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Mechanistic correlations between two itch biomarkers, cytokine interleukin-31 and neuropeptide β-endorphin, via STAT3/calcium axis in atopic dermatitis

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Conflicts of interest
None declared.

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Summary

Background Itch is the cardinal symptom of atopic dermatitis (AD). β-Endorphin, a neuropeptide, is increased in both AD skin and sera. Interleukin (IL)-31, an itch-relevant cytokine, activates IL-31 receptors in keratinocytes. However, how IL-31 and β-endorphin interact in AD skin remains elusive.

Objectives To investigate the mechanistic interaction of IL-31 and β-endorphin in AD.

Methods This was a prospective cross-sectional study. We recruited adult patients with AD and controls according to Hanifin's AD criteria. Serum levels of IL-31 and β-endorphin were measured by enzyme-linked immunosorbent assay. Expressions of IL-31 receptor A (IL-31RA) and β-endorphin in the skin were assessed by immunohistochemistry. Their expression in the skin and blood was compared and correlated in patients with AD and in controls. We also treated primary keratinocytes with IL-31 and measured calcium influx, β-endorphin production and signalling pathways to define their mechanistic interactions.

Results β-Endorphin was increased in the supernatant from IL-31-treated keratinocytes. IL-31 receptor activation resulted in calcium influx and STAT3 activation; pretreatment with STAT3 inhibitor stopped the increase of β-endorphin. Notably, either replacement of extracellular calcium or treatment with 2-aminoethoxydiphenyl borate, an inhibitor for the store-operated channel, blocked STAT3 activation. We found higher levels of blood β-endorphin and IL-31, which were significantly correlated, in patients with AD. Moreover, IL-31RA and β-endorphin were increased and colocalized both in AD human skin and TPA-painted mouse skin.

Conclusions IL-31 receptor activation in keratinocytes induces calcium influx and STAT3-dependent production of β-endorphin. These results might contribute to an understanding of the regulatory mechanisms underlying peripheral itch.

Atopic dermatitis (AD), a common chronic inflammatory skin disease prevalent in 6–9% of the general population, is increasing globally.[1] It is characterized by severe itch and is usually associated with a personal or family history of atopic diseases. The itch affects physical growth, mental development, emotional equanimity and performance at school and work.[2] The predominance of itch in patients with AD makes it ideal for studying the pathophysiology of pruritus-like itches. Antihistamines have little effect on alleviating AD itch, suggesting histamine is not a major mediator of AD itch.[3,4]

Neuropeptides, proteinases, arachidonic derivatives and cytokines may contribute to AD pruritus.[3,5] Opioids such as morphine may induce severe itching.[6] An incidence of pruritus of 10–50% has been reported in people administered opioids intravenously,[7,8] and an incidence of 20–100% with neuraxial administration. Interestingly, naloxone, an antidote for morphine, suppresses itch in patients with chronic renal failure and AD.[9]

Blood levels of β-endorphin, which binds to opioid receptors, have been associated with intensity of subjective itch in patients with AD.[2] β-Endorphin and its receptors are both present in keratinocytes and free nerve endings.[10] Increases in endorphin are also inhibited in patients with AD treated with psoralen plus ultraviolet (UV) A.[10] Furthermore, the cytokine interleukin (IL)-1 and UV radiation, both known to accentuate itch in AD, enhance the release of β-endorphin from keratinocytes.[11,12] Although the peripheral role of endorphins on induction of itch through the peripheral μ-opioid receptor has not been verified in the literature, indirect evidence suggests that β-endorphin might be closely associated with itch in AD.

Cytokines are considered an important mediator in AD, but little is known about how cytokines in AD contribute to the production of peripheral β-endorphin in AD skin. The transgenic overexpression of the cytokine IL-31 in lymphocytes in mice induces severe pruritus and dermatitis.[13] It is expressed preferentially by T helper (Th) 2 cells, and it activates a heterodimeric receptor composed of IL-31 receptor A (IL-31RA) and oncostatin M receptor (OSMR), both found on epithelial cells and keratinocytes.[13,14] The epidermis of patients with AD has an increased expression of IL-31RA and IL-31.[15,16] IL-31 can induce the production of several proinflammatory mediators, including epidermal growth factor, vascular endothelial growth factor and monocyte chemotactic protein-1, in bronchial alveolar cells.[17] Blood IL-31 level has been correlated to disease severity in patients with AD.[18] Although both IL-31 and opioid pathways are enhanced in AD skin, no study has investigated their relationship in AD. To do this, we performed an *in vitro* study in which we added various doses of IL-31 into primary keratinocytes of normal foreskins and measured the release of endorphins using enzyme-linked immunosorbent assay (ELISA) and the expression of STAT (signal transducer and activator of transcription) 3, ERK (extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinase) by Western blotting. We also performed two *in vivo* studies, one to measure blood levels of IL-31 and β-endorphin in patients with AD recruited from a dermatological clinic in a tertiary centre and normal controls, and the other to measure the colocalization of IL-31RA and β-endorphin in skin samples from the study group and normal controls. In addition, we measured the colocalization of IL-31RA and β-endorphin in the skin of TPA (12-*O*-tetradecanoylphorbol 13-acetate)-painted mice, a model for irritant contact dermatitis. The results of this study might further advance our understanding of the regulatory mechanisms underlying peripheral itch in AD.

Materials and methods

Reagents

IL-31 was purchased from R&D (Minneapolis, MN, U.S.A.), and ATP and thapsigargin from Sigma-Aldrich (St Louis, MO, U.S.A.). 2-Aminoethoxydiphenyl borate (2-APB) was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). ELISA kits for endorphin, calcitonin gene-related peptide (CGRP) and substance P were obtained from Bachem Corp. (Torrance, CA, U.S.A.) and were assayed following the manufacturer's instructions for their levels in conditioned media from IL-31-treated keratinocytes (sensitivity at 0·5 ng mL^{-1}). Dr Tohru Yoshioka provided Fluo-4 for the calcium propagation experiments. Antibodies for STAT3, ERK and JNK used in Western blotting were from Cell Signaling Technology (Boston, MA, U.S.A.). STAT3 inhibitor S31-201 or NSC 74859 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), ERK inhibitor (U0126) was from Promega (Madison, WI, U.S.A.), mitogen-activated protein kinase (MAPK) p38 inhibitor (indole-5-carboxamide) was from Merck (Whitehouse Station, NJ, U.S.A.).

Flow cytometry and immunofluorescent staining of interleukin-31 receptors

Human keratinocytes were obtained from adult foreskins through routine circumcisions. The method for keratinocyte cultivation was described in a previous report.[19] Keratinocytes at the third passage were then grown in keratinocyte-SFM medium free of supplements 24 h before the experiments. For IL-31RA measurements, keratinocytes were stained with 1 : 100 mouse anti-IL-31RA (R&D) for 30 min followed by fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (R&D). We also stained keratinocytes with phycoerythrin (PE)-conjugated antibody for OSMR or its corresponding isotype antibody (both E-Bioscience, San Diego, CA, U.S.A.). Flow cytometry was performed with a FACScan (BD Biosciences, San Jose, CA, U.S.A.). Keratinocytes were stained with the same antibodies for immunofluorescence (1 : 500) and examined by fluorescent microscope (Cell-R; Olympus, Tokyo, Japan).

Immunofluorescence for β-endorphin and interleukin-31 receptor A in skin

Immunofluorescent studies for β-endorphin were performed on 5-μm serial tissue sections described previously.[19] Briefly, all the sections were blocked using 10% goat serum or 3% bovine serum albumin at room temperature for 2 h. For single staining, the sections were incubated with rabbit antihuman β-endorphin (1 : 200; Millipore, Billerica, MA, U.S.A.) followed by goat antirabbit Alexa® 488 (1 : 1000; Invitrogen, Carlsbad, CA, U.S.A.). For double staining, cryostat sections were stained with goat antihuman IL-31RA antibody and stored at 4 °C overnight (1 : 500; R&D). The next day we

incubated the cryostat sections with rabbit antigoat IgG-FITC (1 : 1000; Sigma) for 1 h at room temperature. They were also incubated with rabbit antihuman β-endorphin (1 : 200; Millipore) followed by goat antirabbit Alexa 568 (1 : 1000; Invitrogen). Image analysis was performed by NIH IMAGEJ (http://rsbweb.nih.gov/ij/). Fluorescent intensity index (0–255) was calculated in five random mid-power fields above the dermoepidermal junction. A plugin of colocalization was used and estimated by coefficient of Spearman's correlation (−1 to 1).

Measurement of interleukin-31 receptor A and β-endorphin expressions in TPA-painted mice

To determine the colocalization of IL-31RA and β-endorphin in the inflammatory mouse skin, we measured their colocalizations in the skin of TPA-painted mice, a model for irritant contact dermatitis. TPA painting to induce skin inflammation was adopted from our previous study.[20] TPA or acetone as solvent control was given four times to each animal at an interval of 24 h (8·1 nmol in 100 μL acetone; Sigma-Aldrich). The mouse skin was obtained 1 h after the fourth application of TPA or acetone. Cryostat sections were stained with goat antimouse IL-31RA antibody and stored at 4 °C overnight (1 : 500; R&D). The next day we incubated the sections with rabbit antigoat IgG-FITC (1 : 1000; Sigma) for 1 h at room temperature. They were also incubated with rabbit antimouse β-endorphin (1 : 200; Millipore) followed by goat antirabbit Alexa 568 (1 : 1000; Invitrogen).

Western blotting

Methods for Western blotting have been described previously.[19] An enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) was used to detect specific proteins. The visualized films were recorded on a digital imaging system (Alpha Imager 2000; Alpha Innotech Corp., San Leandro, CA, U.S.A.).

Real-time polymerase chain reaction

The RNA was extracted and assessed for purity by A260/A280 absorption. Only samples with ratios over 1·7 were used for further amplification. A 1-μL sample of the products underwent real-time polymerase chain reaction (PCR) amplification using FastStart Universal SYBR Green Master (ROX) (Roche Applied Science, Mannheim, Germany) to measure proopiomelanocortin (POMC), a precursor of β-endorphin, according to the manufacturer's instructions. The primers were: POMC forward CTACGGCGGTTTCATGACCT, reverse CCCTCACTCGC CCTTCTTG; 18SrRNA forward TTCGGAACTGAGGCCATGAT, reverse TTTCGCTCTGGTCCGTCTTG. Reactions were performed using SYBR Green PCR Master Mix, and the data were collected by an ABI Prism 7700 and analysed using a Sequence Detector 1·9·1 (Applied Biosystems, Foster City, CA, U.S.A.).

Calcium propagation

Human keratinocytes were incubated with 2·5 μmol L^{-1} Fluo-4 (a calcium-sensitive dye) for 20 min at 37 °C, and then incubated with basal salt solution for 1 min at 37 °C. We measured the intracellular Ca^{2+} dynamics in individual live cells sequentially using a Cell-R microscope (Olympus) attached to image analysis software. IL-31 (200 nmol L^{-1}), ATP (1 μmol L^{-1}) or IL-8 (500 ng mL^{-1}) was added as indicated.

Collection of blood from patients with atopic dermatitis

The patients with AD were recruited from a dermatological clinic in a tertiary centre from 2008 to 2009. AD was defined based on diagnostic criteria of Hanifin and Rajka.[21] To be included, the patients must not have received any topical treatments for at least 2 weeks or any systemic treatment for 2 months before the study. They were excluded if their disease was considered acute, or if they had a history of diabetes, cancer or human immunodeficiency virus. Eighty-one of the patients with AD (40 men and 41 women, aged 32·2 ± 9·4 years, range 1–71) were enrolled. A dermatologist took a medical history and generally surveyed the skin of each patient with AD and each normal control for skin lesions. Extrinsic AD was defined as blood IgE level ≥ 100 IU mL^{-1}, while intrinsic AD was defined as blood IgE level < 100 IU mL^{-1}. Venous peripheral blood was collected from all patients and controls. After centrifugation, the serum was stored at −70 °C until assayed for β-endorphin and IL-31. The protocol for this study was approved by the Institutional Review Board of the Kaohsiung Medical University Hospital. All clinical assessments and specimen collections were conducted according to principles stipulated in the Declaration of Helsinki.

Determination of serum β-endorphin by radioimmunoassay and interleukin-31 by enzyme-linked immunosorbent assay

The concentration of β-endorphin in sera was determined by radioimmunoassay (RIA) with the use of the ^{125}I-RIA kit (Phoenix Pharmaceuticals, Inc., Belmont, CA, U.S.A.) as previously described.[2] The concentration of IL-31 was measured using a commercial ELISA kit (R&D). Although no sensitivity data were provided in the product instructions, the detection limit was about 50 pg mL^{-1} based on our standard curves.

Statistical analysis

P-values were two-tailed and considered significant at 0·05 without adjusting by multiple testing. Kruskal–Wallis test with Dunn's *post hoc* test was used for comparisons of β-endorphin, IL-31, SCORAD index, and IgE levels between patients with extrinsic AD, intrinsic AD and controls. It was also used for the comparisons of β-endorphin level and POMR expression between different IL-31-treated groups at different chronological points. The difference in fluorescent intensity of IL-31RA and

β-endorphin of skin from AD and normal controls was compared using the Mann–Whitney test. Spearman's correlation test was used to examine the association between blood β-endorphin and blood IL-31 as well as the colocalization of IL-31RA and endorphin in skin. All statistical operations were performed using SAS version 8·01 (SAS Inc., Cary, NC, U.S.A.).

Results

Activation of interleukin-31 receptors in cultured keratinocyte-induced calcium propagation

To measure the expression of IL-31RA and OSMR in keratinocytes *in vitro*, we harvested primary human keratinocytes and measured the IL-31RA expression by flow cytometry and immunofluorescence. IL-31RA and OSMR levels were both increased in the cultured keratinocytes (Fig. 1a). To ensure that the receptor was functional, we measured calcium propagation in IL-31-treated keratinocytes, and first found a marked increase in calcium influx after treatment with IL-31 treatment followed by a second smaller increase in calcium 5 min later (Fig. 1b). The second increase suggests that other membrane channels, including store-operated channels (SOC), mediate calcium influx. Moreover, IL-8, which binds to CXCR8 in keratinocytes, also induces the propagation of calcium to a similar extent.

Interleukin-31 enhanced the release of β-endorphin but not calcitonin gene-related peptide or substance P from keratinocytes

We investigated whether IL-31 would induce the production of certain neuropeptides, β-endorphin, CGRP and substance P, through epidermal keratinocytes. Keratinocytes were treated with IL-31, and the release of β-endorphin, substance P and CGRP was measured by ELISA 24 h later. Only β-endorphin was increased (Fig. 2a). IL-31 300, 500 and 1000 ng mL^{-1} caused a two to threefold increase in β-endorphin ($P = 0{\cdot}03$, 0·03 and 0·02, respectively). We also investigated whether IL-31 would upregulate POMC, the precursor of β-endorphin, using real-time PCR. IL-31 300 and 500 ng mL^{-1} induced a two to fourfold increase in POMC (Fig. 2b; $P = 0{\cdot}02$ and $P = 0{\cdot}01$, respectively). Kinetic analysis revealed that the upregulation of POMC preceded the increase of β-endorphin,

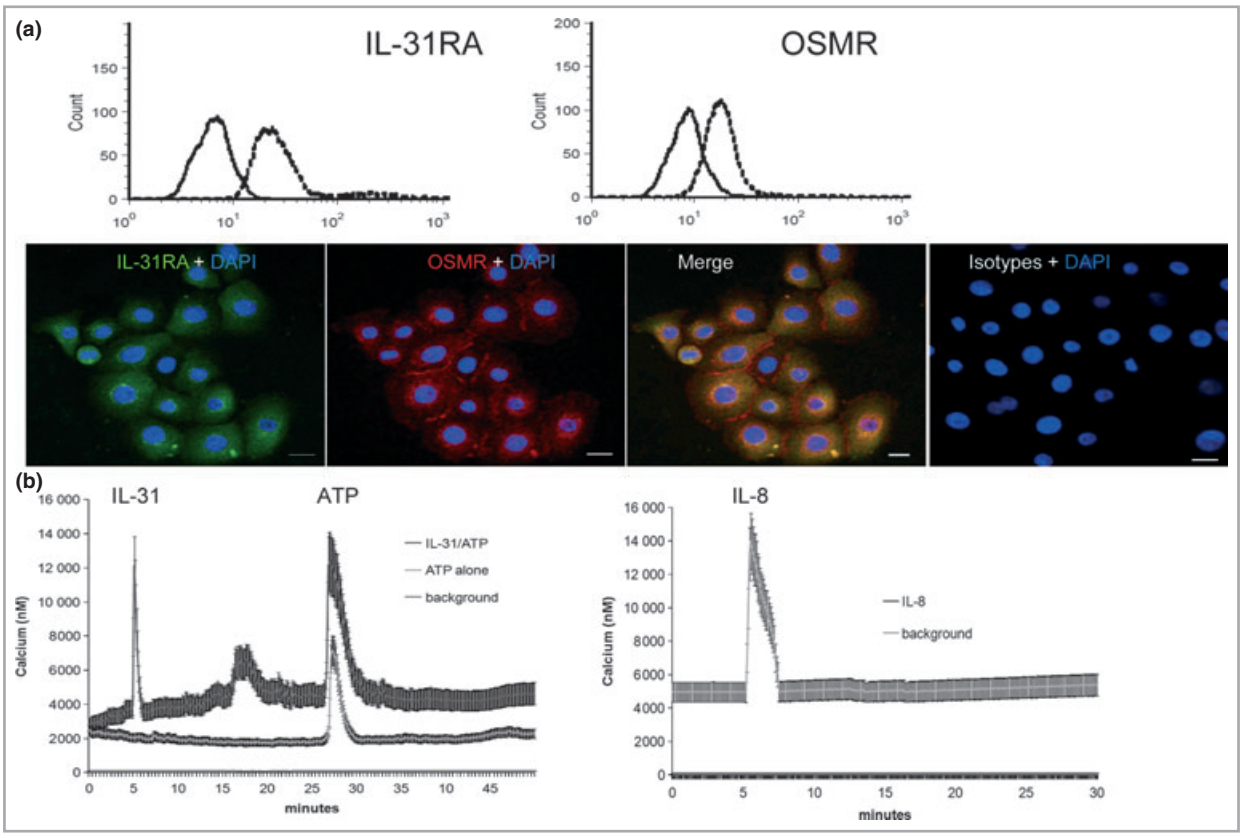

Fig 1. Expression of functional interleukin (IL)-31 receptors in primary keratinocytes. (a) Expression of IL-31 receptor A (IL-31RA) and oncostatin receptor (OSMR) in primary keratinocytes. The solid line represents isotype control staining while the dashed line area shows IL-31RA-fluorescein isothiocyanate or OSMR-phycoerythrin staining in flow cytometry (upper panel). Corresponding results from immunofluorescence examination are shown (lower panel, bar = 5 μm). (b) Live cultured keratinocytes were preloaded with Fluo-4® to visualize calcium propagation in real time under Cell-R microscopy (Olympus). Keratinocytes were treated with IL-31 at 500 ng mL^{-1}, ATP at 1 μmol L^{-1}, and IL-8 at 500 ng mL^{-1} at the indicated time on the *x*-axis. The *y*-axis shows the average optical intensity with SDs from a preselected group of keratinocytes and a preselected group of baseline backgrounds. Three independent experiments were performed with consistent results. DAPI, 4,6-diamino-2-phenylindole.

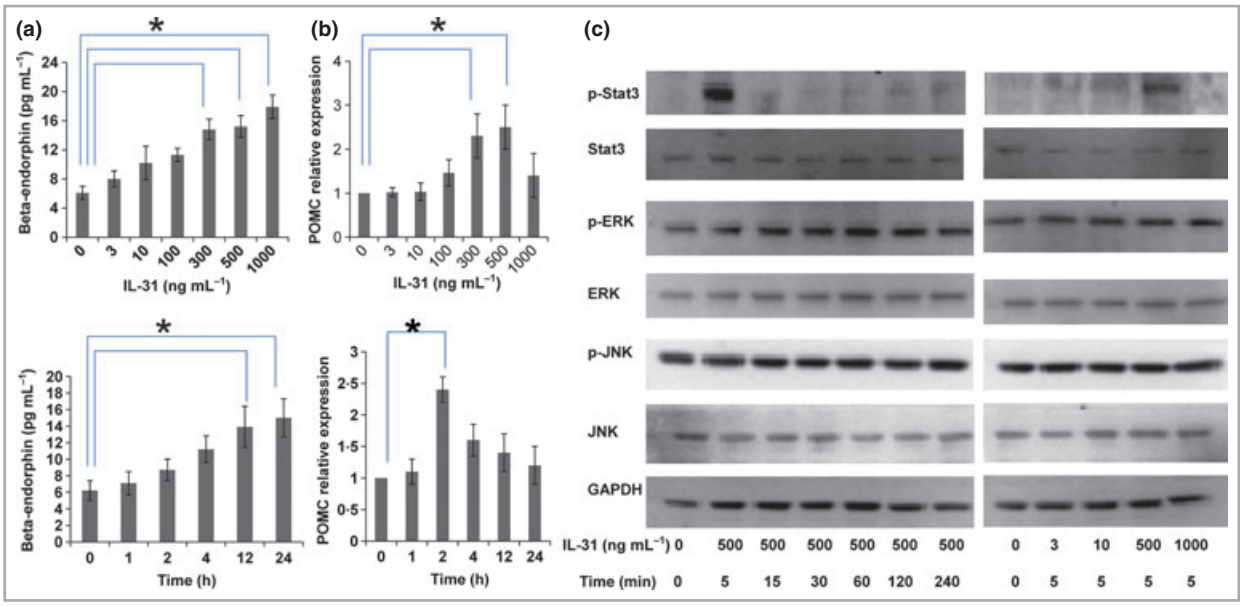

Fig 2. IL-31 induced proopiomelanocortin (POMC) upregulation and β-endorphin production, along with STAT3 activation and ERK activation. (a) Keratinocytes were incubated with interleukin (IL)-31 at indicated concentrations for 24 h (upper panel). For kinetic analysis, 500 ng mL^{-1} IL-31 was added into keratinocytes for 1, 2, 4, 12 and 24 h (lower panel). β-Endorphin was measured by enzyme-linked immunosorbent assay and POMC expression was determined by real-time polymerase chain reaction normalized with β-actin (b). (c) Activation of STAT3, ERK and JNK was measured by Western blotting. Kinetic analysis was performed with 500 ng mL^{-1} IL-31 for 5, 15, 30, 60, 120 and 240 min. In addition, keratinocytes were incubated with IL-31 at indicated concentrations for 30 min. Three independent experiments were performed with consistent results. *$P < 0{\cdot}05$ by Kruskal–Wallis with *post hoc* Dunn's test.

suggesting that IL-31-induced production of endorphin involved the initial transcription of POMC.

Interleukin-31 triggered the activation of STAT3 and ERK but not JNK

To investigate how IL-31-induced endorphin release was triggered in keratinocytes, we treated keratinocytes with 3, 10, 500 or 1000 ng mL^{-1} of IL-31 for 5 min and 500 ng mL^{-1} IL-31 from 5 to 240 min and observed the effect on its signalling pathway by Western blotting. The concentrations of IL-31 used were decided based on the result that IL-31 at 500 and 1000 ng mL^{-1} had optimal effects on β-endorphin production at 24 h (Fig. 2a). Only 500 ng mL^{-1} IL-31 markedly increased the activation of phosphorylated (p) STAT3, which peaked at 5 min (Fig. 2c). Only 500 ng mL^{-1} IL-31 gradually increased ERK phosphorylation starting from 15 min and continuing to increase up to 60 min, at which time it stabilized (Fig. 2c). The discrepancy of the concentration of IL-31 required to induce peak STAT3 activation at 5 min (500 ng mL^{-1}) and peak β-endorphin production at 24 h (1000 ng mL^{-1}) might result from an even earlier activation (< 5 min) of STAT3 by IL-31 at 1000 ng mL^{-1} or another undisclosed STAT3-independent pathway.

Capacitative calcium entry mediated STAT3 activation in IL-31-treated keratinocytes

We further investigated the role of capacitative calcium entry in IL-31-induced STAT3 activation. Because 2-APB is a blocker of store-operated calcium entry[22] and thapsigargin, an inhibitor of intracellular calcium pumps, mediates capacitative calcium entry by emptying the endoplasmic reticulum store of calcium, we used 2-APB and thapsigargin to inhibit and activate capacitive calcium entry, respectively. 2-APB blocked STAT3 activation in IL-31-treated keratinocytes (Fig. 3a), while thapsigargin and ATP treatment alone substantially increased STAT3 activation (Fig. 3a), indicating that capacitive calcium entry mediated the STAT3 activation by IL-31. Further, replacement of culture medium with calcium-free medium abrogated the activation of STAT3 (Fig. 3c), suggesting that calcium entry may play a role in IL-31-mediated STAT3 activation.

Interleukin-31 increased β-endorphin release from keratinocytes via STAT3

Having found that IL-31 induced STAT3 activation through calcium influx and that IL-31 induced β-endorphin release, we next investigated whether the release of β-endorphin was dependent on STAT3 activation. We pretreated the keratinocytes with a p38-MAPK inhibitor, an ERK inhibitor, a STAT3 inhibitor or a SOC inhibitor, 2 h before IL-31 treatment and measured the release of β-endorphin (Fig. 3b). The increase of β-endorphin was abrogated only by STAT3 inhibition ($P = 0{\cdot}02$) or SOC inhibition ($P = 0{\cdot}03$) by 2-APB, indicating IL-31-mediated release of β-endorphin depended on the activation of STAT3 and influx of calcium. Considered with the previous results, this blocking test provided evidence that in keratinocytes IL-31 first

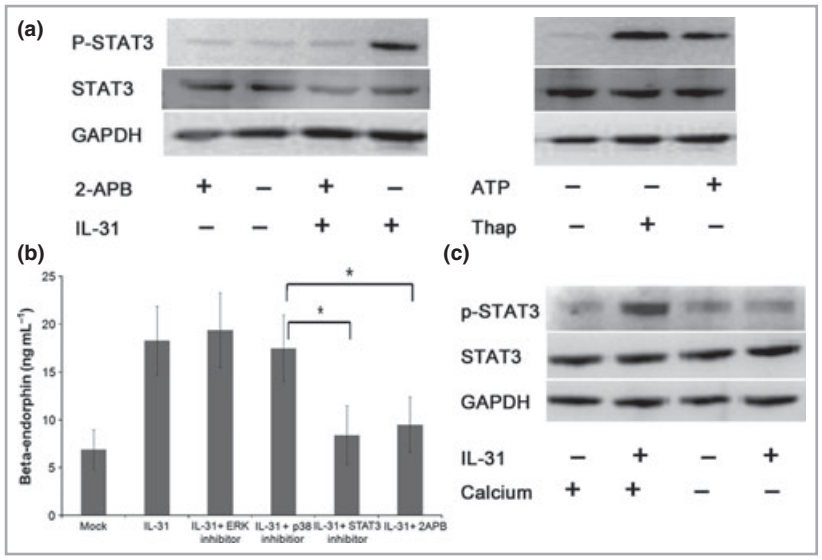

Fig 3. Interleukin (IL)-31 induced STAT3 activation, calcium propagation and β-endorphin release. (a) Keratinocytes were treated with IL-31 at 500 ng mL^{-1} with or without 2-aminoethoxydiphenyl borate (2-APB) at 50 μmol L^{-1} for 5 min. STAT3 and its phosphorylation were analysed by Western blot. Keratinocytes were also treated with ATP at 1 mmol L^{-1} or thapsigargin (Thap) at 300 nmol L^{-1} for 5 min. Three independent experiments were performed and produced consistent findings. One set of representative data is shown. (b) Keratinocytes were treated with IL-31 at 500 ng mL^{-1} for 24 h with or without pretreatment of a p38-mitogen-activated protein kinase inhibitor (30 μmol L^{-1}), an ERK inhibitor (30 μmol L^{-1}), a STAT3 inhibitor (30 μmol L^{-1}), and a store-operated calcium channel inhibitor (2-APB, 50 μmol L^{-1}) for 2 h. Cultured supernatants were collected and assayed for β-endorphin by enzyme-linked immunosorbent assay. *$P < 0{\cdot}05$, Kruskal–Wallis with *post hoc* Dunn's test. (c) IL-31-induced STAT3 activation depended on the presence of extracellular calcium. Keratinocytes were treated with IL-31 at 300 ng mL^{-1} for 5 min with regular calcium (120 nmol L^{-1}) (lane 2). Replacement of the regular calcium medium with calcium-free medium abolished the activation of phosphorylated (p)-STAT3 (lane 4). Three independent experiments were performed and produced consistent results. One representative set of data is shown.

induced the influx of calcium which, in turn, activated STAT3, leading to the release of β-endorphin.

Interleukin-31 was significantly correlated with β-endorphin in sera from patients with atopic dermatitis

To determine further the biological significance of our findings and extrapolate our data *in vivo*, we studied the possible correlation of the blood levels of IL-31 and β-endorphin in patients with AD and controls. We recruited 81 patients with chronic AD (age 41·3 ± 22·9 years) and 70 normal controls (age 38·6 ± 20·4 years) from our dermatology clinic (Fig. 4). Although blood IL-31 and β-endorphin levels were higher in patients with AD ($P = 0{\cdot}02$ and 0·03, respectively), they were not significantly different in extrinsic vs. intrinsic AD ($n = 65$ and 16; $P = 0{\cdot}27$ and 0·35, respectively). We did find, however, a significant association between serum β-endorphin levels and IL-31 levels in patients with AD and controls by Spearman's correlation analysis ($P = 0{\cdot}02$, $r = 0{\cdot}82$ and $P = 0{\cdot}03$, $r = 0{\cdot}78$, respectively) (Fig. 4).

Interleukin-31 receptor A and endorphin were both increased and colocalized in atopic dermatitis skin

Having observed an increase of β-endorphin in IL-31 keratinocytes *in vitro*, we investigated whether β-endorphin and IL-31RA were increased and colocalized in skin. We took four samples from the skin of patients with extrinsic AD (aged 19–52 years), four samples from skin of patients with intrinsic AD (aged 20–55 years), and five from the skin of normal controls (22–49 years). Using immunofluorescence for IL-31RA and β-endorphin, we found a significantly greater increase in IL-31RA in the intercellular space in epidermis from both extrinsic and intrinsic AD skin than in control skin, based on fluorescent intensity indices represented as median (lower–upper quartiles): 60 (14–78) and 53 (12–79) vs. 14 (7–25) ($P = 0{\cdot}02$, Mann–Whitney test) (Fig. 5). Similarly, fluorescent intensity indices indicated significantly greater increase in β-endorphin: 75 (25–89) and 76 (27–85) vs. 16 (8–35) ($P = 0{\cdot}03$, Mann–Whitney test). Notably, IL-31RA expression and β-endorphin expression were strongly colocalized in both AD and normal skin (patients with extrinsic AD $P = 0{\cdot}01$, $r = 0{\cdot}90$, patients with intrinsic AD $P = 0{\cdot}0$, $r = 0{\cdot}89$, and controls $P = 0{\cdot}02$, $r = 0{\cdot}83$, Spearman's correlation).

Interleukin-31 receptor A and β-endorphin are increased in parallel in TPA-painted mice

As we observed the colocalization of IL-31RA and β-endorphin in AD skin and the correlation of IL-31 and β-endorphin in the blood, we looked at whether this association could be reproduced in mice. Balb/c mice were painted with TPA to

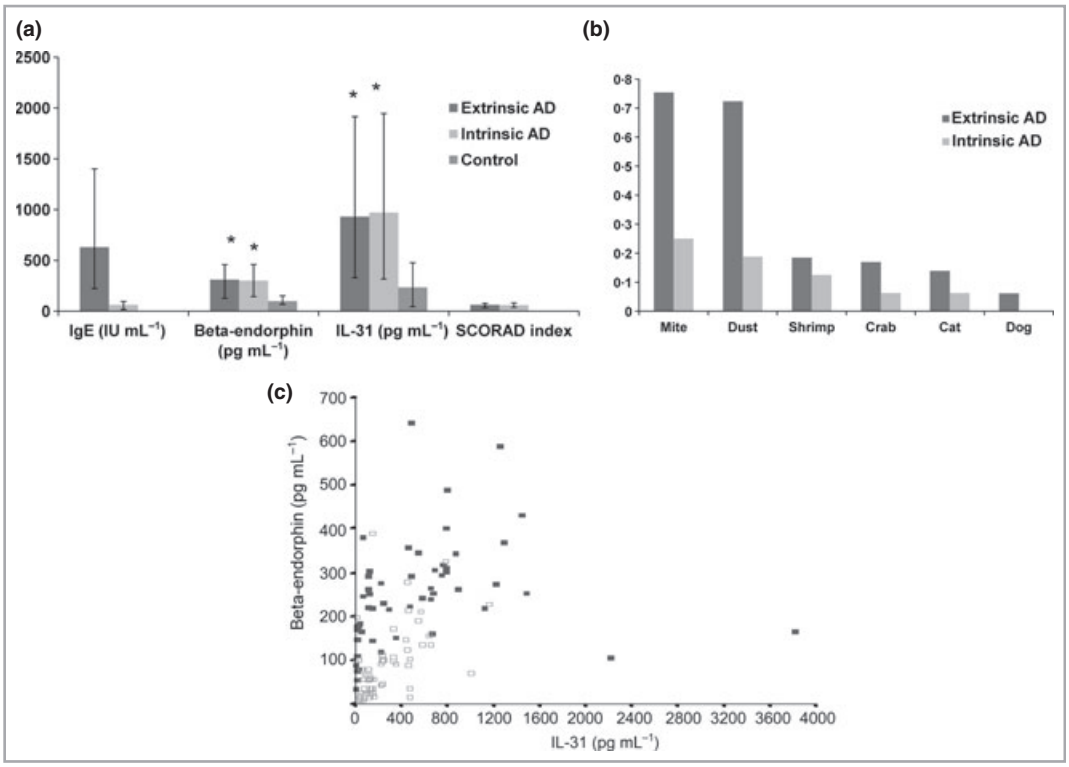

Fig 4. There was a significant correlation between serum β-endorphin and interleukin (IL)-31. (a) Patients [n = 81, including 65 with extrinsic atopic dermatitis (AD) and 16 with intrinsic AD] and controls (n = 70) were defined and recruited. IL-31 and β-endorphin were measured in the blood, by enzyme-linked immunosorbent assay and radioimmunoassay, respectively, of both groups and compared. Values of median and lower and upper quartiles are shown.*$P < 0{\cdot}05$, Kruskal–Wallis with *post hoc* Dunn's test when compared with controls. (b) Percentiles of selective allergen-specific IgE from extrinsic and intrinsic AD were recorded. (c) A dot plot graph showing β-endorphin and IL-31 levels is shown. Closed squares, values from patients with AD; open squares, normal controls. There was good correlation between IL-31 and β-endorphin levels in both patients with AD and controls.

induce skin inflammation and itch. Using immunofluorescence (Fig. 6), we found a significantly greater increase in IL-31RA in the intercellular space in epidermis from TPA-treated mouse skin than from acetone-treated control skin based on fluorescent intensity indices represented as median (lower–upper quartiles): 67 (11–77) vs. 10 (5–18) ($P = 0{\cdot}02$, Mann–Whitney test). Similarly, β-endorphin expression was also focally increased in the epidermal keratinocytes from skin painted with TPA: 32 (12–56) vs. 7 (3–15) ($P = 0{\cdot}02$, Mann–Whitney test). In the staining, some of the β-endorphin and IL-31RA expression was colocalized in the epidermis.

Discussion

This study demonstrated that IL-31 can induce the release of β-endorphin from keratinocytes through the entry of capacitative calcium and activation of STAT3 (Fig. 7). Blood levels of IL-31 and β-endorphin were significantly correlated in both patients with AD and normal controls. IL-31RA and β-endorphin levels were increased and colocalized in AD skin.

Calcium influx follows shortly after the activation of the membrane receptor. Chemokines, for example, are reported to induce calcium entry to induce activation of eosinophils.[23] The second phase of calcium propagation after IL-31 treatment in our study suggests that capacitative influx of calcium is involved in IL-31 signalling. Capacitative calcium influx is very important in cell signalling.[24] In T lymphocytes, the master protein for capacitative calcium influx, STIM1, is required for the production of IL-2 and interferon-γ.[25] Studies of keratinocytes report capacitative calcium influx to be important for cell differentiation,[26,27] and to be downregulated in psoriatic skin.[28] In the present study, capacitative calcium influx was needed for IL-31 to induce STAT3 activation. Although the signalling pathways after IL-31 receptor activation have been reported in several cell lines from bronchial and colonic epithelium,[17,29–31] this study represents the first to uncover the IL-31 signalling pathway in primary keratinocytes. More importantly, we demonstrate that keratinocyte production of β-endorphin is mediated sequentially as follows: IL-31–calcium–STAT3–β-endorphin. Although ERK was also activated by IL-31 in keratinocytes, blocking of ERK activation did not abrogate the release of β-endorphin, suggesting that ERK is not directly involved in this process.

Between 200 and 300 ng mL^{-1} IL-31 was required to induce β-endorphin production *in vitro*; blood concentrations of 1 ng mL^{-1} were hundreds of times lower than those used

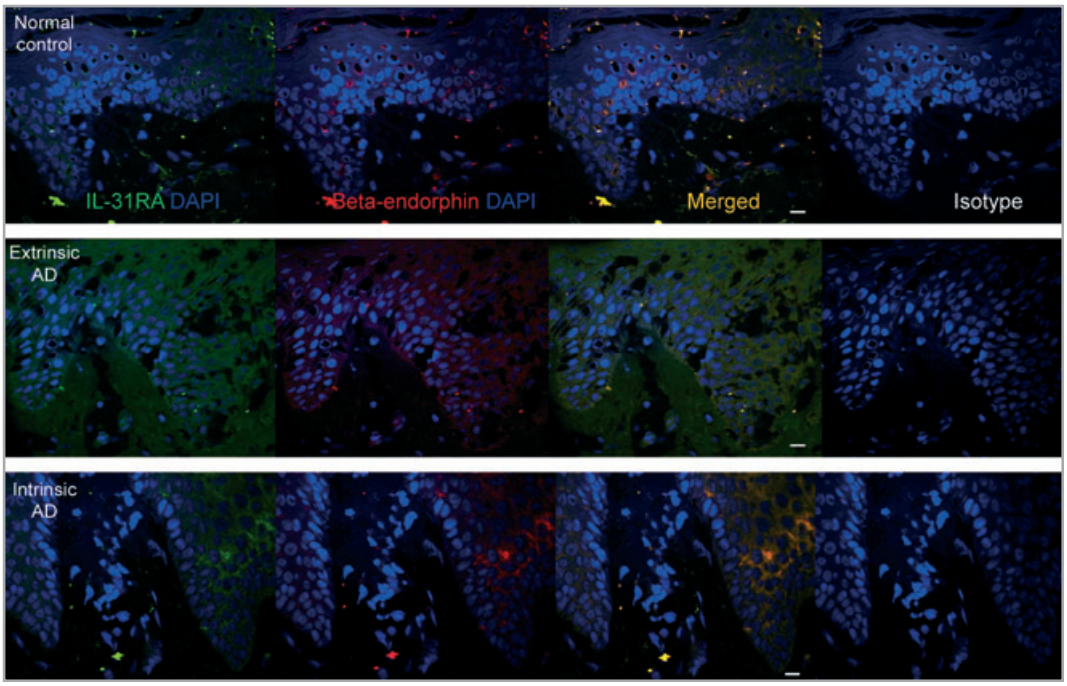

Fig 5. Colocalization of β-endorphin and interleukin (IL)-31 receptor A (IL-31RA) in human atopic dermatitis (AD) skin. Skin sections from patients with extrinsic AD and intrinsic AD, and normal controls (n = 4, 4 and 5, respectively) were stained with antibodies for β-endorphin and IL-31RA or corresponding isotype antibodies. Fluorescence intensity per unit area of epidermal β-endorphin or IL-31RA was measured in each group, and median and quartile values were calculated. There was more β-endorphin and IL-31RA in AD skin than normal skin. Spearman's correlations are given for both types of patient with AD and normal controls in terms of the colocalization for β-endorphin and IL-31RA in skin samples [4,6-diamino-2-phenylindole (DAPI) in blue, β-endorphin in red, and IL-31RA in green, scale bar = 20 μm, representative fields are shown].

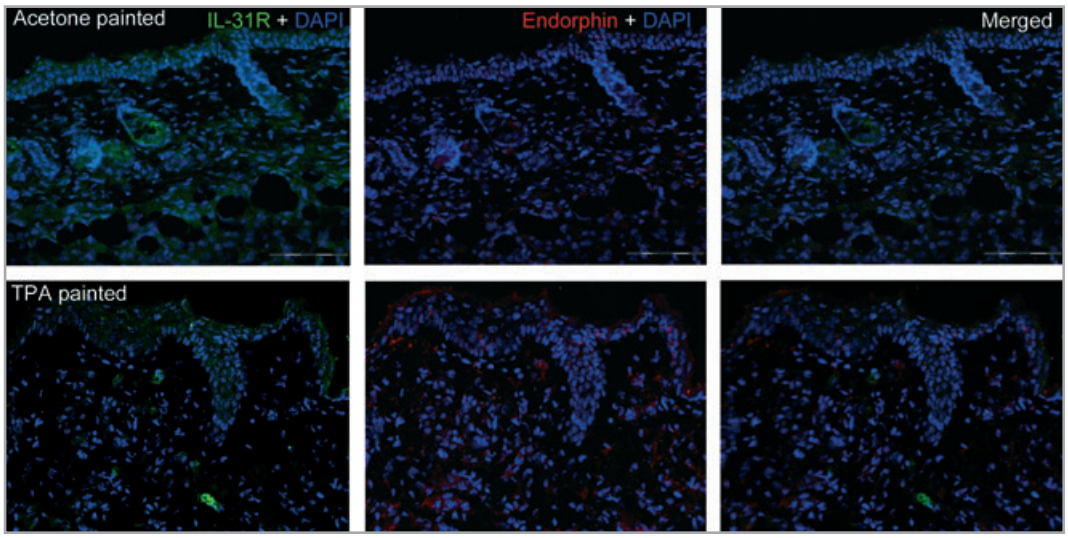

Fig 6. Increased expressions of β-endorphin and interleukin (IL)-31 receptor A (IL-31RA) in mouse skin painted with TPA. Skin sections from Balb/c mice were painted with TPA or acetone solvent only (n = 6 and 6, respectively). The sections were stained with antibodies for β-endorphin and IL-31-RA [4,6-diamino-2-phenylindole (DAPI) in blue, β-endorphin in red, and IL-31RA in green, scale bar = 100 μm, representative fields are shown].

in vitro. Thus, the pathological significance of IL-31 in AD may be debatable. However, the existence of certain other IL-31 releasers, such as superantigens,[32] might contribute to the differences in IL-31 concentrations *in vitro* and in AD skin *in vivo*. Moreover, in intestinal[29] and bronchial epithelial cells,[17] the IL-31 level required to activate intracellular signalling was 100 ng mL^{-1}, only one-third of 300 ng mL^{-1}. In addition, other cells might also express IL-31 and the blood level of

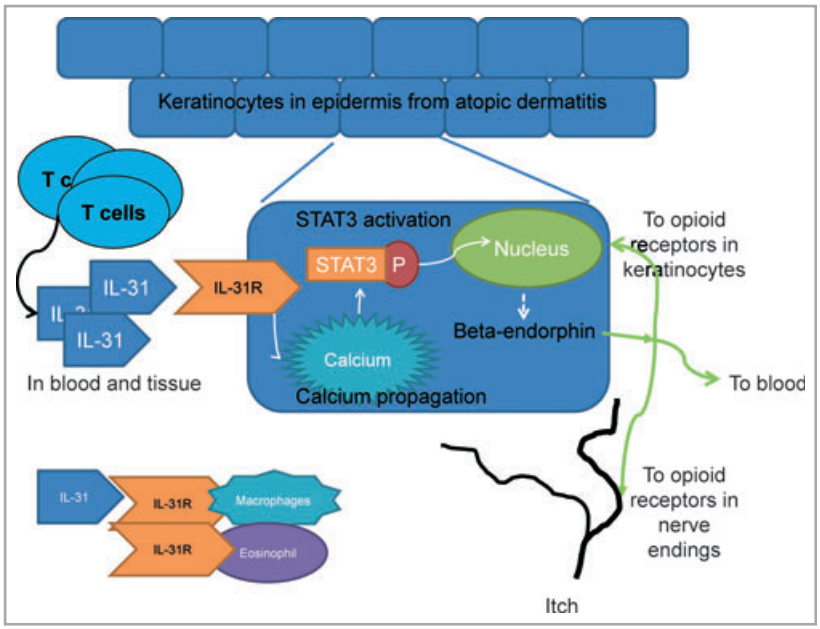

Fig 7. The interleukin (IL)-31/STAT3/β-endorphin axis plays a possible role in the regulation of peripheral itch in atopic dermatitis (AD). IL-31, produced by activated T helper (Th) cells in skin, activated IL-31 receptor (IL-31R) in keratinocytes, mobilized calcium and STAT3 activation, leading to the release of β-endorphin, which might bind to μ-opioid receptors in keratinocytes and free nerve endings. This axis might be important in the regulation of peripheral itch in AD.

IL-31 might not directly reflect IL-31 level in the skin microenvironment.

Keratinocytes are not the sole source of blood β-endorphin. Blood β-endorphin mostly comes from the pituitary gland (central) and also from adrenals, leucocytes and epidermal keratinocytes (peripheral). Although we found a correlation between blood levels of IL-31 and β-endorphin in patients with AD, confounder(s) such as stress and related factors might also result in an increase of IL-31 and β-endorphin in patients with AD. Nevertheless, the levels and correlations between IL-31 and β-endorphin serve as good biomarkers for itch in patients with AD. β-Endorphin is also released by tumour necrosis factor-α-stimulated fibroblasts.[33] It can activate mast cells, leading to degranulation and immediate weals.[34] Human epidermal keratinocytes and nerve endings express functionally active μ-opiate receptors, and the keratinocytes can influence free nerve endings by secreting β-endorphin and vice versa.[35] Thus, during neurogenic inflammation, a reciprocal opioid axis communication exists between keratinocytes and nearby nerve endings. Interestingly, there is a significant downregulation of the μ-opiate receptor in the epidermis of patients with AD,[36] possibly resulting from internalization of the receptor.[36]

We found that only endorphin, and not substance P or CGRP, was upregulated by IL-31. Only a few studies have investigated STAT3 activation and production of substance P, CGRP or β-endorphin, including one reporting a correlation, not a causal relationship, between STAT3 activation and CGRP in erythropoietin-activated axons.[37] In addition, POMC-specific STAT3 mutants diminish POMC expression, further supporting the notion that STAT3 is required for POMC transcription[38] and supporting our results showing IL-31 induced POMC upregulation.

In summary, IL-31 increases β-endorphin in keratinocytes through capacitative calcium influx and STAT3 activation. The correlations of IL-31 and β-endorphin in the blood and the colocalization of IL-31RA and β-endorphin in the skin suggest that the IL-31 and opioid axes may be related to pruritus in AD. These results might further our understanding of the regulatory mechanisms underlying peripheral itch in AD. Further studies are needed to elucidate the direct role of opioids in peripheral itch in AD.

What's already known about this topic?

- In patients with atopic dermatitis (AD), IL-31 expression is increased both in skin and in blood.
- β-endorphin, a neuropeptide, is increased in the blood from AD patients.
- Both IL-31 and β-endorphin are considered as biomarkers for itch. However, how IL-31 and β-endorphin interact peripherally in AD skin remains elusive.

What does this study add?

- Activation of IL-31R in keratinocytes led to β-endorphin release via capacitive calcium influx and STAT3 activation.
- Expressions of IL-31 and beta-endorphin are increased in AD blood, and expressions of IL-31RA and β-endorphin are increased in AD skin. There is a significant correlation between IL-31 and β-endorphin in the human blood and colocalization of IL-31RA and β-endorphin in AD skin.
- These results might contribute to an understanding of regulatory mechanisms underlying the peripheral itch.

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