ORIGINAL ARTICLE

Role of SNAREs in Atopic Dermatitis–Related Cytokine Secretion and Skin-Nerve Communication

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The role of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors in atopic dermatitis (AD) is unknown. This study identifies the function of soluble *N*-ethylmaleimide sensitive factor attachment protein receptor in AD-related cytokine secretion and epidermis-nerve communication. Herein, we report that various cytokines were simultaneously upregulated and coreleased in innate immunity–activated primary human keratinocytes. AD-related cytokines thymic stromal lymphopoietin, endothelin-1, and inflammatory tumor necrosis factor-α activated distinct but overlapping sensory neurons. Tumor necrosis factor-α potentiated thymic stromal lymphopoietin–induced Ca^{2+}-influx, whereas endothelin-1 caused itch-selective B-type natriuretic peptide release. In primary human keratinocytes, B-type natriuretic peptide upregulated genes promoting dermatological and neuroinflammatory diseases and conditions. VAMP3, SNAP-29, and syntaxin 4 proved important in driving cytokine release from primary human keratinocytes. Depletion of VAMP3 inhibited nearly all the cytokine release including thymic stromal lymphopoietin and endothelin-1. Accordingly, VAMP3 co-occurred with endothelin-1 in the skins of patients with AD. Our study pinpoints the pivotal role of soluble *N*-ethylmaleimide sensitive factor attachment protein receptors in mediating cytokine secretion related to AD. VAMP3 is identified as a suitable target for developing broad-spectrum anticytokine therapeutics for controlling itch and atopic skin inflammation.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin condition with eczematous lesions, pruritus, sometimes prurigo, and dry skin (Steinhoff et al., 2012; Weidinger and Novak, 2016; Weidinger et al., 2018). Because the prevalence of AD has increased steadily, investigating the cause of this chronic skin disease and developing new treatment strategies continue to be crucial (Boguniewicz and Leung, 2011; Buddenkotte and Steinhoff, 2010; Silverberg, 2017). In AD, cytokine gene transcripts are highly upregulated and their release elevated (Brunner et al., 2018; Homey et al., 2006). The contribution of keratinocytes (KCs) to the pathogenesis of AD is clear by the secretion of cytokines (Pastore et al., 2006). In addition, certain cytokines can activate sensory fibers to transduce itch signals, leading to the vicious cycle of itching, scratching, and inflammation. However, the molecular mechanisms regulating neuroimmune and neuroepidermal communication in AD are still poorly understood. KC-derived thymic stromal lymphopoietin (TSLP) is a critical immune regulatory cytokine for the induction of chronic pruritus in patients with AD (Wilson et al., 2013). Endothelin-1 (ET-1) is also secreted by KCs and activates sensory neurons, immune cells, and feedback on KCs (Kido-Nakahara et al., 2014). It remains unknown whether these two important pruritogens activate communal or discrete neuronal subpopulations. Proinflammatory tumor necrosis factor-α (TNF-α) is oversecreted in AD (Behniafard et al., 2012), and its receptors have been detected on dorsal root ganglion neurons (DRGs). However, the function of TNF-α in potentiation of pruriceptive primary sensory fibers in response to other pruritogens, such as TSLP or ET-1, remains unclear.

The abnormality of the responses associated with oversecretion of cytokines from KCs in AD might be related to certain soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs; Rothman, 2014). Typically, SNAREs consist of synaptosomal-associated protein, syntaxin (STX), and vesicle-associated membrane protein, known to form complexes that mediate vesicle transport and vesicle fusion. Each of them has multiple isoforms, depending on the cell type (Meng and Wang, 2015). So far, information is

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Abbreviations: AD, atopic dermatitis; BNP, B-type natriuretic peptide; DRG, dorsal root ganglion neuron; ET-1, endothelin-1; KC, keratinocyte; KD, knockdown; phKC, primary human keratinocyte; shRNA, short hairpin RNA; SNARE, soluble N-ethylmaleimide sensitive factor attachment protein receptor; STX, syntaxin; TGN, trigeminal ganglionic sensory neuron; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α; TSLP, thymic stromal lymphopoietin

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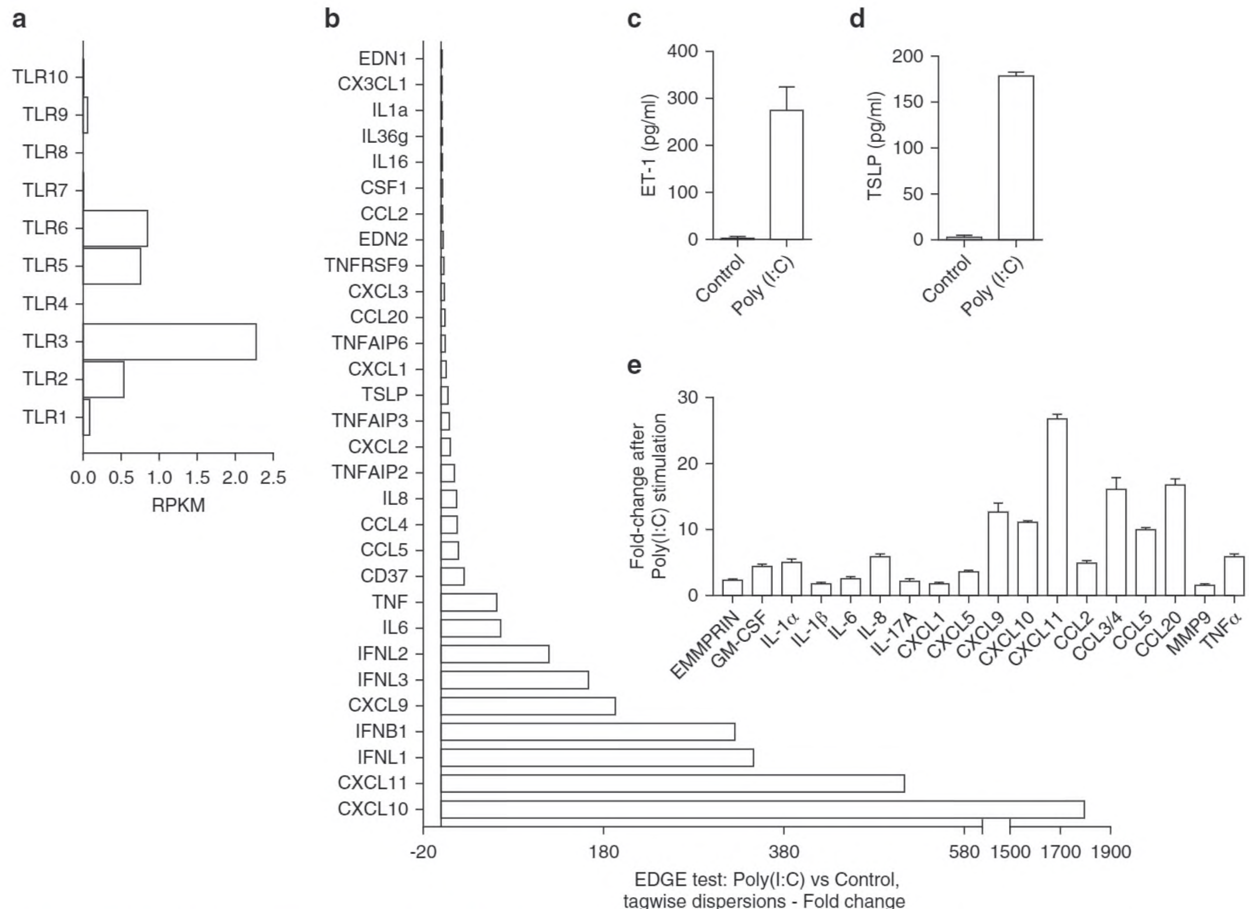

Figure 1. TLR3 activation elevated cytokine transcripts and elicited robust cytokine release in phKCs. (**a**) RNA-Seq of cultured phKCs from three different donors analyzed relative transcripts RPKM of TLR family members in unstimulated cells. (**b**) The EDGE fold-change for cytokines in Poly(I:C)-treated cells versus control. (**c, d**) ET-1 (c) or TSLP (d) content was detected in the supernatant of phKCs treated with medium (basal), alone (control), or with supplement of Poly(I:C). The amount in pg/ml of basal release was as follows: 5.67 $\pm$ 0.9 for ET-1 and 4.5 $\pm$ 0.7 for TSLP. (**e**) Cytokine antibody array was used to semiquantify the released cytokine; fold-change after Poly(I:C) stimulation (stimulated/basal) was plotted. For each graphical representation (c–e), data are presented as mean $\pm$ standard error of the mean (n $\geq$ 3). EDGE, extraction of differential gene expression; ET-1, endothelin-1; phKC, primary human keratinocyte; RPKM, reads per kilobase of exon model per million mapped reads; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α; TSLP, thymic stromal lymphopoietin.

lacking on the involvement of SNAREs in AD related to regulating cytokine release in KCs.

Innate immunity toll-like receptors (TLRs) are important players in AD-associated inflammation, skin barrier dysfunction, and pruritus (Kubo et al., 2014; Stowell et al., 2009). TLR3 upregulation contributes to dry skin which is essential for the development of chronic itch, such as in AD. TLR3 agonist, Poly(I:C), is a pruritogen that induces pruritus upon intradermal or intrathecal injection in mice (Liu et al., 2012). Activation of TLR3 serves as a useful platform for assaying SNARE function in the inflammatory cellular response. The aim of this study is to (i) investigate the release mechanisms of pruritogens or cytokines involved in the pathogenesis of AD; (ii) use RNA-Seq to identify AD-associated cytokine transcripts in primary human keratinocytes (phKCs); (iii) use calcium imaging on DRGs to validate the functional consequences of key cytokines; (iv) pinpoint the key SNAREs involved in the release of AD-associated cytokines from phKCs; (v) immunostain and image SNAREs and pruritus-related cytokines in healthy and AD skin specimens to highlight the unique properties of SNARE in AD.

RESULTS

TLR3 activation induced robust upregulation of cytokine transcripts and AD-related cytokine release

TLR3 activation in phKCs produces proinflammatory cytokines in inflammatory skin diseases, including AD (Yasuike et al., 2017); consequently, phKCs are a reasonable model to investigate the function of SNAREs in regulating AD-associated cytokine secretion. Using RNA-Seq, the functional significance of TLR3 was confirmed on cultured phKCs because its relative transcript levels in reads per kilobase of exon model per million mapped reads were higher than that of other TLR isoforms (Figure 1a). Treatment with Poly(I:C) resulted in the upregulation of numerous cytokines (Figure 1b). We observed 1,800-, 64-, 21.1-, 9.5-, and 2.5-fold upregulation of CXCL10, TNF, CCL5, TSLP, and ET-1, respectively (Figure 1b). Among the 120 gene transcripts co-upregulated, 30 cytokine genes were elevated significantly ($P \leq 0.05$).

After incubation of phKCs with Poly(I:C), the coreleased cytokines in the supernatant were measured using ELISAs or cytokine array. Remarkably, Poly(I:C) elicited ~49-fold of ET-1 (Figure 1c) and ~40-fold of TSLP release (Figure 1d). Using

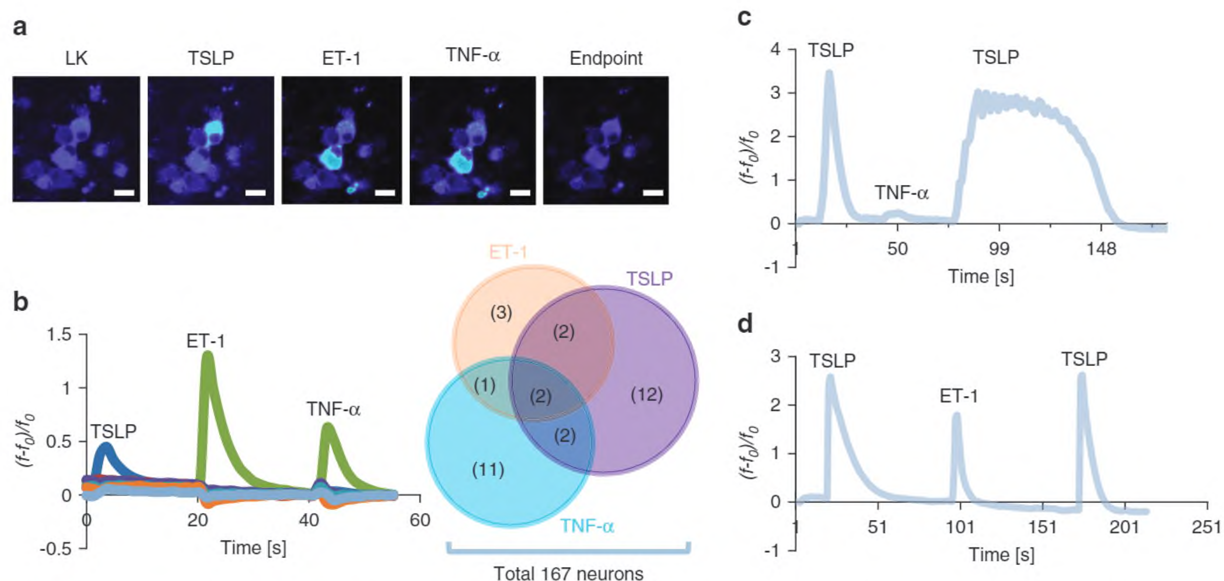

Figure 2. Cytokines cooperatively activate DRGs. (**a**) Ca^{2+}-mobilization in response to sequentially applied TSLP, ET-1, and TNF-α in DRGs showed that each cytokine activates distinct or overlapping DRG subsets. DRGs were washed between stimulations. Scale bars = 20 μm. (**b**) A representative trace of sequentially applied cytokines on the Ca^{2+} transients is shown. Venn diagram shows the number of neurons activated by each cytokine; in all, 167 neurons were recorded. (**c**) Representative trace showing that TNF-α potentiates TSLP-induced Ca^{2+}-mobilization in DRGs. (**d**) Representative trace showing that ET-1 does not influence TSLP-induced Ca^{2+}-mobilization in DRGs. DRG, dorsal root ganglion neuron; ET-1, endothelin-1; LK, low potassium basal release buffer; TNF-α, tumor necrosis factor-α; TSLP, thymic stromal lymphopoietin.

the semiquantitative cytokine array, stimulation enhanced the secretion of EMMPRIN and 17 other cytokines (Figure 1e). Among them, IL-1/-6/-8/-17A, TNF, CCL2/3/4/5, GM-CSF, and CXCL10 are related to the inflammatory response and appeared to be upregulated in patients with AD (Czarnowicki et al., 2014; Fedenko et al., 2011; Giustizieri et al., 2001; Gros et al., 2009). The function of EMMPRIN in itch is unknown, but it is involved in chronic inflammation (Yurchenko et al., 2010).

ET-1, TSLP, and TNF-α activated distinct but overlapping sensory neuronal subsets

Despite the importance of ET-1, TSLP, and TNF-α in KC-derived itch or skin inflammation, the distinct or communal biological consequences attributed to neuronal activation are still poorly understood. To address this question, mouse DRGs were treated with TSLP (20 ng/ml), ET-1 (100 nM), or TNF-α (100 ng/ml), sequentially, before being monitored using Ca^{2+}-imaging (Figure 2a). These concentrations were selected based on previously used protocols for activating skin cells and sensory neurons (Jin and Gereau, 2006; Kido-Nakahara et al., 2014; Meng et al., 2018; Rochman et al., 2018; Shin et al., 2016). After analysis of 167 total neurons with sequential application, we observed that ET-1, TNF-α, and TSLP activated 4.8%, 9.6%, and 11% of total neurons, respectively, and the responsive neurons were partially overlapping (Figure 2b and Venn diagram). Subsequently, we asked whether cytokines, such as TSLP, ET-1, or TNF-α, have additive or synergistic effects. DRGs were incubated with TSLP, followed by washout, and then brief incubation with TNF-α or ET-1, before cells were incubated with the same concentration of TSLP again (Figure 2c and d). We found that TNF-α (Figure 2c) but not ET-1 (Figure 2d) caused TSLP to give a greater calcium spike during the second incubation than the first one, suggesting that TNF-α but not ET-1 can potentiate the TSLP-mediated effects on DRGs.

ET-1 induced B-type natriuretic peptide (BNP) release from trigeminal ganglionic sensory neurons (TGNs), and BNP upregulated genes in keratinocytes associated with dermatological diseases

In a previous study, we have shown that T helper type 2−pruritic cytokine IL-31 can induce BNP release from DRGs (Meng et al., 2018). Herein, we reaffirmed that IL-31 also induced BNP release from TGNs (Figure 3a). In addition, treatment of TGNs with ET-1 caused ∼6-fold increment of BNP release over the basal level (Figure 3a). To investigate whether BNP exerts any effects on gene regulation in phKCs, we treated phKCs with 1 μM BNP for 2 hours and performed RNA-Seq. We identified that the BNP receptor NPR2 is transcribed at higher levels than NPR1 and NPR3 in unstimulated phKCs (Figure 3b), and revealed that BNP induced upregulation of many genes (Figure 3c). Canonical pathway analysis revealed that the BNP effect on phKCs is closely related to dermatological diseases and skin conditions (Figure 3d).

TSLP and ET-1 release from phKCs requires distinct SNAREs

We performed knockdown (KD) of SNARE in phKC cultures using specific short hairpin RNA (shRNA) lentiviral particles. Western blotting detected substantial reduction of V3, V8, STX3, STX4, S23, and S29 expression levels by specific shRNA compared with nontargeted shRNA controls (Figure 4a−c). phKCs did not express V1 and V2 (Figure 4a). KD of S23, V3, or STX3 expression resulted in significant reduction of Poly(I:C)-induced ET-1 release, whereas KD of S29 and STX4 only resulted in a minimal, nonsignificant reduction (Figure 4d). KD of S29 caused a more pronounced

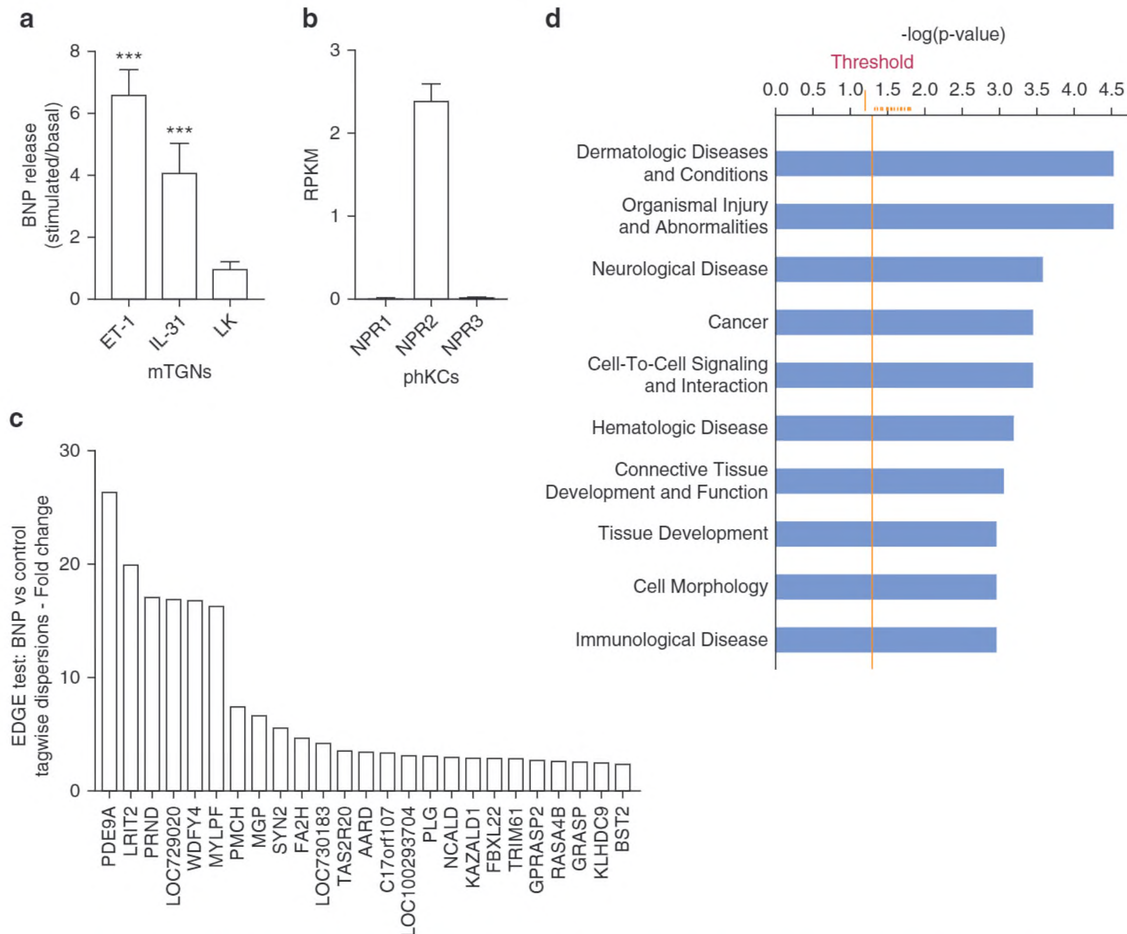

Figure 3. ET-1 and IL-31 induce BNP release from TGNs, and BNP upregulates genes in phKCs linked with dermatologic diseases/conditions. (**a**) ET-1 and IL-31 induce BNP release from cultured TGNs. Stimulated versus basal. (**b**) RNA-Seq from three donors revealed that NPR2 (BNP receptor) transcript in untreated phKCs is predominant. (**c**) The statistically significant upregulated genes after BNP treatment in phKCs were analyzed by RNA-Seq. The fold-change of RPKM for each gene was plotted; fold-change ≥2 was deemed significant. (**d**) Cellular pathways hit by BNP treatment in phKCs analyzed by IPA. For each graphical representation, data are presented as mean ± standard error of the mean (n = 3). ***$P < 0.001$. BNP, B-type natriuretic peptide; ET-1, endothelin-1; LK, low potassium basal buffer; phKC, primary human keratinocyte; RPKM, reads per kilobase of exon model per million mapped reads; TGN, trigeminal ganglionic sensory neuron.

reduction of TSLP release than KD of S23, whereas STX4 KD seemed to result in more inhibition of TSLP release than STX3 KD (Figure 4d). KD of V8 was incomplete (Figure 4a) despite various shRNA clones attempted. Partial KD of V8 did not significantly affect either TSLP or ET-1 release (Figure 4d). Furthermore, KD of V3 resulted in substantial inhibition of both TSLP (∼80%) and ET-1 (∼70%) release (Figure 4d). Thus, V3 seemed to be the most critical SNARE in mediating both ET-1 and TSLP release. In fact, double-immunostaining of ET-1 with V3 (Figure 4e, top panels) or with TSLP (Figure 4e, bottom panels) revealed that the immune signal of ET-1, TSLP, and V3 seemed to be spread out and colocated close to the edge of the plasma membrane in Poly(I:C)-stimulated phKCs, suggesting cotrafficking of ET-1, TSLP, and V3 (Figure 4e).

As a translational approach, we also investigated the localization of V3, ET-1, and TSLP on skin of patients with AD and compared the results with those of healthy controls. In the healthy skin control, the immunosignals of ET-1 and TSLP were weak (Figure 5a), in agreement with previous findings (Aktar et al., 2015; Nakahara et al., 2018; Soumelis et al., 2002). Whereas in AD skin, ET-1 and TSLP signals were enhanced greatly (Figure 5a). In healthy skin, the V3 immunosignal was predominantly located in the cytosol of epidermal KCs, with much less signal detected in the dermis (Figure 5b). In AD skin, however, ET-1 immunosignals appeared colocalized with V3 in the epidermal layer (Figure 5b, right panel). The calculated Pearson correlation coefficient confirmed colocalization of ET-1 with TSLP (Figure 5c) and ET-1 with V3 (Figure 5d) in AD skin. Collectively, our data show the importance of V3 in mediating exocytosis of pruriceptive cytokine-containing vesicles.

V3, STX4, and S29 are important SNAREs in controlling release of various itch-related cytokines from phKCs

Using cytokine arrays, we further analyzed supernatant samples collected for measuring ET-1 and TSLP contents after KD of a specific SNARE isoform (Figure 4a–d). V3 KD resulted in a substantial reduction of Poly(I:C)-induced release of CXCL9/10/11 and CCL2/3/4/5/20, as well as inflammatory mediators, such as EMMPRIN, GM-CSF, IL-1β/-6/-17A, matrix metalloproteinase-9, and TNF-α (Figure 6a).

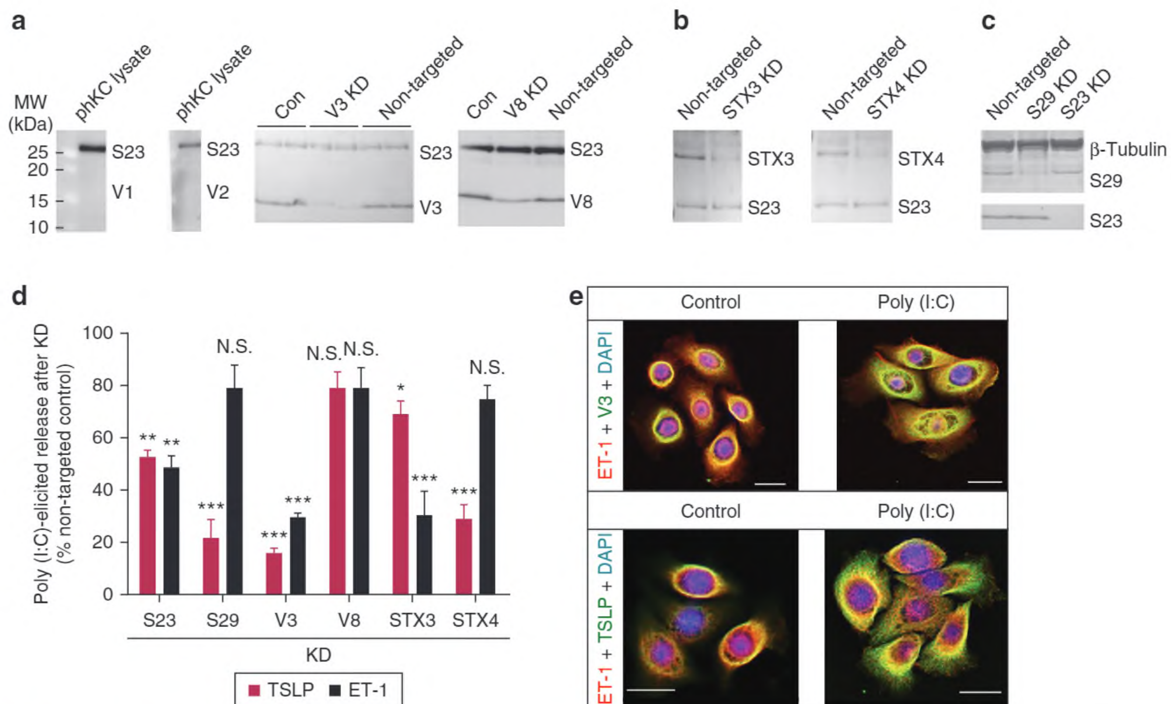

Figure 4. SNAREs control TSLP and ET-1 release from phKCs. (**a**–**c**) Representative blots show KD of (a) V3 or V8, (b) STX3 or STX4, (c) S29 or S23. S23 in (a) and (b) and ß-Tubulin in (c) were probed as internal loading control. V1 and V2 were not detected. (**d**) Poly(I:C)-evoked release after specific KD of SNARE was calculated as percentage of nontargeted control. SNAREs are differentially required for ET-1 and TSLP release. Significant difference between shRNA-treated versus nontargeted control is indicated. (**e**) Dual-labeling of ET-1 and V3 or ET-1 and TSLP in cultured phKCs with or without Poly(I:C) treatment. Scale bars = 20 μm. For each graphical representation, data are presented as mean ± standard error of the mean (n = 3). NS, $P > 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ET-1, endothelin-1; KD, knockdown; NS, nonsignificant; phKC, primary human keratinocyte; shRNA, short hairpin RNA; SNARE, soluble *N*-ethylmaleimide sensitive factor attachment protein receptor; STX, syntaxin; TSLP, thymic stromal lymphopoietin.

Again, partial KD of V8 (Figure 4a) failed to inhibit cytokine release (Figure 6a). KD of STX4 exerted a more pronounced effect than KD of STX3 with respect to the inhibition of CXCL/5/9/11, CCL/2/3/4, GM-CSF, or TNF-α (Figure 6b). In contrast, KD of STX3, but not STX4, substantially reduced EMMPRIN and IL-1α release (Figure 6b). KD of S29 resulted in a greater inhibitory effect than KD of S23 on the release of nearly all cytokines, with the exception of CXCL11 and CCL20 (Figure 6c).

Taken together, our data indicate that V3, STX4, S29, and, to a lesser extent, S23 are important SNAREs for regulating release of various cytokines from phKCs involved in AD. These proteins might be potential targets for designing new antipruritic treatments for AD.

DISCUSSION

AD is a common yet poorly understood chronic inflammatory skin disease with an unmet need for effective therapeutics (Weidinger et al., 2018). Our study identified previously unrecognized molecules involved in AD, hinted at previously unknown neuronal-immune and neuroepidermal circuits, and discovered SNAREs as potential targets for the treatment of AD.

KCs play a critical role in atopic skin inflammation (Giustizieri et al., 2001), adaptive and innate immunity (Guttman-Yassky and Krueger, 2017; Paller et al., 2017), and neuroimmune communication (Frateschi et al., 2011; Kido-Nakahara et al., 2014; Steinhoff et al., 2003, 2006). Investigation of the innate-immune-derived cytokines in KCs with subsequent activation of neuroimmune circuits may help us to better understand AD and to design therapeutics to control neuroinflammation and pruritus simultaneously in AD. First, we demonstrated that activation of TLR3 in phKCs produced a robust upregulation of gene transcripts of TSLP, ET-1, CXCL10, TNF-α, CCL5, and other cytokines. Many cytokines, such as TSLP, ET-1, EMMPRIN, GM-CSF, IL-1/-6/-17A, CXCL1/5/9/10/11, CCL2/3/4/5/20, and TNF-α are coreleased. Neuroepidermal communication is an important pathophysiological process in AD, in which various mediators from KCs (Kido-Nakahara et al., 2014; Steinhoff et al., 2001, 2005; Wilson et al., 2013) affect inflammation and pruritus through sensory neuronal signaling (Kido-Nakahara et al., 2014; Steinhoff et al., 2000). Herein, we show that TSLP, TNF-α, and neuromediators such as ET-1 can activate distinct but overlapping DRG subsets, suggesting that these cytokines may signal either separately or together into sensory neuronal subsets to propagate itch sensation. TSLP, ET-1, and TNF-α expression were upregulated in AD skin samples, with TSLP having increased ∼100-fold that of healthy skin samples (Fornasa et al., 2015). Topical application of vitamin D3 or low-calcemic analog MC903 induced TSLP expression in epidermal KCs and triggered AD. The serum level of TSLP at day 4 in ears treated with MC903 reached ∼7 ng/ml (Li et al., 2006). In fact, a number of different cell types (epithelial, mast, stromal, and dendritic cells) can release these cytokines (Elder et al., 2016; Soumelis et al., 2002).

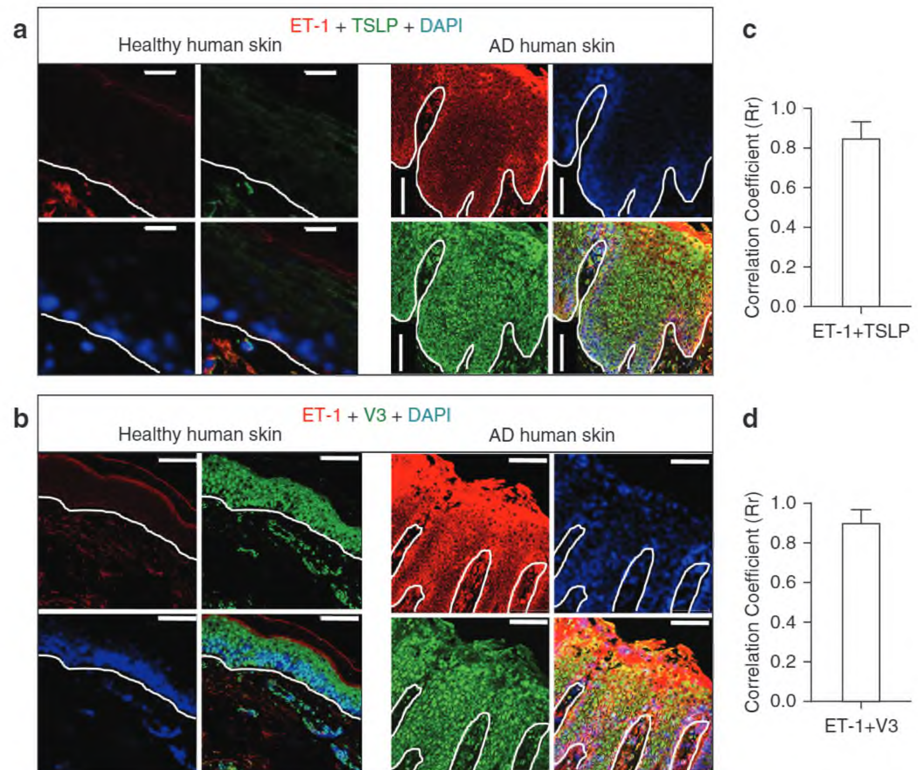

Figure 5. Staining of ET-1, TSLP, and V3 in clinical skin samples. (**a, b**) Representative images showing expression patterns of ET-1 and TSLP (a), or ET-1 and V3 (b) in healthy control and AD skin. Samples from three donors (two specimens/donor). Lines denote the epidermal-dermal junctions. (**c, d**) Pearson correlation coefficient analysis was used to calculate the colocalization level of ET-1 with TSLP (c), or ET-1 with V3 (d) in AD skins using ≥12 images recorded from three donors. Quantitative data indicated that ET-1 largely colocalized with TSLP and V3 in AD skin. As expression levels of TSLP and ET-1 in epidermis of control skins are very low, calculation of the correlation has no biological meaning. Scale bar = 20 μm for healthy skin in (a); other scale bars = 100 μm. For each graphical representation, data are presented as mean ± standard error of the mean. AD, atopic dermatitis; ET-1, endothelin-1; TSLP, thymic stromal lymphopoietin.

This upregulation of proinflammatory cytokines within AD could have an even greater effect in stimulating sensory neurons and propagating the sensation of pathological itch. Our finding of TNF-α potentiation of TSLP-induced Ca^{2+}-transients in DRGs highlights a cooperative, synergistic role of overactive cytokines that may magnify an even greater sensation of itch.

In this study, we found that ET-1 and IL-31 can stimulate TGNs to release BNP. Moreover, BNP upregulated several genes in phKCs including *PDE9A*, *LRIT2*, *PRND*, *WDFY4*, *MYLPF*, *PMCH*, *MGP*, and *FA2H*. Future studies are necessary to further explore the role of these genes in inflammation, itch, and AD. Canonical pathway analysis of RNA-Seq data indicates that the feedback effect of gene regulation induced by BNP on phKCs is highly related to dermatological diseases/conditions. Consistently, our previous study shows that elevated expression of BNP in TGN, DRG, and skin is linked to IL-31−induced itch and AD (Meng et al., 2018). Thus, peripheral BNP may play a significant role in modulating inflammatory response in the skin during AD. Recent papers support this notion by showing that BNP is implicated in AD transmission in dog and mouse itch models (Pitake et al., 2018; Ralvenius et al., 2018). Altogether, our study adds insights into the itch circuits in the neuron-skin communications (Graphical Abstract).

The effective treatment of AD requires designing efficacious therapeutics to intervene in multiple cytokine signaling pathways (Leung and Guttman-Yassky, 2014; Malajian and Guttman-Yassky, 2015). Our results provide evidence that SNARE proteins are critical in releasing cytokines from KCs during inflammation and bridging skin-nerve communication (Graphical Abstract). We show that V3, STX4, S29, and S23 play a pivotal role in regulating the function of phKCs through controlling the release of cytokines, such as GM-CSF, IL-1, IL-6, CXCL5/9/11/2, CCL3/4/5/20, matrix metalloproteinase-9, TSLP, ET-1, and TNF-α. In addition, V3, S29, STX4, and S23 differentially mediate cytokine release, suggesting a selective role of various SNAREs in different inflammatory cascades, a finding that needs to be further explored. For example, V3 and S29 are critical for AD-related cytokine release. A mutation of S29 causes a neurocutaneous syndrome with cerebral dysgenesis, neuropathy, ichthyosis, and palmoplantar keratoderma. S29KO or KC-specific S29KO mice are neonatal lethal, and their skin demonstrates a disturbed epidermal differentiation (Schiller et al., 2016a, 2016b). Notably, V3 was colocalized with ET-1 on the plasma membrane of Poly(I:C)-stimulated phKCs, in great contrast to the unstimulated cells. Likewise, V3 was found to be colocalized with ET-1 in KCs of AD skin, in agreement with the importance of V3 in mediating the release of a broad range of cytokines upon activation of TLR3. These findings are significant because TSLP is closely related to lesional AD, and its signaling is required for IL-13−induced "Atopic March" and AD progression; ET-1 is increased in AD skin but not in pruritic psoriatic skin (Nattkemper et al., 2018). Though the direct link between ET-1 and AD is unclear, it has been reported that ET-1 induces the production of IL-25 to accelerate T helper type 2 immune deviation (Aktar et al., 2015) and polarizes the dendritic cell-T-cell response to promote the chronicity and persistence of inflammatory skin disease (Nakahara et al., 2018). Moreover, ET-1 levels were positively correlated with AD clinical severity, itch intensity, and serum IgE levels during AD exacerbation (Gomes et al., 2012; Katugampola et al., 2000; Nattkemper et al., 2018;

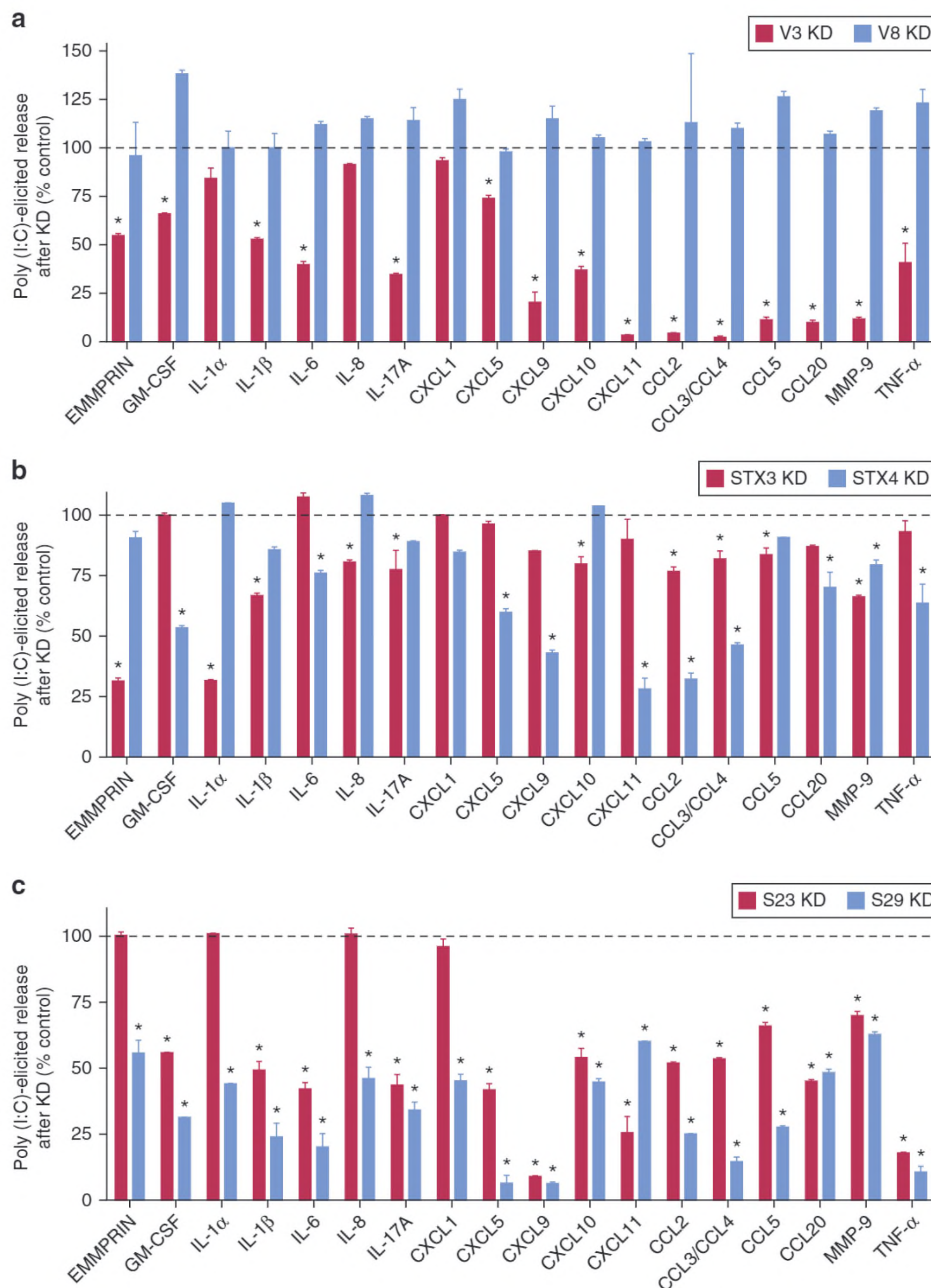

Figure 6. Effect of SNARE isoforms on regulating TLR3 agonist−elicited cytokine release from phKCs. Released samples after KD of specific SNARE isoform (see Figure 4) were processed for semiquantitative cytokine array. (**a**) KD of V3 resulted in extensive inhibition of release of various cytokines involved in AD. (**b**) STX3 and STX4 were differentially required for release of individual cytokines. (**c**) S29 is required for the release of a broader spectrum of cytokines than S23. Significant difference between shRNA-treated versus nontargeted control is indicated. For each graphical representation, data are presented as mean ± standard error of the mean (n = 3). $*P < 0.05$. AD, atopic dermatitis; KD, knockdown; phKC, primary human keratinocyte; shRNA, short hairpin RNA; SNARE, soluble *N*-ethylmaleimide sensitive factor attachment protein receptor; STX, syntaxin; TLR**,** toll-like receptor.

Tsybikov et al., 2015). Thus, it is reasonable to deduce that suppression of elevated release of KC-derived AD-related cytokines by targeting V3 might pose an exciting development in the area of treating AD and other pruritic skin diseases.

In conclusion, our study not only provides evidence of particular SNAREs mediating the release of AD-related cytokines from phKCs but also points out a previously unknown pathophysiological role of certain cytokines in the neuro-immune axis of AD (Graphical Abstract). Rather than targeting just one or a few particular cytokines or single kinase pathways (Brunner et al., 2017), our data provide a perspective for targeting a group of inflammatory and pruritic mediators, as well as neuropeptides and their subsequent cell signaling cascades, to treat AD and probably other chronic inflammatory skin diseases.

MATERIALS AND METHODS

Animal and human rights

Housing, handling, and experimental procedures of C57BL6 mice were approved by the University College Dublin Ethics Committee and the Irish authorities. The human skin tissues were purchased from Tissue Solutions (Glasgow, Scotland); thus, institutional approval and patient consent was not necessary.

Cell cultures

PhKCs provided by Lonza were cultured in the KBM-Gold plus SingleQuots supplements for KCs (Lonza, Basel, Switzerland).

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The procedures for isolation and digestion of mouse dorsal root or trigeminal ganglions were described elsewhere (Eckert et al., 1997). Cells were seeded onto 8-well IBIDI chambers precoated with poly-L-lysine (0.1 mg/ml) and laminin (20 μg/ml) in DMEM with nerve growth factor (50 ng/ml), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 5% fetal bovine serum, with Cytosine-β-D-arabinofuranoside (10 μM) added.

RNA-Seq

Three batches of phKCs from three different human healthy donors were treated with or without 20 μg/ml Poly(I:C) or 1 μM human BNP for 2 hours before cell pellets were harvested and entrusted to IMGM Laboratories GmbH (Martinsried, Germany) for RNA-Seq using NextSeq 500 next-generation sequencing system (Illumina, San Diego, CA). The expression values were processed as reads per kilobase of exon model per million mapped reads. To analyze statistically significant expression results, the CLC Genomics Workbench tool "Empirical analysis of DGE" (Qiagen Bioinformatics, Redwood City, CA) was used. A gene or transcript is classified as induced in a specific comparison if its false discovery rate–corrected *P*-value is no more than 0.05, and if its fold-change value greater than 2. Canonical pathway analysis was performed using Qiagen Bioinformatics software.

Immunofluorescence staining

For ET-1, TSLP, and V3 staining in cultured phKCs, cells were dual-labeled overnight at 4 °C with mouse anti-ET-1 (1:1000; ab2786; Abcam, Cambridge, United Kingdom), rabbit anti-TSLP (1:100, ab47943, Abcam), or rabbit anti-V1/2/3 (1:1000; 104 203; SYSY, Göttingen, Germany), before adding secondary donkey anti-rabbit Alexa 488 (1:2000) and anti-mouse Alexa 594 (1:2000) for 1 hour at room temperature. At the end, specimens were mounted using ProLong Gold antifade reagents (P36935; Invitrogen, Carlsbad, CA) containing DAPI.

Human skins from three donors with AD and three healthy control subjects (Tissue Solutions, Glasgow, Scotland) were sectioned and deparaffinized before being boiled in DAKO antigen retrieval buffer for 25 minutes and permeabilized in 0.2% Triton X-100 in phosphate buffered saline for 45 minutes. Specimens were blocked in phosphate buffered saline with 5% horse serum + 5% donkey serum before primary antibodies were applied overnight at 4 °C as follows: mouse anti-ET-1 with rabbit anti-TSLP or rabbit anti-V1/2/3. Specimens were incubated for 1 hour with secondary antibodies (1:2000; Alexa 594 goat anti-mouse and Alexa 488 goat anti-rabbit; Invitrogen).

Images were taken using the LSM710 confocal microscope (Carl Zeiss MicroImaging, Jena, Germany) and the Zen software (Universal Imaging, Göttingen, Germany).

SNARE KD

Cultured phKCs were incubated with shRNA lentiviral particles (Sigma-Aldrich, Wicklow, Ireland) that specifically target V3, V8, S23, S29, STX3, STX4, or nontargeted particles (400 transducing units/well). After 2 days in culture, 1 μg/ml of puromycin was added for 3 days before adding Poly(I:C) for release. Cells were harvested for western blotting. Rabbit antibodies against STX3 (1:1000; ab4113, Abcam), S23 (1:1000; 111202, SYSY), STX4 (1:1000; 110043, SYSY), V1/2/3 (1:1000), and V2 (1:1000; 104202, SYSY) were used. Validated sequences for used shRNA lentiviruses were the following:

NM_004781,V3:CCGGGCAGCCAAGTTGAAGAGGAAACTCGAGTTTCCTCTTCAACTTGGCTGCTTTTTG

NM_003761,V8:CCGGGATCTGGAAGCCACATCTGAGCTCGAGCTCAGATGTGGCTTCCAGATCTTTTTG

NM_003825,S23:CCGGGCAAGGCTTATAAGACAACATCTCGAGATGTTGTCTTATAAGCCTTGCTTTTTG

NM_004782,S29:GTACCGGCCTTAGAAAGCTGGATGATACCTCGAGGTATCATCCAGCTTTCTAAGGTTTTTTG

NM_004177,STX3:CCGGGCCCGGAAGAAATTGATAATTCTCGAGAATTATCAATTTCTTCCGGGCTTTTTG

NM_004604,STX4:CCGGGCTGCACGACATATTCACTTTCTCGAGAAAGTGAATATGTCGTGCAGCTTTTTG

Release assay

PhKCs were incubated for 24 hours in hydrocortisone-free medium with or without 20 μg/ml Poly(I:C) before culture supernatant was collected for measuring TSLP and ET-1 concentrations using ELISA or for other cytokines using proteome profiler human XL cytokine antibody array (R&D, Minneapolis, MN). Fold-change of cytokine release was calculated as described elsewhere (Meng et al., 2018).

TGNs were cultured for 7 days before adding low potassium basal release buffer (Meng et al., 2009) alone or in the presence of 100 nM ET-1 (Sigma, St. Louis, MO) or 300 nM IL-31 (Zymogenetics, Seattle, WA) for 30 minutes at 37 °C. Released BNP was quantified using the enzyme immunoassay kit (Sigma).

Ca^{2+} imaging

DRGs transfected with Lenti-GCaMP6s (Addgene, Cambridge, MA) were cultured for ~5 days before adding low potassium basal release buffer and cell chambers attached to confocal microscope (Bai et al., 2014; Meng et al., 2009). An argon laser was used to excite the fluorophore at 488 nm. Fluorescent signals were grabbed every 2 seconds, and the superfusate was switched after ~20 seconds from low potassium basal release buffer to low potassium basal release buffer containing TSLP (20 ng/ml), ET-1 (100 nM), or TNF-α (100 ng/ml). Sequential application of these compounds occurred with washout between each cytokine. Fluorescence intensities at 505–530 nm of the baseline (f_0) in low potassium basal release buffer and in stimulation (f) were analyzed on a cell-by-cell basis. The fluorescence change ($f-f_0$) was expressed to f_0.

Statistical data analysis

Data of independent experiments ($n \geq 3$) were expressed as mean ± standard error of the mean. Probability values were determined with two-tailed Student *t* test; *P*-values <0.05 are significant; *P*-values >0.05 are nonsignificant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Data analysis was performed with Prism software.

Data availability statement

Datasets related to this article can be found at https://www.ncbi.nlm.nih.gov/gds/, hosted by GEO database (accession number GSE129346).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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Conceptualization: JM, JW, MS; Data Curation: JM, JW; Funding Acquisition: JM, JW, MS; Resources: JB, TB; Writing - Original Draft Preparation: JM, JW, MS; Writing - Review and Editing: JM, JW, JB, TB, MS

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