Accepted Manuscript

Title: Involvement of thromboxane A_2 in interleukin-31-induced itch-associated response in mice

Authors: Tsugunobu Andoh, Sikai Li, Daisuke Uta

PII: S1734-1140(17)30380-8
DOI: https://doi.org/10.1016/j.pharep.2017.10.001
Reference: PHAREP 801

To appear in:

Received date: 3-6-2017
Revised date: 7-9-2017
Accepted date: 2-10-2017

Please cite this article as: Tsugunobu Andoh, Sikai Li, Daisuke Uta, Involvement of thromboxane A2 in interleukin-31-induced itch-associated response in mice (2010), https://doi.org/10.1016/j.pharep.2017.10.001

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Involvement of thromboxane A_2 in interleukin-31-induced itch-associated response in mice

Tsugunobu Andoh*, Sikai Li, Daisuke Uta

Department of Applied Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan

Short title: TXA_2-mediated IL-31-induced itching

*Corresponding author: Tsugunobu Andoh

Department of Applied Pharmacology

Graduate School of Medicine and Pharmaceutical Sciences

University of Toyama

2630 Sugitani, Toyama 930-0194

Japan

Tel: +81-76-434-7511

Fax: +81-76-434-5045

E-mail: andoht@pha.u-toyama.ac.jp

Approve of this animal experiment

Procedures in the animal experiments were approved by the Committee for Animal Experiments at the University of Toyama.

Acknowledgements

This research is supported by the Practical Research Project for Allergic Diseases and Immunology (Research on Allergic Diseases and Immunology) from Japan Agency for Medical Research and Development (AMED) (16ek0410011h0003) and JSPS KAKENHI (JP16K15337)..

Highlights

- Interleukinin-31 (IL-31) induced scratching, an itch-associated response, in mice.
- IL-31-induced scratching was inhibited by TP thromboxane (TX) receptor antagonist.
- IL-31 receptors were expressed mainly in epidermal keratinocytes.
- IL-31 induced TXA_2 production in the skin and primary cultures of keratinocytes.
- The results suggest that IL-31 elicits scratching via epidermal TXA2 production.

ABSTRACT

Background: Atopic dermatitis is a chronic and severe pruritic skin disease. Interlukin-31 (IL-31) has been recently demonstrated to be one of the key pruritogens in atopic dermatitis. However, the mechanisms underlying IL-31-induced itching remains unclear. In our previous study, we have shown that thromboxane (TX) A_2 is involved in itch-associated responses in mice with atopy-like skin diseases.

Methods: IL-31 was given intradermally into the rostral back of ICR mice and the hind-paw scratching to the injection site were counted. Expression of TX synthase and IL-31 receptors were analyzed using immunohistochemical staining or RT-PCR in mouse skin or primary cultures of mouse keratinocytes. The concentration of TXB_2, a metabolite of TXA_2, in the skin

and the culture medium of primary cultures of mouse keratinocytes was measured using enzyme immunoassay. The concentration of intracellular Ca^{2+} ions in mouse keratinocytes was measured using the calcium imaging method.

Results: An intradermal injection of IL-31 elicited scratching, an itch-related response, in mice. The scratching was inhibited by TP TXA_2 receptor antagonist DCHCH. The distribution of TX synthase and IL-31RA receptor was mainly epidermal keratinocytes in the skin. The primary cultures of keratinocytes expressed the mRNAs of TX synthase and IL-31 receptors. IL-31 increased the concentration of TXB_2, which was inhibited by TX synthase inhibitor sodium ozagrel and EGTA, in the skin and the culture medium of primary cultures of keratinocytes. IL-31 increased the concentration of intracellular Ca^{2+} ions in mouse keratinocytes.

Conclusion: It is suggested that IL-31 elicits itch-associated responses through TXA_2 produced from keratinocytes.

Keywords: interleukin-31; thromboxane A_2; itch; thromboxane synthase; keratinocytes

Introduction

Interleukin 31 (IL-31) is a cytokine released from T cells, such as $CD4^+$-Th2 cells, and signals through a receptor complex comprising IL-31 receptor A (IL-31RA) and oncostatin M receptor β (OSMRβ) [1,2]. IL-31 is involved in several allergic diseases, such as dermatitis, allergic rhinitis and asthma [3]. Recent studies have shown that the expression of IL-31 mRNA is increased in the skin of patients with atopic dermatitis [4,5] and in animal models of atopic dermatitis [6]. In addition, it has been reported that an anti-IL-31 antibody and an anti-IL-31 receptor antibody attenuated itching in atopic dermatitis patients [7] and scratching in an animal model of atopic dermatitis [8-10]. An intradermal injection of IL-31 elicits an itch-related response in animals [11-15]. These findings suggest that IL-31 is an itch mediator.

Although intradermal IL-31 elicits itching, the peripheral underlying mechanisms of the itching remains unclear. IL-31 elevates the concentration of intracellular Ca^{2+} ions in the primary cultures of dorsal root ganglia, suggesting that IL-31 directly activates primary afferents and elicits itching [12]. IL-31 receptors are also expressed in epidermal keratinocytes [2,14,16]. A recent study suggests that IL-31 induces itching through the itch mediators (such as leukotriene (LT) B_4 [14]) released from keratinocytes rather than the direct stimulation to primary sensory neurons [16].

Thromboxane (TX) A_2 is produced by metabolizing arachidonic acid with cyclooxygenase and TX synthase (TXSyn) and is spontaneously inactivated to TXB_2. Although TXA_2 exerts several pharmacological effects (e.g., platelet aggregation and inflammation) [17], recent studies have shown that TXA_2 is a potent itch mediator [18] and TXA_2 released from epidermal keratinocytes is involved in atopic dermatitis-related itching [19]. However, the underlying mechanisms on the production of TXA_2 in keratinocytes remains unclear. IL-31 is a key cytokine for itch in atopic dermatitis [7,8,9,10] and the receptors are expressed in keratinocytes [14]. In this study, therefore, we investigated whether TXA_2 released from keratinocytes is involved in IL-31-induced itching-related responses in mice.

Materials and methods

Animals

Male ICR mice (neonatal or 4-8 weeks old) were used in this study. Adult and pregnant mice were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in a room under controlled temperature (21–23°C), humidity (45–65%), and light (lights on from 08:00 to 20:00 h). Food and water were freely available.

Chemical preparation and administration

Mouse IL-31 (BioVision, Milpitas, CA, USA) was dissolved in phosphate-buffered saline (PBS) and injected intradermally into the interscapular region in a volume of 50 μL. DCHCH (7-[2α, 4α-(dimethylmethano)-6β-(2-cyclohexyl-2β-hydoxyacetamino)-1α-cyclohexyl]-5(Z)-heptanoic acid) (Ono Pharmaceutical, Osaka, Japan) [14] and sodium ozagrel (Wako Pure Chemical Industries, Osaka, Japan) were dissolved in tap water and 0.5% sodium carboxymethyl cellulose, respectively. These agents were administered orally 1 h before the IL-31 injection. For the *in vitro* studies, mouse IL-31 (BioVision) and sodium ozagrel (Wako Pure Chemical Industries) were dissolved in basal medium (CnT-Prime Basal Medium, CELLnTEC Advanced Cell Systems AG, Bern, Switzerland). O,O'-Bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetraacetic acid (EGTA) (Dojindo Laboratories, Kumamoto, Japan) was dissolved in distilled water (pH 8.0) and diluted with CnT-Prime Basal Medium. Sodium ozagrel and EGTA were administered 30 min before IL-31 application.

Behavioral experiments

The hair on the rostral back of the mouse was removed using hair clippers. The following day, the behavioral experiments were conducted. Mice were placed individually in acrylic cages (13 × 9 × 35 cm) composed of four compartments for at least 1 h for acclimation. Immediately after intradermal injection of IL-31 or the vehicle, the mice were returned to the same cells, and their behavior was recorded using a digital video camera (HDC-TM25, Panasonic CO., Osaka, Japan) for 1 h with personnel kept out of the observation room. The digital recording was reviewed to count the number of injection site scratches by the hind paws. A series of scratching movements for about 1 s was considered as one bout of scratching [20].

Primary cultures of murine keratinocytes

The skin obtained from neonatal mice was treated with 0.05% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) in serum-free MCDB 153 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 0.67% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Dojindo, Kumamoto, Japan), 0.12% sodium bicarbonate (Wako Pure Chemical Industries Ltd., Osaka, Japan), 0.01% penicillin G (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), and 0.006% kanamycin (Wako Pure Chemical Industries Ltd.) at 4 °C overnight. The epidermal sheet was removed from the dermis. The serum-free MCDB 153 medium including the epidermal sheets

were gently shaken and the keratinocytes were dissociated. The dissociated keratinocytes were cultured in the keratinocyte growth medium (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) on a collagen-coated 24–well plate, 6-cm-diameter plate, or glass bottom culture plate.

Immunostaining

Mice were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal) and were transcardially perfused with PBS and then with 4% paraformaldehyde (PFA). The fixed skin was immersed in 30% sucrose solution for more than 2 days. The tissue was embedded in Tissue-Tek1 O.C.T. Compound (Sakura Fineteck Co., Ltd., Tokyo, Japan) and the frozen samples were sectioned at 10 μm with a cryostat (Leica, Wetzlar, Germany). With respect to the primary cultures of murine keratinocytes, the cells were washed with PBS and fixed with 4% PFA. The skin sections and keratinocytes were washed with PBS and then treated with Protein Block® (DAKO Co., Hamburg, Germany) followed by 0.3% Triton X-100 in PBS. These samples were treated with rabbit anti-TXSyn antibody (1:500, Cayman Chemical, Ann Arbor, MI, USA) [19] and goat anti-IL-31 receptor A (IL-31RA) antibody (1:500, R&D Systems, Inc., Minneapolis, MN, USA) [14] for absorption at 4 °C overnight. As negative control, normal

rabbit IgG (Whole molecule) and normal goat IgG (Whole molecule) (Wako Pure Chemical., Osaka, Japan) were used for 1st antibody. After washing with PBS, the preparations were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (1:1000, Life Technologies, Carlsbad, CA, USA) and Cy3-conjugated antigoat-IgG (1:1000, J Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature. Fluorescence signals were observed using a fluorescence microscope (BX-61/DP70, Olympus, Tokyo, Japan).

Reverse transcription and polymerase chain reaction

The total RNA from the primary cultures of mouse keratinocytes was extracted using TRIzol reagent® (Invitrogen Co., Carlsbad, CA, USA) and treated with DNase I (Takara Bio Inc., Otsu, Japan). The total RNA (1 μg) was reverse transcribed using oligo $(dT)_{16}$ primer and Reverscript III® (Wako Pure Chemical Industries Ltd.). cDNA was amplified using GoTaq® Flexi DNA Polymerase (Promega Co., Madison, WI, USA) and the following primer pairs: TXSyn (sense) 5'-ccctgtcctcttctgagtgc-3'; TXSyn (anti-sense) 5'-gcctctgctgtgaacctttc-3', IL-31RA (sense) 5'-tgctgctatggcatgaagtc-3'; IL-31RA (antisense) 5'-tcaagtgccaacaagacagc-3', OSMRβ (sense) 5'-taaaccaggcgtccgttac-3'; OSMRβ (antisense) 5'-aaggttttccacggtgagtg-3', glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense) 5'-ccaaggtcatccatgacaac-3';

GAPDH (antisense) 5'-ttactccttggaggccatgt-3'. The reaction product was separated on a 2% agarose gel and stained with ethidium bromide.

Enzyme immunoassay for TXB_2

Mice were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal) and were transcardially perfused with PBS. The skin (8 mm in diameter) punched out was immediately shredded with scissors. The shredded skin sample was added to 1 mL of ice-chilled ethanol containing indomethacin (10 μM) and zileuton (10 μM) to inhibit cyclooxygenase and 5-lipoxygenase, respectively. After homogenization and centrifugation (600 × *g* at 4 °C for 10 min), the supernatant (0.8 mL) was diluted with 5 mL of distilled water (pH3.0). The diluted supernatant was applied to a C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA) equilibrated with methanol. The cartridges were washed with distilled water and then hexane. The lipids were eluted with ethanol and the elution was evaporated. The residue was resuspended with an enzyme immunoassay buffer (Cayman Chemical, Ann Arbor, MI, USA) for the TXB_2 assay. The amount of TXB_2 was determined using an enzyme immunoassay kit (Cayman Chemical). To measure the concentration of TXB_2 in mouse keratinocyte growth medium (CnT-Prime basal medium; CELLnTEC Advanced Cell Systems AG) in a 24-well plate, the medium for the TXB_2

assay was obtained 20 min after IL-31 administration. Similar to the situation for the skin samples, the concentration of TXB_2 in the culture medium was measured using the enzyme immunoassay kit (Cayman Chemical). Cultured keratinocytes were washed with PBS and treated with 1% Triton X-100 to solubilize the cell proteins. The protein concentration was determined using a Bio-Rad Protein Assay Kit (Hercules, CA, USA). The concentration of TXB_2 in the skin and the medium was normalized to a piece of skin (8 mm in diameter) and the amount of protein in keratinocytes, respectively.

Measurement of intracellular Ca^{2+} concentration

Primary cultures of murine keratinocytes were incubated with 10 μM fluo-3/AM (Dojindo, Kumamoto, Japan) in Opti-MEM® (Life Technologies) containing 0.05% poloxamer (Calbiochem, Dermstadt, Germany) at 37 °C for 30 min. The cells were washed with the buffer [115 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 0.4 mM KH_2PO_4, 0.4 mM Na_2HPO_4, 20 mM HEPES, 10 mM glucose, pH 7.4] for calcium imaging. Intracellular Ca^{2+} concentration was measured fluorometrically at an excitation of 488 nm and an emission between 515–545 nm using a laser-scanning microscope system (MiCAM02, Brainvision Inc., Tokyo, Japan).

Statistical analysis

Data were presented as means ± SEM. Statistical significance was analyzed using one-way analysis of variance followed by a *post-hoc* Holm–Šidák test. $p < 0.05$ was considered statistically significant.

Results

Effects of TP thromboxane receptor antagonist on IL-31-induced scratching in mice

Our previous study has shown that an intradermal injection of IL-31 at doses of 10 to 100 ng per site elicits dose-dependently hind-paw scratching in mice [14]. Thus, in this study, we used the dose of 100 ng/site of IL-31.

An intradermal injection of IL-31 (100 ng/site) significantly increased the amount of scratching in mice, compared with the vehicle control (Fig. 1). The TP receptor antagonist DCHCH (30 and 100 mg/kg) [19] significantly inhibited IL-31-induced scratching (Fig. 1). Additionally, DCHCH (100 mg/kg) did not affect scratching induced by vehicle for IL-31 (Fig.

1).

Distribution of thromboxane synthase (TXSyn) and IL-31 receptor A (IL-31RA) in murine skin

The immunoreactivities of TXSyn and IL-31RA were mainly localized in the epidermis (Fig. 2a). The immunoreactivity of IL-31RA was also observed the fibrous-like immunoreactivity in the dermis, but not in the epidermis (Fig. 2a). In negative control using non-specific IgG, any fluorescent signal was not shown (Fig. 2a). In primary cultures of dermal fibroblasts, OSMRβ mRNA, but not TXSyn mRNA and IL-31RA mRNA, were expressed (Fig. 2b).

IL-31 induced production of TXA_2 in murine skin

An intradermal injection of IL-31 (100 ng/site) induced TXA_2 production, as determined by an increase in TXB_2 concentration, in murine skin (Fig. 3). The production of TXA_2 induced by IL-31 was inhibited by the TXSyn inhibitor sodium ozagrel (10 and100 mg/kg) [21] (Fig. 3). Moreover, sodium ozagrel (100 mg/kg) did not affect TXA_2 production in the skin treated with vehicle for IL-31 (Fig 3).

Effect of sodium ozagrel on IL-31-induced scratching

An intradermal injection of IL-31 (100 ng/site) significantly increased the amount of scratching in mice, compared with the vehicle control (Fig. 4). The TXSyn inhibitor sodium ozagrel (100 mg/kg) was enhanced the scratching (Fig. 4). Additionally, sodium ozagrel (100 mg/kg) did not affect scratching induced by vehicle for IL-31 (Fig. 4).

IL-31 induced production of TXA_2 in primary cultures of murine keratinocytes

TXSyn and IL-31 receptor (IL-31RA and OSMRβ) mRNA were expressed in primary cultures of mouse keratinocytes (Fig. 5a). In addition, the immunoreactivities of both TXSyn and IL-31RA were observed in mouse keratinocytes (Fig. 5b). IL-31 (2 μg/ml) significantly increased the production of TXA_2, as determined by an increase in TXB_2 concentration, in mouse keratinocytes (Fig. 5c). IL-31-induced TXA_2 production was inhibited significantly by TXSyn inhibitor sodium ozagrel (10 μM) [22] and Ca^{2+} ion chelator EGTA (1 mM) [23]. In addition, anti-IL-31RA antibody (10 μg/mL) [14] significantly inhibited IL-31-induced TXA_2 production (Fig. 5c). Since IL-31-induced TXA_2 production was inhibited by EGTA, it was examined whether IL-31 increased the concentration of intracellular Ca^{2+} ions. IL-31 (2 μg/mL) significantly increased the concentration of intracellular Ca^{2+} ions in mouse keratinocytes,

compared with the vehicle control (Fig. 5d). EGTA (1 mM) inhibited the concentration of intracellular Ca^{2+} ions induced by IL-31 (Fig. 5d).

Discussion

An intradermal injection of IL-31 elicited scratching, an itch-related response, and increased cutaneous TXA_2 production in mice (Fig. 1 and 3). IL-31-induced scratching was inhibited by TP receptor antagonist DCHCH (Fig. 1). IL-31 also increased TXA_2 production in murine skin (Fig. 3). Therefore, these findings suggest that TXA_2 plays an important role in IL-31-induced scratching.

TXSyn is a key enzyme involved in the production of TXA_2 [24]. In the skin, TXSyn was mainly expressed in epidermal keratinocytes (Fig. 2). The distribution of TXSyn in this study is supported by our previous reports [18,19]. We also detected TXSyn in primary cultures of murine keratinocytes (Fig. 5). Although cutaneous mast cells also expressed TXSyn and induced TXA_2 production via an immunological stimulation, the production is very low, compared with other arachidonic acid metabolites, such as prostaglandin (PG) D_2 and leukotriene (LT) C_4 [25]. In addition, we did not observe both TXSyn (Fig. 2) in the dermis and TXSyn mRNA in

fibroblasts (Fig. 2b). Taken together, the results suggest that TXA_2 is mainly produced in epidermal keratinocytes in the skin.

IL-31 binds to a heterodimer composed of IL-31RA and OSMRβ [2]. In this study, IL-31RA was mainly expressed in the epidermal keratinocytes of the skin (Fig. 2a). In addition, IL-31RA protein expression and the expression of IL-31 receptors (IL-31RA and OSMRβ) mRNA was demonstrated in primary cultures of murine keratinocytes (Fig. 5). This coincides with several reports that have shown that IL-31 acts on keratinocytes [2,14,26]. Our previous report has also shown that IL-31 receptors are expressed in primary afferents, based on the fact that the receptors were expressed in murine skin and that their mRNA was detected in primary cultures of murine dorsal root ganglia [14]. Thus, in this study, our results suggested that the fibrous forms showing immunoreactivity for IL-31RA in the dermis were primary afferents. Transient Receptor Potential Vanilloid 1 (TRPV1) -positive C-fibers are involved in scratching induced by pruritogens [27,28] and allergies [29]. IL-31 elicits scratching through IL-31RA(+)/TRPV1(+) neurons [12]. Thus, a part of IL-31 may act directly to primary afferents in the skin. Mast cells are wildly distributed in the skin and are involved in itch. It has been reported that mast cells express IL-31 receptor [2]. However, we did not detect immunoreactivity of IL-31RA in cutaneous mast cells and IL-31 elicited scratching in mast cell-deficient mice [14]. Furthermore, IL-31-induced scratching was not inhibited by H_1 histamine receptor antagonist [14]. Therefore,

the role of mast cells may be small on IL-31-induced scratching. In addition, there are fibroblasts in dermis. Fibroblasts are known to express OSMRβ [30], and primary cultures of mouse fibroblasts expressed OSMRβ mRNA, but not IL-31RA mRNA (Fig. 2b). Taken together, it is suggested that IL-31 acts mainly keratinocytes and primary afferents.

TXSyn and IL-31 receptors were co-localized in epidermal keratinocytes and IL-31 increased TXA_2 production, which was inhibited by the TXSyn inhibitor sodium ozagrel and anti-IL-31RA antibody (Fig. 5). Thus, it is suggested that keratinocytes are the main TXA_2 producing cells in the skin. The mechanisms underlying IL-31-induced TXA_2 production in keratinocytes remain unclear, but may stem from signaling through one of the Il-31-related pathways (i.e., the PI3K/AKT, Jak/STAT, and MAPK pathways) [31]. PGH_2 is a substrate of TXSyn for the production of TXA_2, and is produced through the oxidation of arachidonic acid catalyzed by cyclooxygenase. Arachidonic acid is produced by phospholipase A_2, which is activated by an increase in intracellular Ca^{2+} ions [32]. In this study, IL-31 increased intracellular Ca^{2+} ions in primary cultures of murine keratinocytes, and this effect was inhibited by the Ca^{2+} ion chelator EGTA (Fig. 5). In addition, IL-31-induced TXA_2 production was also inhibited by EGTA. Thus, as a possible mechanism, IL-31-induced Ca^{2+} influx may be involved in the production of TXA_2.

TP receptors are expressed in primary afferent neurons [18,19]. In primary cultures of

dorsal root ganglion neurons, TP receptors are expressed in small-size neurons [18], which are characterized by unmyelinated C-fibers [33,34]. C-fibers play an important role in itch transmission [35,36]. An intradermal injection of TXA_2 analogue U-46619 elicits itch-related responses in mice, but not in TP receptor-deficient mice [18]. U-46619 also increases intracellular Ca^{2+} ions through TP receptors and L-type calcium channel in primary cultures of dorsal root ganglion neurons [18], suggesting that the activation of TP receptors induces membrane depolarization [37]. These findings suggest that TXA_2 released from keratinocytes acts directly on primary afferent neurons and elicits scratching.

In this study, the TXSyn inhibitor sodium ozagrel inhibited IL-31-induced TXA_2 production in the skin and primary cultures of murine keratinocytes. However, sodium ozagrel did not inhibit IL-31-induced scratching but rather enhanced scratching (Fig. 4). TXA_2 is produced by metabolizing PGH_2 with TXSyn. PGH_2 is also a substrate for PGE_2 synthase. It is well known that PGE_2 is an itch mediator and enhancer [38]. Thus, the inhibition of TXSyn may increase PGE_2 production and enhance scratching. In our preliminary experiments using a mouse model of atopic dermatitis, sodium ozagrel inhibited cutaneous TXA_2 production, but enhanced spontaneous scratching (data not shown). Furthermore, a similar phenomenon was observed when a cyclooxygenase inhibitor was used. LTB_4, which is produced when 5-lipoxygenase metabolizes arachidonic acid, is a potent pruritogen [39]. Substance P-induced

scratching is inhibited by glucocorticoids, phospholipase A_2 inhibitor, LTB_4 receptor antagonist and 5-lipoxygenase inhibitor, but is enhanced by cyclooxygenase inhibitors [40]. This phenomenon may be due to increased LTB_4 production by the inhibition of cyclooxygenase. Taken together, it appears that a receptor antagonist rather than an enzyme inhibitor is more suitable for regulation of the itching response involving arachidonic acid metabolites.

In conclusion, IL-31 elicits an itch-associated response not only through direct action on primary sensory neurons, but also through TXA_2 production in keratinocytes.

Conflicts of interest

None of the authors have any conflicts of interest to declare.

References

[1] Dillon SR, Sprecher C, Hammond A, Bilsborough J, Rosenfeld-Franklin M, Presnell SR, et al. Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. Nat Immunol 2004;5(7):752–60.

[2] Cornelissen C, Lüscher-Firzlaff J, Baron JM, Lüscher B. Signaling by IL-31 and functional consequences. Eur J Cell Biol 2012;91(6-7):552–66.

[3] Rabenhorst A, Hartmann K. Interleukin-31: a novel diagnostic marker of allergic diseases. Curr Allergy Asthma Rep 2014;14(4):423.

[4] Bilsborough J, Leung DY, Maurer M, Howell M, Boguniewicz M, Yao L, et al. IL-31 is associated with cutaneous lymphocyte antigen-positive skin homing T cells in patients with atopic dermatitis. J Allergy Clin Immunol 2006;117(2):418–25.

[5] Sonkoly E, Muller A, Lauerma AI, Pivarcsi A, Soto H, Kemeny L, et al. IL-31: a new link between T cells and pruritus in atopic skin inflammation. J Allergy Clin Immunol 2006;117(2): 411–7.

[6] Takaoka A, Arai I, Sugimoto M, Yamaguchi A, Tanaka M, Nakaike S. Expression of IL-31 gene transcripts in NC/Nga mice with atopic dermatitis. Eur J Pharmacol 2005;516(2):180–1.

[7] Nemoto O, Furue M, Nakagawa H, Shiramoto M, Hanada R, Matsuki S, et al. The first trial of CIM331, a humanized antihuman interleukin-31 receptor A antibody, in healthy volunteers and patients with atopic dermatitis to evaluate safety, tolerability and pharmacokinetics of a single dose in a randomized, double-blind, placebo-controlled study. Br J Dermatol 2016;174(2):296–304.

[8] Grimstad O, Sawanobori Y, Vestergaard C, Bilsborough J, Olsen UB, Grønhøj-Larsen C, et al. Anti-interleukin-31-antibodies ameliorate scratching behaviour in NC/Nga mice: a model of atopic dermatitis. Exp Dermatol 2009;18(1):35–43.

[9] Kasutani K, Fujii E, Ohyama S, Adachi H, Hasegawa M, Kitamura H, et al. Anti-IL-31 receptor antibody is shown to be a potential therapeutic option for treating itch and dermatitis in mice. Br J Pharmacol 2014;171(22):5049–58.

[10] Michels GM, Walsh KF, Kryda KA, Mahabir SP, Walters RR, Hoevers JD, et al. A blinded, randomized, placebo-controlled trial of the safety of lokivetmab (ZTS-00103289), a caninized anti-canine IL-31 monoclonal antibody in client-owned dogs with atopic dermatitis. Vet Dermatol 2016;27(6):505–e136.

[11] Arai I, Tsuji M, Takeda H, Akiyama N, Saito S. A single dose of interleukin-31 (IL-31) causes continuous itch-associated scratching behaviour in mice. Exp Dermatol 2013;22(10):669–71.

[12] Cevikbas F, Wang X, Akiyama T, Kempkes C, Savinko T, Antal A, et al. A sensory neuron-expressed IL-31 receptor mediates T helper cell-dependent itch: Involvement of TRPV1 and TRPA1. J Allergy Clin Immunol 2014;133(2):448–60.

[13] Oyama S, Kitamura H, Kuramochi T, Higuchi Y, Matsushita H, Suzuki T, et al. Cynomolgus monkey model of interleukin-31-induced scratching depicts blockade of human interleukin-31 receptor A by a humanized monoclonal antibody. Exp Dermatol. 2016. doi: 10.1111/exd.13236.

[14] Andoh T, Harada A, Kuraishi Y. Involvement of leukotriene B_4 released from keratinocytes in itch-associated response to intradermal interleukin-31 in mice. Acta Derma Venereol 2017; DOI: 10.2340/00015555-2697

[15] Lewis KE, Holdren MS, Maurer MF, Underwood S, Meengs B, Julien SH, et al. Interleukin (IL) 31 induces in cynomolgus monkeys a rapid and intense itch response that can be inhibited by an IL-31 neutralizing antibody. J Eur Acad Dermatol Venereol 2017;31(1):142–50.

[16] Hawro T, Saluja R, Weller K, Altrichter S, Metz M, Maurer M. Interleukin-31 does not induce immediate itch in atopic dermatitis patients and healthy controls after skin challenge. Allergy 2014;69(1):113–7.

[17] Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and

functions. Physiol Rev 1999;79(4):1193–226.

[18] Andoh T, Nishikawa Y, Yamaguchi-Miyamoto T, Nojima H, Narumiya S, Kuraishi Y. Thromboxane A_2 induces itch-associated responses through TP receptors in the skin in mice. J Invest Dermatol 2007;127(8):2042–7.

[19] Andoh T, Yamamoto A, Haza S, Yuhki K, Ushikubi F, Narumiya S, et al. Thromboxane A_2 is involved in itch-associated responses in mice with atopic dermatitis-like skin lesions. Acta Derma Venereol 2016;96(7):899–904.

[20] Kuraishi Y, Nagasawa T, Hayashi K, Satoh M. Scratching behavior induced by pruritogenic but not algesiogenic agents in mice. Eur J Pharmacol 1995;275(3):229–33.

[21] Shinagawa K, Kojima M, Ichikawa K, Hiratochi M, Aoyagi S, Akahane M. Participation of thromboxane A(2) in the cough response in guinea-pigs: antitussive effect of ozagrel. Br J Pharmacol 2000;131(2):266–70.

[22] Matsunobu T, Okuno T, Yokoyama C, Yokomizo T. Thromboxane A synthase-independent production of 12-hydroxyheptadecatrienoic acid, a BLT2 ligand. J Lipid Res 2013;54(11):2979–87.

[23] Qu W, Ikejima K, Zhong Z, Waalkes MP, Thurman RG. Glycine blocks the increase in intracellular free Ca2+ due to vasoactive mediators in hepatic parenchymal cells. Am J Physiol Gastrointest Liver Physiol 2002;283(6):G1249–56.

[24] Needleman P, Moncada S, Bunting S, Vane JR, Hamberg M, Samuelsson B. Identification of an enzyme in platelet microsomes which generates thromboxane A_2 from prostaglandin endoperoxides. Nature 1976;261(5561):558–60.

[25] Mita H, Ishii T, Akiyama K. Generation of thromboxane A_2 from highly purified human sinus mast cells after immunological stimulation. Prostaglandins Leukot Essent Fatty Acids 1999;60(3):175–80.

[26] Kasraie S, Niebuhr M, Baumert K, Werfel T. Functional effects of interleukin 31 in human primary keratinocytes. Allergy 2011;66(7):845–52.

[27] Andoh T, Nagasawa T, Satoh M, Kuraishi Y. Substance P induction of itch-associated response mediated by cutaneous NK1 tachykinin receptors in mice. J Pharmacol Exp Ther 1998;286(3):1140–5.

[28] Yamaguchi T, Nagasawa T, Satoh M, Kuraishi Y. Itch-associated response induced by intradermal serotonin through 5-HT2 receptors in mice. Neurosci Res 1999;35(2):77–83.

[29] Nakano T, Andoh T, Sasaki A, Nojima H, Kuraishi Y. Different roles of capsaicin-sensitive and H1 histamine receptor-expressing sensory neurons in itch of mosquito allergy in mice. Acta Derm Venereol 2008;88(5):449–54.

[30] Blanchard F, Wang Y, Kinzie E, Duplomb L, Godard A, Baumann H. Oncostatin M regulates the synthesis and turnover of gp130, leukemia inhibitory factor receptor alpha,

and oncostatin M receptor beta by distinct mechanisms. J Biol Chem 2001;276(50):47038–45.

[31] Zhang Q, Putheti P, Zhou Q, Liu Q, Gao W. Structures and biological functions of IL-31 and IL-31 receptors. Cytokine Growth Factor Rev 2008;19(5-6):347–56.

[32] Clark JD, Milona N, Knopf JL. Purification of a 110-kilodalton cytosolic phospholipase A_2 from the human monocytic cell line U937. Proc Natl Acad Sci USA 1990;87(19):7708–12.

[33] Harper AA, Lawson SN. Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurons. J Physiol 1985;359:31–46.

[34] Lee KH, Chung K, Chung JM, Coggeshall RE. Correlation of cell body size, axon size, and signal conduction velocity for individually labelled dorsal root ganglion cells in the cat. J Comp Neurol 1986;243(3):335–46.

[35] Handwerker HO, Forster C, Kirchhoff C. Discharge patterns of human C-fibers induced by itching and burning stimuli. J Neurophysiol 1991;66(1):307–15.

[36] Schmelz M, Schmidt R, Bickel A, Handwerker HO, Torebjörk HE. Specific C-receptors for itch in human skin. J Neurosci 1997;17(20): 8003–8.

[37] Arshad M, Vijay V, Floyd BC, Marks B, Sarabu MR, Wolin MS, et al. Thromboxane receptor stimulation suppresses guanylate cyclase-mediated relaxation of radial arteries.

Ann Thorac Surg 2006;81(6):2147–54.

[38 Fjellner B, Hägermark O. Pruritus in polycythemia vera: treatment with aspirin and possibility of platelet involvement. Acta Derm Venereol 1979;59(6):505–12.

[39] Andoh T, Kuraishi Y. Intradermal leukotriene B_4, but not prostaglandin E_2, induces itch-associated responses in mice. Eur J Pharmacol 1998;353(1):93–6.

[40] Andoh T, Katsube N, Maruyama M, Kuraishi Y. Involvement of leukotriene B_4 in substance P-induced itch-associated response in mice. J Invest Dermatol 2001;117(6):1621–6.

Figure legends

Fig. 1. Effects of TP receptor antagonist on IL-31-induced scratching in mice. IL-31 (100 ng/site) or the vehicle (VH2) was injected intradermally. TP receptor antagonist DCHCH or the vehicle (VH1) was administered orally 60 min before IL-31 or VH2 injection. Data are presented as mean ± SEM. (n = 8). *$p < 0.05$ (Holm–Šidák test).

Fig. 2. Distribution of thromboxane synthase (TXSyn) and IL-31 receptor A (IL-31RA) in mouse skin. (a) The typical examples of the distribution of TXSyn and IL-31RA in the mouse skin. TXSyn (green) and IL-31RA (red) were immunostained in the rostral back skin. Right panels show negative control using non-specific IgG for rabbit or goat. Nuclei were counterstained in blue with DAPI. Scale bar: 50 μm. (b) Expression of thromboxane synthase (TXSyn), IL-31 receptor A (IL-31RA), oncostatin M receptor β (OSMRβ), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mouse fibroblasts. Typical examples of the bands of TXSyn, IL-31RA, OSMRβ, and GAPDH mRNAs are shown. These mRNAs were detected by RT-PCR.

Fig. 3. IL-31-induced thromboxane (TX) A_2 production in the skin. Since TXA_2 is altered

spontaneously to form inactive TXB_2, the TXB_2 content in the assay medium was measured by an enzyme immunoassay. IL-31 (100 ng/site) or the vehicle (VH2) was injected intradermally. Sodium ozagrel (OZG: 10 and 100 mg/kg) and the vehicle (VH1) were administered orally 60 min before IL-31 or VH2 injection. The concentration of TXB_2 in the tested medium was determined 30 min after IL-31 application. Data are presented as mean ± SEM. (n = 6-7). *p < 0.05 (Holm–Šidák test).

Fig. 4. Effect of sodium ozagrel on IL-31-induced scratching. IL-31 (100 ng/site) or the vehicle (VH2) was injected intradermally. Sodium ozagrel (OZG: 100 mg/kg) and the vehicle (VH1) were administered orally 60 min before IL-31 or VH2 injection. Data are presented as mean ± SEM, (n = 6). *p < 0.05 (Holm–Šidák test).

Fig. 5. IL-31-induced thromboxane (TX) A_2 production in primary cultures of mouse keratinocytes. (a) Expression of thromboxane synthase (TXSyn), IL-31 receptor A (IL-31RA), oncostatin M receptor β (OSMRβ) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mouse keratinocytes. Typical examples of the bands of TXSyn, IL-31RA, OSMRβ and GAPDH mRNAs are shown here. These mRNA were detected by RT-PCR. (b) TXSyn and IL-31RA immunoreactivity in mouse keratinocytes. Scale bar: 100 μm. (c) IL-31-induced TXA_2

production in mouse keratinocytes. Mouse keratinocytes were treated with sodium ozagrel (OZG: 10 μM), EGTA (1 mM), the vehicle (VH1), non-specific IgG (nIgG: 10 μg/mL) and anti-IL-31RA (IL-31RAab: 10 μg/mL), and were treated 30 min before IL-31 (2 μg/mL) or VH2 exposure. The culture medium was collected 10 min after IL-31 or VH2 treatment. Since TXA_2 is altered spontaneously to form inactive TXB_2, the TXB_2 content in the assay medium was measured by an enzyme immunoassay. The TXB_2 content was normalized by the protein extracted form keratinocytes. Data are presented as mean ± SEM. (n = 6). *$p < 0.05$ (Holm–Šidák test). (d) Increase in intracellular Ca^{2+} ions in mouse keratinocytes treated with IL-31. IL-31 (2 μg/mL) or the vehicle (VH) was applied to mouse keratinocytes and the concentration of intracellular Ca^{2+} ions was measured using a laser-scanning microscope system (see Materials and methods). EGTA (1 mM) was applied 5 min before IL-31 application. The left panel shows a typical example of a time-course of IL-31- or VH-induced changes in the concentration of intracellular Ca^{2+} ions (fluorescence) in mouse keratinocytes, and the right panel shows maximum changes in the increase of intracellular Ca^{2+} ions. Data are presented as mean ± SEM, (n = 15 cells). *$p < 0.05$ (Holm–Šidák test). These experiments were repeated more than three times, with similar results.

Figure 1

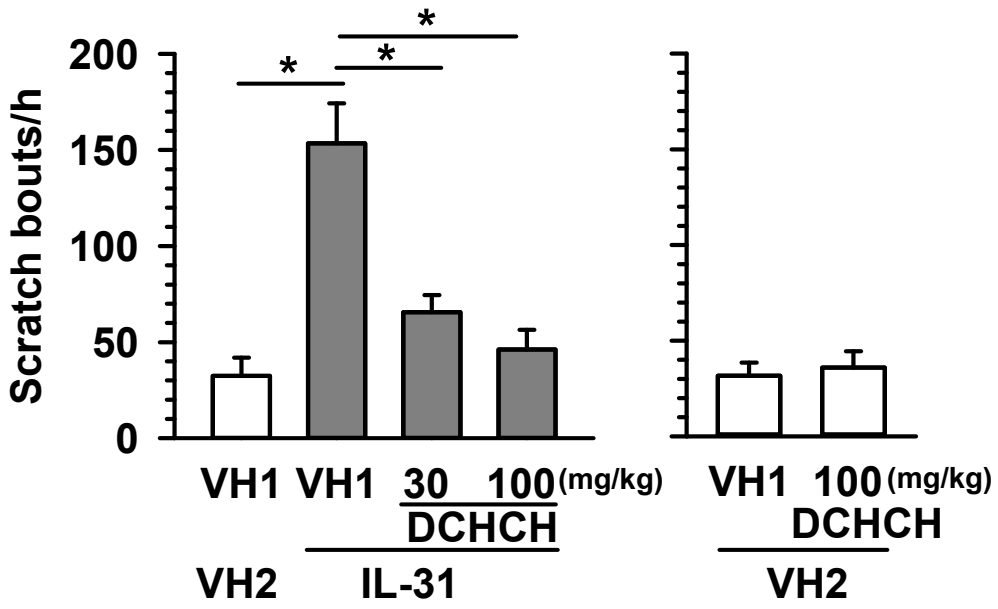

Scratch bouts/h
200
150
100
50
0
*
*
*
VH1
VH1
30
100
(mg/kg)
DCHCH
VH2
IL-31
VH1
100
(mg/kg)
DCHCH
VH2

Figure 2

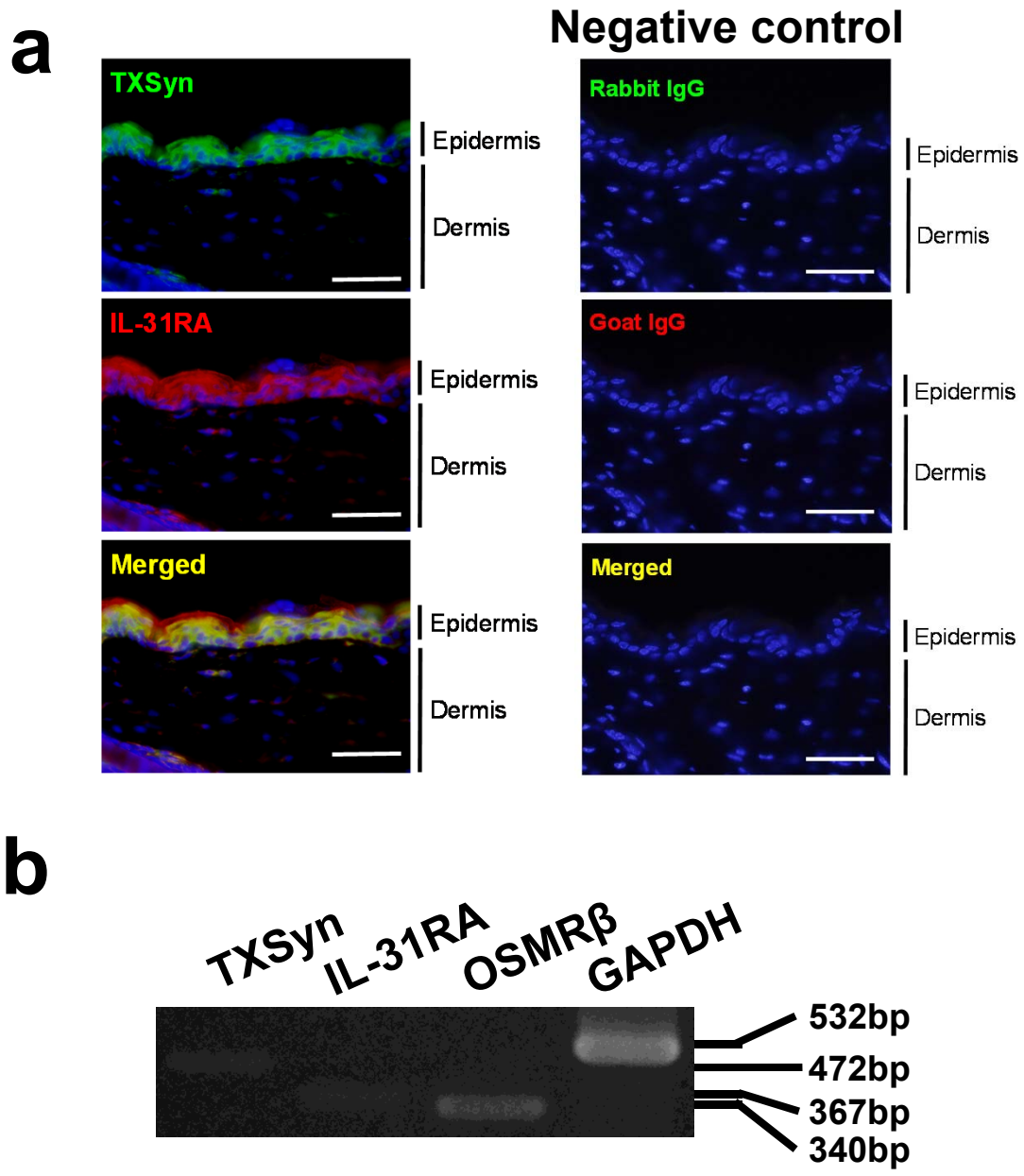

a
Negative control
TXSyn
Rabbit IgG
IL-31RA
Goat IgG
Merged
Merged
Epidermis
Dermis
b
TXSyn
IL-31RA
OSMRβ
GAPDH
532bp
472bp
367bp
340bp

Figure 3

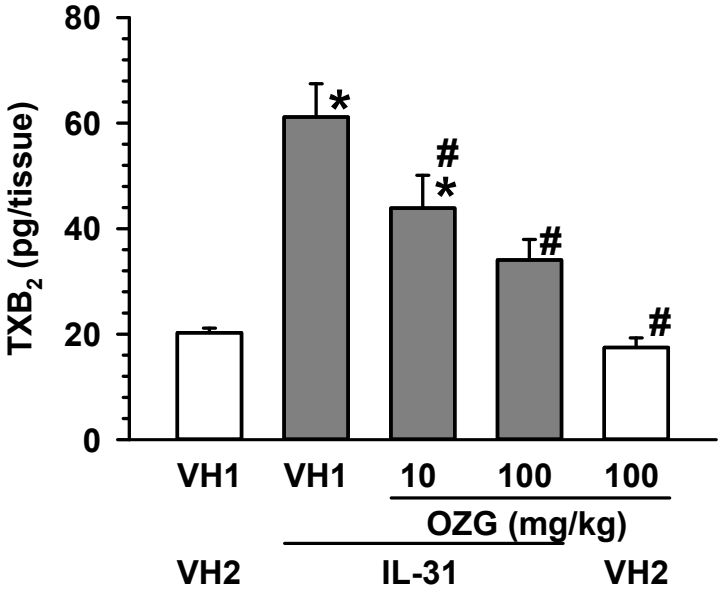

80
60
40
20
0
TXB2 (pg/tissue)
*
#
*
#
#
VH1
VH1
10
100
100
OZG (mg/kg)
VH2
IL-31
VH2

Figure 4

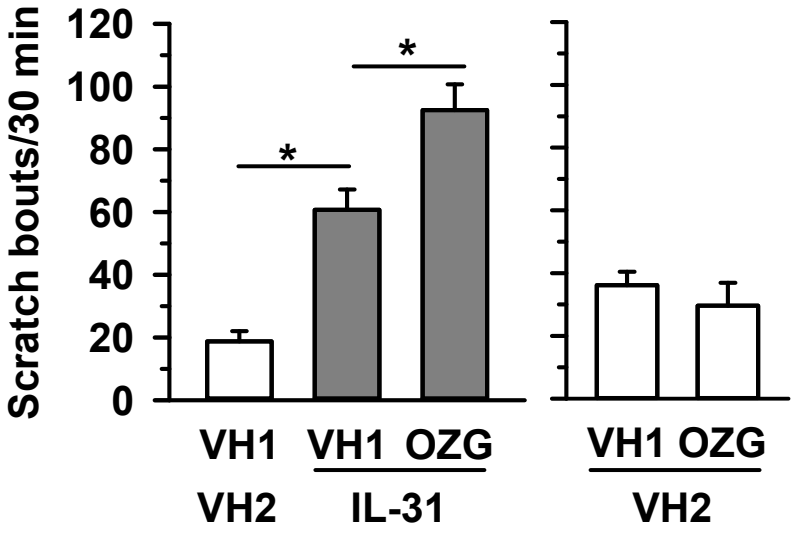
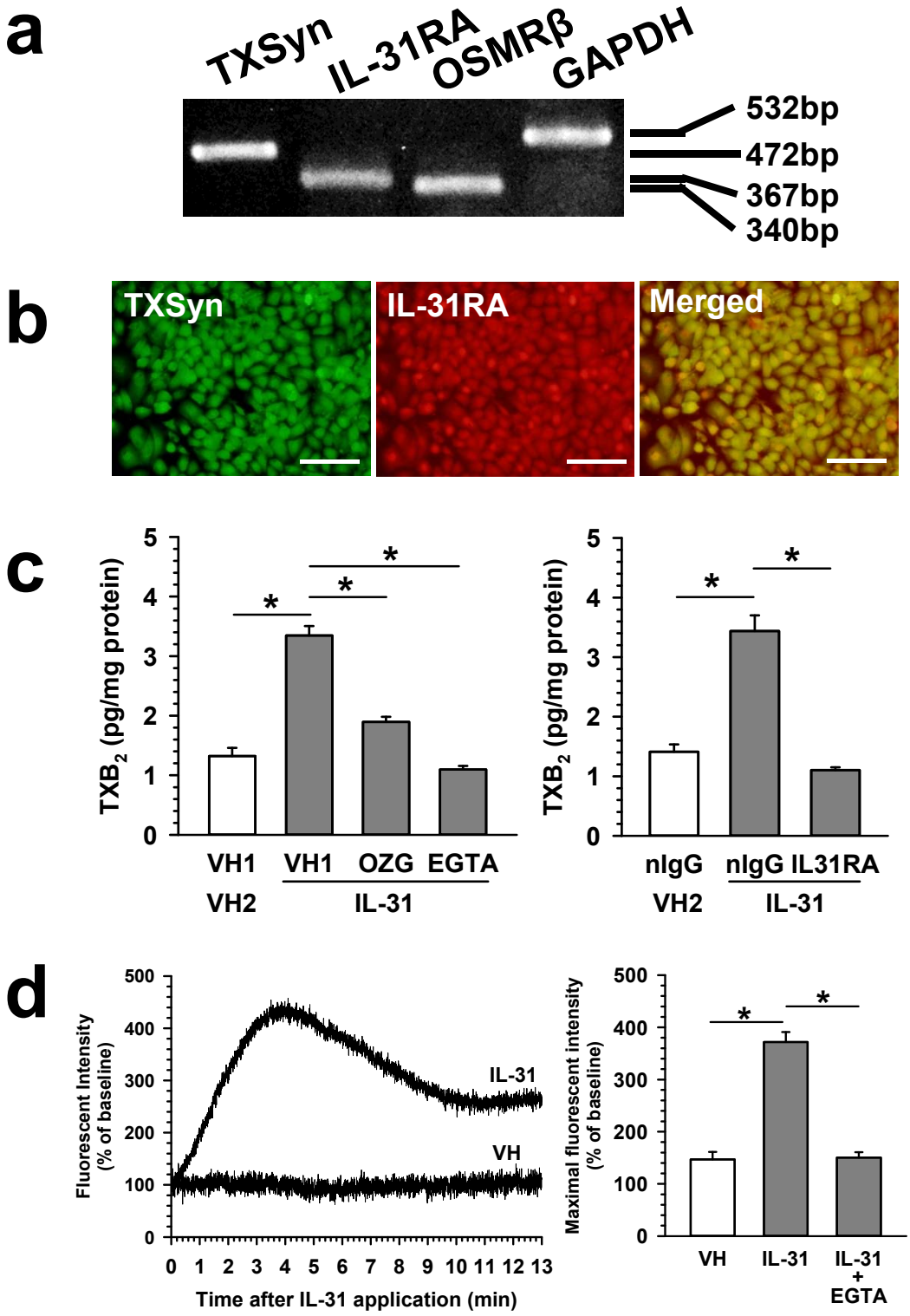

Scratch bouts/30 min
120
100
80
60
40
20
0
*
*
VH1
VH2
VH1 OZG
IL-31
VH1 OZG
VH2

Figure 5


a
TXSyn
IL-31RA
OSMRβ
GAPDH
532bp
472bp
367bp
340bp
b
TXSyn
IL-31RA
Merged
c
TXB2 (pg/mg protein)
VH1
VH1
OZG
EGTA
VH2
IL-31
nIgG
nIgG
IL31RA
VH2
IL-31
d
Fluorescent Intensity (% of baseline)
IL-31
VH
Time after IL-31 application (min)
Maximal fluorescent intensity (% of baseline)
VH
IL-31
IL-31 + EGTA