhibit MEK-dependent ERK activation. Hence, we examined the expression of IL-12p40 upon IL-31 and staphylococcal exotoxins' stimulation in the presence or absence of PD98059 or U0126, respectively. Inhibition of MEK1/2 by PD98059 or U0126 led to an increase in IL-31

suppressed IL-12p40 mRNA levels (Fig. 4A). SB202474 was used as a negative control for the MAPK inhibition studies (15) and did not show an increase in IL-12p40 expression (data not shown).

To ensure that the effect of the inhibitors is not within SEB or α -toxin pathways, IL-31RA expression following SEB or α -toxin stimulation in absence of IL-31 but presence of the inhibitors was measured by flow cytometry. Our data show that IL-31RA levels are unaffected by the inhibitors (Fig. S3).

IL-31 inhibits the induction of IL-12p40 production in activated human macrophages at the protein level

Interleukin-31 significantly down-regulated IL-12p40 secretion following up-regulation of the IL-31 receptor with staphylococcal α -toxin (Fig. 5A) or SEB in a dose-dependent manner (Fig. 5B).

Interleukin-12p40 can be secreted as IL-12p70, IL-23 (p19/p40) and IL-12p40 homodimer (18). In addition, we screened other IL-12-related subunits of interest (IL-12p70, IL-23p19/p40). These molecules were found not to be significantly regulated by IL-31 in SEB or α -toxin prestimulated cells (Fig. S2B).

Discussion

Interleukin-31 is a newly characterized T-cell-derived cytokine, which is involved in allergic diseases such as AD (1). 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 (19). 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, 2). In humans, IL-31 has been detected in the skin mainly in acute eczema (20). 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. There are a limited number of investigations regarding the effects of IL-31 on inflammatory mechanisms.

We recently showed that IL-31 induces pro-inflammatory effects in activated human monocytes and macrophages following up-regulation of the receptor with staphylococcal exotoxins (9). In this study, we demonstrate for the first time signalling pathways of IL-31 in human macrophages. Interleukin-31 induces pro-inflammatory cytokines in activated human macrophages via STAT-1 and 5 as well as ERK1/2 phosphorylation.

More recently, we demonstrated that IL-31 induces inflammatory effects (CCL2 secretion) via STAT-3 activation in human primary keratinocytes as another target cells for IL-31 (10). Human eosinophils express the functional active IL-31RA (6) and are activated by IL-31 with induction of chemotaxis, mobilization of Ca^{2+} , release of CCL 26 and reactive oxygen species via phosphorylation of STAT-3 and ERK 1/2 (unpublished data).

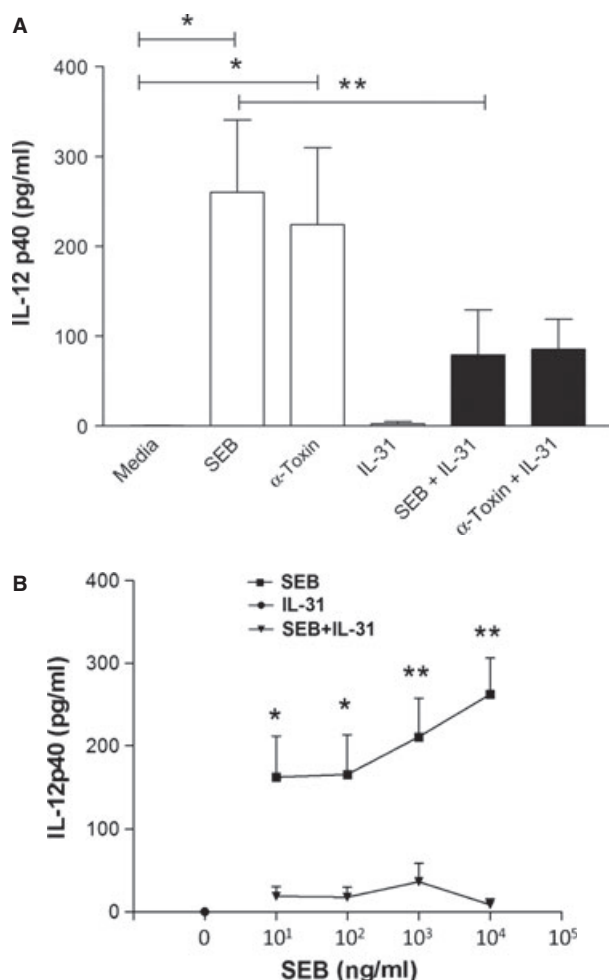


Figure 5 Suppressive effect of interleukin (IL)-31 on IL-12p40 production following up-regulation of the IL-31R with (A) α -toxin or staphylococcal exotoxins (SEB) in a dose-dependent manner (B) in human macrophages at the protein level. Approximately 2×10^5 macrophages were left either unstimulated or stimulated with α -toxin (100 ng/ml) or SEB (10, 1 μ g/ml, 100 and 10 ng/ml) for 24 h to up-regulate IL-31RA and subsequently with and without IL-31 (100 ng/ml) for 24 h. Cell-culture supernatants were measured for IL-12p40 secretion, using an ELISA. The mean value + SEM of $n = 10$ (A), $n = 5$ (B) independent experiments is shown. * $P < 0.05$.

Our data show that IL-31 actions in respect to cytokine and chemokine production as well as signalling pathways are clearly cell dependent and show a distinct pattern in IL-31R positive cells in the skin.

As STAT-1 and STAT-5 have been implicated in the induction of the cell cycle inhibitor p21/WAF/Cip1 (11, 12), we measured the expression of p21/WAF/Cip1 by IL-31 in human activated macrophages. We found that IL-31 induced p21/WAF/Cip1 expression in macrophages following the up-regulation of the IL-31 receptor with staphylococcal exotoxins.

p21/WAF/Cip1 is a cyclin-dependent kinase (CDK) inhibitor that directly inhibits the activity of cyclin E/CDK2 and

cyclin D/CDK4/6 complexes. p21/WAF/Cip1 acts as a regulator of cell cycle progression at S phase and prevents cell proliferation (11, 12).

A recent study demonstrated that IL-31 is highly effective in suppressing proliferation by altering expression of cell cycle proteins including up-regulation of p27^{Kip1} and down-regulation of cyclin B1, CDC2, CDK6, MCM4, and retinoblastoma in lung epithelial cells (5).

Dambacher et al. (8) also showed that IL-31 at high doses and low cell density significantly decreases cell proliferation in the colorectal cancer cell line HCT116. However, they indicated that the antiproliferative effect of IL-31 in HCT116 cells is lost in dense cell cultures and under certain conditions even reverses into a proliferation stimulating effect (8).

Our data provide some signalling pathways and regulation of downstream molecules by IL-31 in macrophages, which might be important in functional effects of IL-31.

This study shows that IL-31 negatively regulates the Th1 cytokine IL-12p40 via ERK phosphorylation following up-regulation of the receptor with staphylococcal exotoxins, which contribute to the exacerbation and maintenance of atopic skin inflammation.

Interleukin-12 is mainly produced by monocytes, macrophages and dendritic cells in response to bacterial products such as LPS and intracellular pathogens or upon interaction with activated T cells (21, 22). Interleukin-12p40 is the common subunit for two heterodimeric pro-inflammatory cytokines, IL-12p70 and IL-23 (21, 22). Both IL-12p70 and IL-23 activate NK cells and promote their secretion of IFN- γ . While IL-12p70 is required for Th1 cell differentiation, IL-23 is critical for the generation of Th17 cells. Interleukin-12p40 is also secreted as a homodimer, which regulates IL-12p70 and IL-23 signalling and acts as a chemoattractant for macrophages and dendritic cells (23–25).

Interleukin-12p40 production is tightly controlled by multiple negative regulatory mechanisms. Production of IL-12p40 can be inhibited by anti-inflammatory cytokines such as IL-10 and TGF- β , by PI3K activation, by the activation of mitogen-activated protein (MAP) kinase family member ERK and by increase in intracellular cyclic AMP (26–29).

Another study indicates that JNK negatively affects LPS-induced IL-12 production from human macrophages (30). There is also evidence for the negative regulatory effect of Th2 cytokines such as IL-4 and IL-13 in IL-12 production (31, 32). For example, IL-4 inhibits the LPS-induced IL-12p70 production (32). Interleukin-4 enhances the production of IL-12p70 in CD40L-stimulated dendritic cell (DC), but inhibits the production of the p40 subunit of IL-12 (32).

Human monocytes/macrophages produce high amounts of bioactive IL-12 when a priming signal (IFN- γ or GM-CSF) precedes a second signal (e.g., LPS). We and others have previously shown that pre-incubation with LPS before this stimulation procedure can efficiently and selectively suppress the production of IL-12 by human monocytes. In former study, we also showed that an almost complete suppression of IL-12 production can also be observed after pre-incubation of monocytes with co-stimulatory cell surface molecules that bind to members of the TNFR superfamily [CD40 ligand,

TNF-related activation-induced cytokine (TRANCE)] via ERK1/2 signalling pathway (8, 33).

In this study, we could also conclude that IL-31-induced ERK 1/2 activation contributes to the underlying mechanism of IL-12 suppression in macrophages. Our findings add a new regulatory mechanism for IL-12p40 by IL-31.

Furthermore, regulation of IL-12p40 but not IL-23 and IL12p70 by IL-31 might point to the distinct regulation of IL-12 subsets. The opposite roles by IL-12p70 and IL-12p40 and the different requirements for the induction of either factor in human antigen-presenting cells (APCs) have been documented (34).

For instance, Kalinski et al. (34) reported that prostaglandin E2 (PGE2), an inflammatory mediator with a previously known Th2-driving function, enhances the IL-12p40 expression in human TNF- α -stimulated immature DCs. This effect is selective and is not accompanied by the induction of IL-12p35 expression or by secretion of IL-12p70 heterodimer, which supporting the distinct and independent regulation of IL-12 subunits.

It has been shown that the expression of IL-12 appears to be regulated primarily at the level of transcription. The p40 gene is regulated at the level of transcription and is highly inducible by microbial products. Multiple regulatory elements, such as NF κ B and the F1 complex, including Ets-2, IFN-regulatory factor-1, c-Rel and Ets-related factors, are implicated in p40 regulation (30).

Interleukin-12 plays a pivotal role in the regulation of cell-mediated immunity, exerting pleiotropic effects on T and NK cells. It also induces the development of Th1 responses, leading to IFN- γ and IL-2 production and has an important role in maintaining the balance between Th1 and Th2 responses *in vivo* (35).

Our data indicate that IL-31 modulates Th1 cytokine IL-12 production via ERK1/2 phosphorylation. 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 due to the high levels of *Staphylococcus aureus* colonization in patients with AD.

Taken together, this mechanism may be relevant in Th2 inflammatory responses and further therapeutic considerations may include IL-31 as a target in IL-31-associated diseases such as AD.

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Conflict of interest

The authors state no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. STAT-3 (A), MAPK p38 (B), SAPK/JNK (C), PI3K-Akt (D) and NF κ B (E) activation in human macrophages following up-regulation of the receptor with staphylococcal exotoxins.

Figure S2. Regulation of IL-12p35 (A) and IL-23p19/p40 (B) in human macrophages following up-regulation of the receptor with staphylococcal exotoxins.

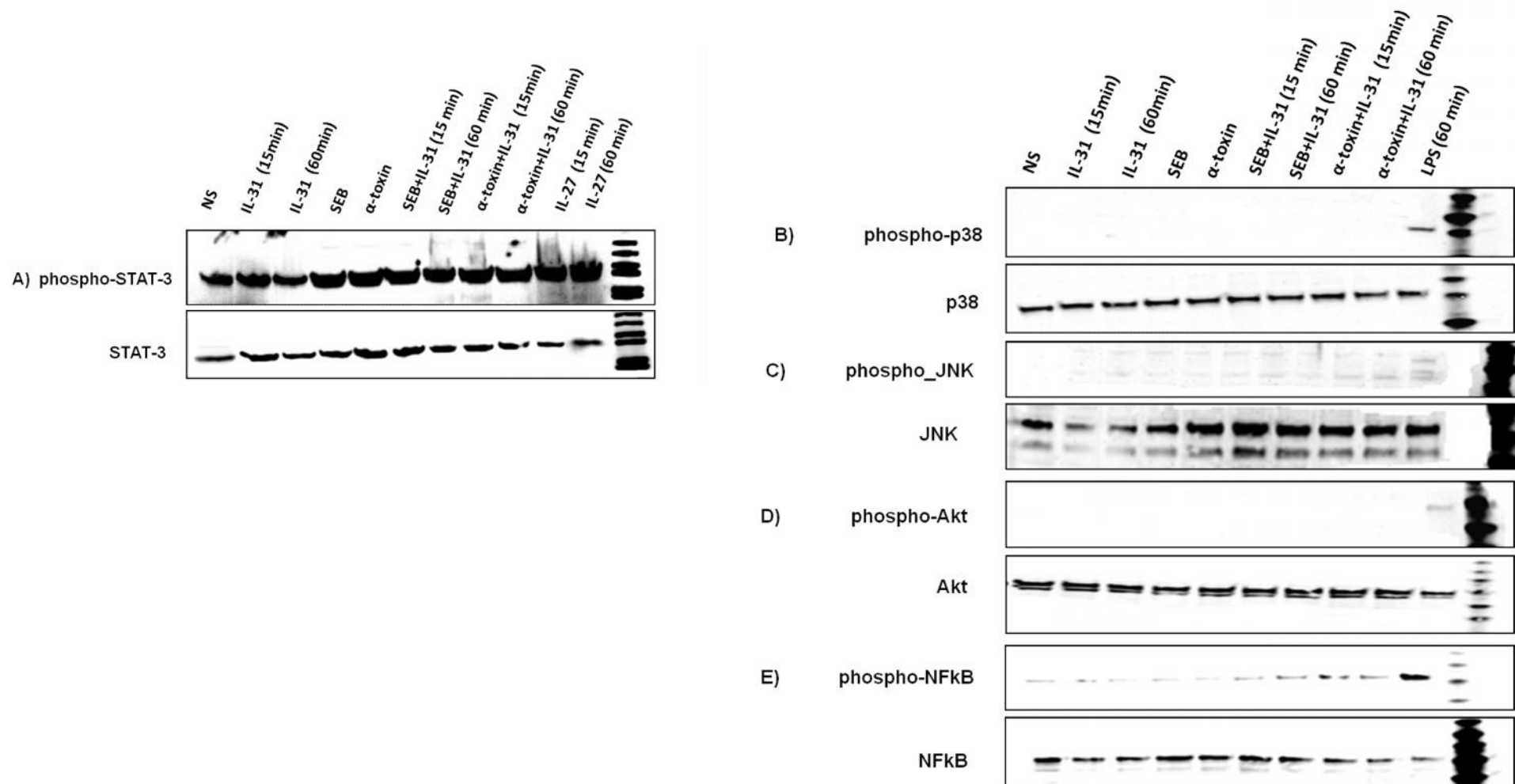
Figure S3. Effect of MEK-ERK inhibitors on IL-31RA expression in human macrophages following up-regulation of the receptor with staphylococcal exotoxins.

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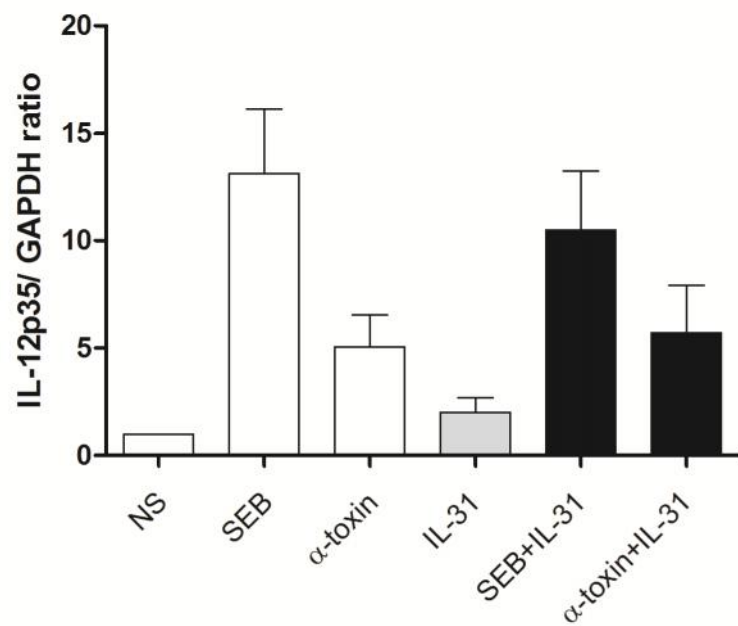
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Supplementary Figure 1:

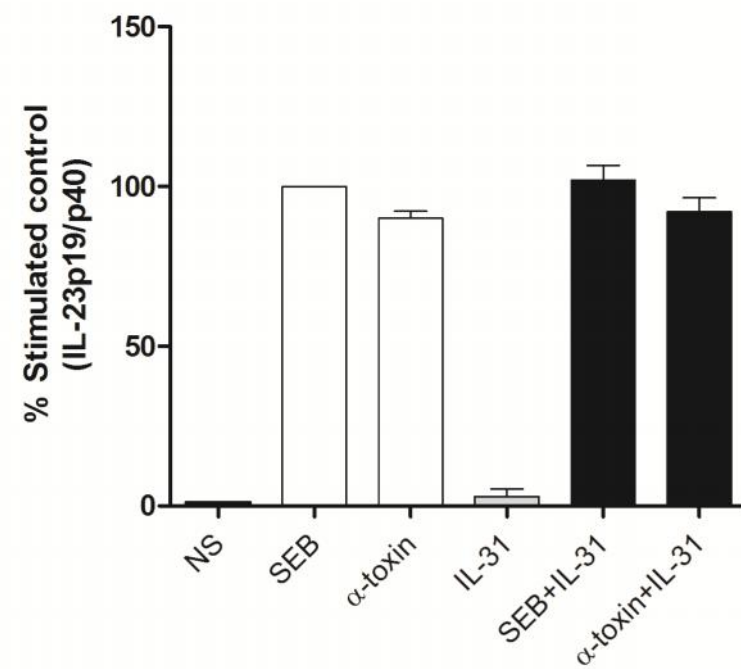


Supplementary Figure 2:

A)



B)



Supplementary Figure 3:

