New mechanism underlying IL-31–induced atopic dermatitis

Jianghui Meng, PhD,[a,b] Masaki Moriyama, PhD,[c] Micha Feld, PhD,[d] Joerg Buddenkotte, MD, PhD,[e,f] Timo Buhl, MD,[g] Attila Szöllösi, PhD,[b] Jingming Zhang, PhD,[h] Paul Miller, PhD,[h] Andre Ghetti, PhD,[h] Michael Fischer, MD,[i] Peter W. Reeh, MD,[j] Chunxu Shan, PhD,[a,b] Jiafu Wang, PhD,[a] and Martin Steinhoff, MD, PhD[b,c,e,f] *Dublin, Ireland; San Francisco and San Diego, Calif; Dusseldorf, Gottingen, and Erlangen, Germany; Doha, Qatar; and Vienna, Austria*

Background: T_H2 cell–released IL-31 is a critical mediator in patients with atopic dermatitis (AD), a prevalent and debilitating chronic skin disorder. Brain-derived natriuretic peptide (BNP) has been described as a central itch mediator. The importance of BNP in peripheral (skin-derived) itch and its functional link to IL-31 within the neuroimmune axis of the skin is unknown.

Objective: We sought to investigate the function of BNP in the peripheral sensory system and skin in IL-31–induced itch and neuroepidermal communication in patients with AD.

Methods: Ca^{2+} imaging, immunohistochemistry, quantitative real-time PCR, RNA sequencing, knockdown, cytokine/phosphokinase arrays, enzyme immune assay, and pharmacologic inhibition were performed to examine the cellular basis of the IL-31–stimulated, BNP-related itch signaling in dorsal root ganglionic neurons (DRGs) and skin cells, transgenic AD-like mouse models, and human skin of patients with AD and healthy subjects.

Results: In human DRGs we confirmed expression and co-occurrence of oncostatin M receptor β subunit and IL-31 receptor A in a small subset of the neuronal population. Furthermore, IL-31 activated approximately 50% of endothelin-1–responsive neurons, and half of the latter also responded to histamine. In murine DRGs IL-31 upregulated *Nppb* and induced soluble N-ethylmaleimide–sensitive factor activating protein receptor–dependent BNP release. In Grhl3PAR2$^{/+}$ mice house dust mite–induced severe AD-like dermatitis was associated with *Nppb* upregulation. Lesional IL-31 transgenic mice also exhibited increased *Nppb* transcripts in DRGs and the skin; accordingly, skin BNP receptor levels were increased. Importantly, expression of BNP and its receptor were increased in the skin of patients with AD. In human skin cells BNP stimulated a proinflammatory and itch-promoting phenotype.

Conclusion: For the first time, our findings show that BNP is implicated in AD and that IL-31 regulates BNP in both DRGs and the skin. IL-31 enhances BNP release and synthesis and orchestrates cytokine and chemokine release from skin cells, thereby coordinating the signaling pathways involved in itch. Inhibiting peripheral BNP function might be a novel therapeutic strategy for AD and pruritic conditions. (J Allergy Clin Immunol 2018;141:1677-89.)

Key words: *Atopic dermatitis, pruritogens, brain-derived natriuretic peptide, pruritus, skin, dorsal root ganglion, keratinocytes, dendritic cells, soluble N-ethylmaleimide–sensitive factor activating protein receptors*

Atopic dermatitis (AD) is one of the most prevalent chronic inflammatory skin diseases worldwide. It is characterized by dysregulation of immunity and skin barrier and nerve function, resulting clinically in eczema and itch.[1-4] The mediators of histamine-independent itch in patients with AD are poorly understood.[5] Candidates include endothelin-1 (ET-1), thymic stromal lymphopoietin, and the cytokines IL-4, IL-13, and IL-31.[1] IL-31 is a critical cytokine in the pathophysiology of AD,[1,6,7] playing a role in eczema,[7] itch,[8] and nerve growth.[9] Increased serum levels of IL-31 were found to correlate with disease severity in patients.[6,10] IL-31 serves as a critical neuron-immune link between T_H2 cells and sensory nerves in the generation of T cell–mediated itch.[8] Cutaneous and intrathecal injections of IL-31 evoked robust itch behavior in mice.[8] IL-31 injection in the mouse cheek induces itch but not pain.[8] IL-31 transgenic (Tg) mice that overexpress IL-31 have severe pruritus and skin lesions similar to AD.[7] In mice IL-31 receptor A (IL-31RA) associates with oncostatin M

From [a]the International Center for Neurotherapeutics, Dublin City University, Dublin; [b]the Department of Dermatology and UCD Charles Institute for Translational Dermatology, Dublin; [c]the Department of Dermatology, University of California, San Francisco; [d]the Department of Dermatology, University Hospital Düsseldorf; [e]the Department of Dermatology and Venereology, Hamad Medical Corporation, Qatar University, Doha; [f]the School of Medicine, Weill Cornell University–Qatar and Qatar University, Doha; [g]the Department of Dermatology, University Medical Center Göttingen; [h]AnaBios, San Diego; [i]the Center for Physiology and Pharmacology, Medical University of Vienna; and [j]Institut für Physiologie & Pathophysiologie, Universität Erlangen-Nürnberg, Erlangen.

Science Foundation Ireland is thanked for funding this research through a Starting Investigator Award (to J.M.; grant no. 15/SIRG/3508T), a Principle Investigator Award (to M.S.) and a Career Development Award (13/CDA/2093 to J.W.). M.S. received Excellence seed funding from Hamad Medical Corporation, Qatar.

Disclosure of potential conflict of interest: J. Meng has received a grant from Science Foundation Ireland (15/SIRG/3508T). M. Moriyama has received a grant from the National Institutes of Health. J. Zhang, P. Miller, and A. Ghetti have received fees for service from UCD. J. Wang has received a grant from Science Foundation Ireland (13/CDA/2093). The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication June 12, 2017; revised November 18, 2017; accepted for publication December 21, 2017.

Available online February 7, 2018.

Corresponding author: Jianghui Meng, PhD, International Centre for Neurotherapeutics, Dublin City University, Glasnevin Avenue, Dublin 9, Dublin, Ireland. E-mail: jianghui.meng@dcu.ie. Or: Martin Steinhoff, MD, PhD, Department of Dermatology, PO Box 3050, Hamad Medical Corporation, Rumailah Hospital, Qatar University, Doha, Qatar. E-mail: MSteinhoff@hamad.qa.

The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections

0091-6749/$36.00

© 2018 American Academy of Allergy, Asthma & Immunology

https://doi.org/10.1016/j.jaci.2017.12.1002

Abbreviations used

AD: Atopic dermatitis
BNP: Brain-derived natriuretic peptide
CGRP: Calcitonin gene–related peptide
DRG: Dorsal root ganglionic neuron
ET-1: Endothelin-1
FPKM: Fragments per kilobase of transcript per million mapped reads
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GSK3: Glycogen synthase kinase 3
hDC: Human dendritic cell
HDM: House dust mite
hDRG: Human dorsal root ganglionic neuron
H&E: Hematoxylin and eosin
hKC: Human primary keratinocyte
IL-31RA: IL-31 receptor A
JNK: c-Jun N-terminal kinase
mDRG: Mouse dorsal root ganglionic neuron
MMP9: Matrix metalloproteinase 9
NeuN: Neuronal nuclei
NPR1: Natriuretic peptide receptor A
NPR2: Natriuretic peptide receptor B
OSMRβ: Oncostatin M receptor β subunit
PGP9.5: Protein gene product 9.5
RNA-Seq: RNA sequencing
shRNA: Short hairpin RNA
SNAP-25: Synaptosomal associated protein 25k
SNARE: Soluble N-ethylmaleimide–sensitive factor activating protein receptor
SP: Substance P
TG: Trigeminal ganglion
Tg: Transgenic
V1: Vesicle-associated membrane protein isoform 1
V7: Vesicle-associated membrane protein isoform 7
VAMP: Vesicle-associated membrane protein
WT: Wild-type

receptor β (OSMRβ) to form the IL-31RA heterodimeric complex that binds to IL-31.[8]

Targeting the IL-31 pathway therapeutically has proved effective in patients with AD.[11,12] For example, IL-31 neutralization in AD models was effective in the treatment of IL-31–induced itch and dermatitis.[13-17] The anti–IL-31RA antibody nemolizumab was evaluated in a phase II study with satisfactory efficacy and safety for the treatment of patients with moderate-to-severe AD.[11] Despite this, it is not known whether IL-31 stimulates neuropeptide release from central and/or peripheral primary afferent neurons to modulate itch transmission in the skin, spinal cord, or both. The importance of IL-31 in the regulation and release of neuropeptides from peripheral sensory nerves in the skin and the resulting neurogenic inflammation in patients with AD also remains elusive.

Brain-derived natriuretic peptide (BNP) is a 32-amino-acid cyclic peptide expressed in a subset of primary afferent neurons.[18-20] It binds to natriuretic peptide receptor A (NPR1) and, to a lesser extent, natriuretic peptide receptor B (NPR2).[21] BNP is the *Nppb* gene product and has been identified as an important neuropeptide for itch transmission from the sensory level to the spinal cord.[19] Intrathecal injection of BNP in mice induced a robust itch phenotype, whereas *Nppb*$^{-/-}$ mice exhibited lack of scratching responses to many pruritogens.[19] In contrast to substance P (SP) and calcitonin gene–related peptide (CGRP), which facilitate pain processing and painful neurogenic vasodilation in mice, BNP has been characterized as a negative regulator of nociceptive transmission.[20,22] *Nppb* has been detected in the murine primary pruritogenic sensory dorsal root ganglionic neurons (DRGs), which overlapped with IL-31RA^{+} neurons.[23] Despite its importance in itch, it is unknown whether BNP can be released from peripheral sensory neurons in response to pruritogens (ie, IL-31 or histamine). The cellular and molecular basis for its pruritic action on skin and immune cells also remains unknown. Understanding the downstream signaling pathways of IL-31 and its potential interacting mediators, such as BNP, CGRP, or SP, is significant because it will not only expand our current knowledge and theoretical repertoire but might also aid in the development of a more efficacious treatment for chronic skin inflammation and pruritus.

Here we investigated the possible mechanistic relationship between IL-31 and BNP in itch. The aim of the current study was to (1) examine the expression and function of IL-31 receptor in human dorsal root ganglionic neurons (hDRGs); (2) investigate the role of BNP in patients with AD by using mice models and human subjects; (3) investigate IL-31–induced release of BNP and other neuropeptides in relation to itch; (4) dissect the molecular components of the exocytotic machinery in IL-31–induced BNP release; (5) study the alteration of BNP receptor in AD skin; and (6) screen distinct intracellular kinases activated and cytokines induced by BNP in human skin cells. Overall, our results reveal a novel IL-31–mediated downstream target in itch induction and highlight the importance of BNP as a potential target for the treatment of AD and other itch disorders.

METHODS

Materials

Human skin samples from 3 donors with AD and 3 healthy control donors were bought from Tissue Solutions (Glasgow, United Kingdom). hDRG paraffin sections were purchased from Amsbio (local distributor of Zyagen Laboratories, San Diego, Calif). Antibodies, cells, and reagents for tissue culture and others are detailed in the Methods section in this article's Online Repository at www.jacionline.org.

Human and animal rights

hDRGs were isolated, as previously described,[24] with full legal consent by Anabios (San Diego, Calif). DRGs were isolated and cultured from 1 donor, a 25-year-old Hispanic woman. Housing/handling of mice (C57BL6), IL-31Tg mice, and experimental procedures were approved by the University College Dublin Ethics Committee and the Irish Authorities. Housing/handling of the Grhl3PAR2$^{/+}$ Tg mouse model and experimental procedures following federal guidelines were approved by the University of California, the San Francisco Ethics Committee, and local authorities.

House dust mite application for the Grhl3PAR2$^{/+}$ mouse model

Grhl3PAR2$^{/+}$ mice were maintained in C57BL6/J-129X1/SvJ mixed strain and used for the experiments as an AD model. For details of house dust mite (HDM) treatment, see the Methods section in this article's Online Repository.

RNA sequencing

For gene expression experiments, trigeminal ganglia (TGs) were harvested from wild-type (WT) and HDM-treated Grhl3PAR2$^{/+}$ mice and processed for RNA sequencing (RNA-Seq). For details, see the Methods section in this article's Online Repository.

Quantitative real-time PCR

RNA was isolated from cultured mouse DRGs (mDRGs) by using the RNeasy kit (Qiagen, Hilden, Germany). TRIzol Reagent was used to isolate RNA from dorsal root ganglions and the skin of WT and IL-31Tg mice. mRNA quantitation was performed by using real-time fluorescence detection with the SYBR Green ROX mix (ABI). For details, see the Methods section in this article's Online Repository.

BNP, CGRP, and SP release assays

For detailed preparation of mDRG culture and neuropeptide release, see the Methods section in this article's Online Repository.

Measurement of intracellular Ca^{2+} concentration

hDRGs and mDRGs in culture were loaded with calcium indicator before measurement of Ca^{2+} mobilization in response to various pruritogens. For details, see the Methods section in this article's Online Repository.

Immunofluorescence staining

Cultured mDRGs, paraffin-embedded sections of hDRGs, and skin samples (15 μm) were stained and imaged with a Zeiss LSM710 confocal microscope (Carl Zeiss MicroImaging, Oberkochen, Germany). For details, see the Methods section in this article's Online Repository.

Lentivirus-mediated knockdown of soluble N-ethylmaleimide–sensitive factor activating protein receptors

mDRGs cultured for 7 days *in vitro* were treated with nontargeted control lentiviral particles or short hairpin RNA (shRNA) lentivirus specifically targeting soluble N-ethylmaleimide–sensitive factor activating protein receptors (SNAREs) for 7 to 10 days before measuring BNP release and protein expression. For details, see the Methods section in this article's Online Repository.

Culture of human primary epidermal keratinocytes and human monocyte-derived dendritic cells, cytokine antibody arrays, and phosphokinase arrays

Human primary keratinocytes (hKCs) were cultured in KBM-Gold medium with KBM-Gold SingleQuot KC supplement (Lonza, Basel, Switzerland). Human dendritic cells (hDCs) were cultured by means of *in vitro* differentiation of $CD14^+$ monocytes with LGM-3 Lymphocyte Growth Medium plus 50 ng/mL GM-CSF and 50 ng/mL IL-4 (Lonza). Cells were maintained in the above medium for 3 days before use. For detailed cytokine antibody and phosphokinase arrays, see the Methods section in this article's Online Repository.

Statistical data analysis

Data are expressed as means ± SEMs ($n \geq 3$ independent experiments). *P* values were determined by using the Student 2-tailed *t* test; *P* values of less than .05 are considered significant. Data analysis was performed with Prism software (GraphPad Software, La Jolla, Calif).

RESULTS

IL-31RA and OSMRβ are coexpressed in a small subset of hDRGs

In human patients with AD, IL-31 levels correlate with disease severity.[6,10] However, subpopulations responding to IL-31 and distribution of its receptors (IL-31RA and OSMRβ) in hDRGs have not been fully characterized. Our study will help define the functional contribution of neuronal subpopulations to the skin inflammation seen in patients with AD.

Using double immunohistochemistry, we observed IL-31RA and OSMRβ expression in hDRG sections (Fig 1, *A-D*). IL-31RA was detected in approximately 3% of total neurons visualized with the neuronal marker neuronal nuclei (NeuN) antibody, which stained strongly neuronal nuclei and distal cytoplasm (Fig 1, *A*). IL-31RA also occurred in a small proportion of cells immunoreactive to protein gene product 9.5 (PGP9.5) antibody (Fig 1, *B*), another marker for sensory nerves. Similarly, OSMRβ was found in a small subset of $PGP9.5^+$ neurons (Fig 1, *C*). Costaining of IL-31RA and OSMRβ confirmed that in hDRGs all of the $IL\text{-}31RA^+$ neurons were $OSMR\beta^+$ (Fig 1, *D* and *E*). However, approximately 5% of $OSMR\beta^+$ neurons did not appear to be strongly labeled by anti–IL-31RA (Fig 1, *D*, arrowed neuron).

IL-31 induces intracellular calcium mobilization in a distinct subset of hDRGs, which also respond to ET-1

Functional identities of neuronal subsets were further assayed in hDRGs by using intracellular Ca^{2+} imaging on application of individual or combined pruritogens to cultured hDRGs. We found that approximately 4% of hDRGs were activated directly by ET-1 ($n = 100$ neurons; Fig 2, *A* and *B*, top panels). The response consisted of a transient increase in intracellular calcium levels. In comparison, glial cells consistently exhibited large responses with slow kinetics. In several cases after ET-1 application, glial cells generated oscillatory calcium signals that persisted even after ET-1 washout (Fig 2, *A* and *B*, top panels). In our observation, approximately 50% of neurons pre-exposed to ET-1 also responded to histamine (Fig 2, *A*, middle panel, and *C*). After application of ET-1 and histamine, some neurons exhibited spontaneous calcium transients. Coapplication of ET-1 and histamine did not potentiate the response. On the contrary, desensitization was apparent in some neurons (Fig 2, *A*, bottom panel). For the IL-31–induced response, 1 of 50 neurons pre-exposed to ET-1 responded with a rapid calcium transient to IL-31 (Fig 2, *B*, top and middle panels). Likewise, coapplication of ET-1 and IL-31 did not potentiate the response, except in one neuron with a delayed response (Fig 2, *B*, bottom panel).

In summary, approximately 50% of neurons responded to histamine and approximately 4% of total neurons responded to ET-1. Half of the ET-1–responsive neurons also responded to histamine (Fig 2, *C*). Overall, in hDRGs 2% of neurons responded to IL-31, and these cells also responded to ET-1 (Fig 2, *C*). Because of the importance of IL-31 and ET-1 in the pathogenesis of AD, it is tempting to postulate that this neuronal subset mediates itch transmission in human subjects.

We investigated further whether mDRGs exhibit similar phenomena in response to IL-31 and ET-1. Cultured mDRGs uploaded with Fluo-4 AM were treated with IL-31 and ET-1 sequentially. In a total of 335 neurons recorded, IL-31 activated 9 neurons (approximately 2.7%), and ET-1 activated 14 neurons (approximately 4.2%). Five IL-31–responsive neurons did not respond to ET-1 (Fig 2, *D*). Thus both similarities and differences were observed between hDRGs and mDRGs with respect to IL-31 and ET-1 stimulation.

PAR2Tg mice treated with HDM and IL-31Tg mice are associated with *Nppb* upregulation in sensory nerves

We investigated the possible involvement of BNP in patients with AD by using $Grhl3PAR2^{/+}$ mice.[25] These mice

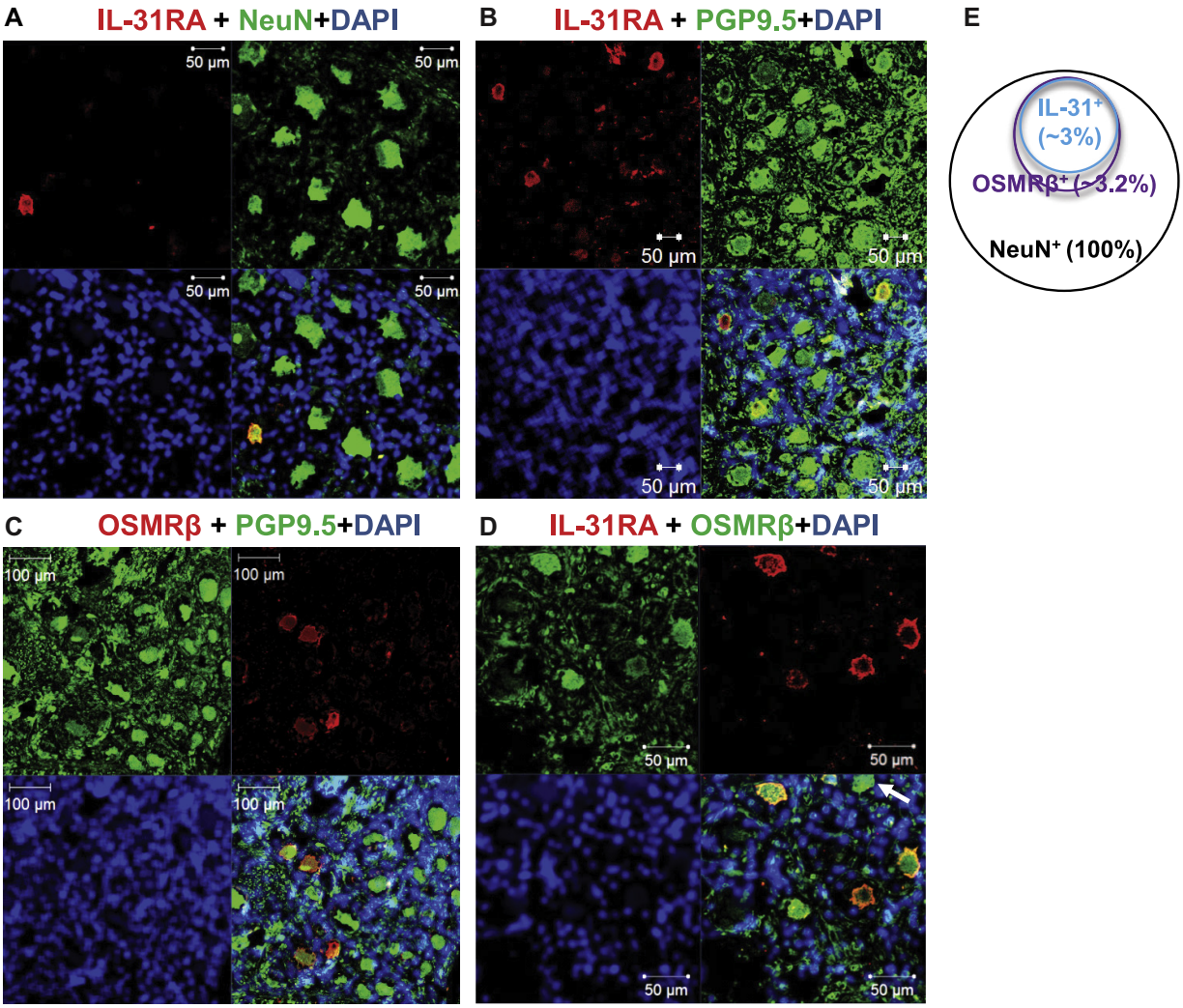

FIG 1. IL-31RA and OSMRβ co-occurred in a small population of hDRGs. **A** and **B,** Representative immunofluorescence confocal images showing that IL-31RA protein immunoreactivity occurred only in a subpopulation of neuronal cells in hDRG sections. Neuronal cells were stained with antibody against NeuN (Fig 1, *A*) or PGP9.5 (Fig 1, *B*). **C,** Costaining of OSMRβ with PGP9.5 in hDRG sections. **D,** Immunofluorescence staining of hDRG sections showing IL-31RA and OSMRβ largely colocalized in a subset of neurons. Specimens were counterstained with 4′,6-diamidino-2-phenylindole *(DAPI)* to highlight all cell nuclei. The *arrow* indicates a neuron expressing OSMRβ but not stained strongly by IL-31RA antibody. **E,** Venn diagram depicting the relationship of IL-31RA$^+$, OSMRβ$^+$, and NeuN$^+$ populations in hDRG sections. Total neuron numbers were counted based on dual labeling of both NeuN and DAPI. A total of 573 neurons were imaged.

have atopic-like inflammation, scaly dry skin, epidermal hyperplasia, and itch behaviors, all characteristics of human AD.[25] We applied HDM on the right cheek to induce severe dermatitis because HDMs have abundant proteases that can activate PAR2 constantly and stably. Ipsilateral and contralateral TGs from each mouse were analyzed by using RNA-Seq (Fig 3, *A*). Clinical scores for pathologic diagnosis (skin lesion severity) were evaluated by using hematoxylin and eosin (H&E) staining on cheek skin biopsy specimens from each of the 8 HDM-applied mice and vehicle-treated WT mice (representative images shown in Fig 3, *B*). Notably, in HDM-treated mice the correlation coefficient between clinical score and fold change of fragments per kilobase of transcript per million mapped reads (FPKM) for *Nppb* transcripts (ipsilateral/contralateral) is about 0.8 (Fig 3, *A*). The average fold change of *Nppb* for these 8 mice is approximately 2.3. In contrast, HDM did not induce significant changes in TGs for NPR1, NPR2, IL-31, IL-31RA, and OSMR in Grhl3PAR2$^{/+}$ mice (Fig 3, *A*). After comparing FPKM from TGs of ipsilateral (right cheek) versus contralateral (left cheek) in 3 Grhl3PAR2$^{/+}$ mice with high clinical scores (scores were 8, 7, and 7, respectively), we found that *Nppb* transcripts were upregulated by 3.5 times by mean of application of HDM and ranked the third position among the highly upregulated genes (Fig 3, *C*). Notably, the *Nppb* FPKM values in the contralateral (left) cheeks of Grhl3PAR2$^{/+}$ mice were not significantly different from those of WT mice with or without vehicle (Vaseline) treatment (see Fig E1 in this article's Online Repository at www.jacionline.org).

Because IL-31Tg mice show skin phenotypes closely resembling those of human AD,[7] we used RT-PCR to compare *Nppb* transcripts in whole DRG tissue isolated from IL-31Tg skin

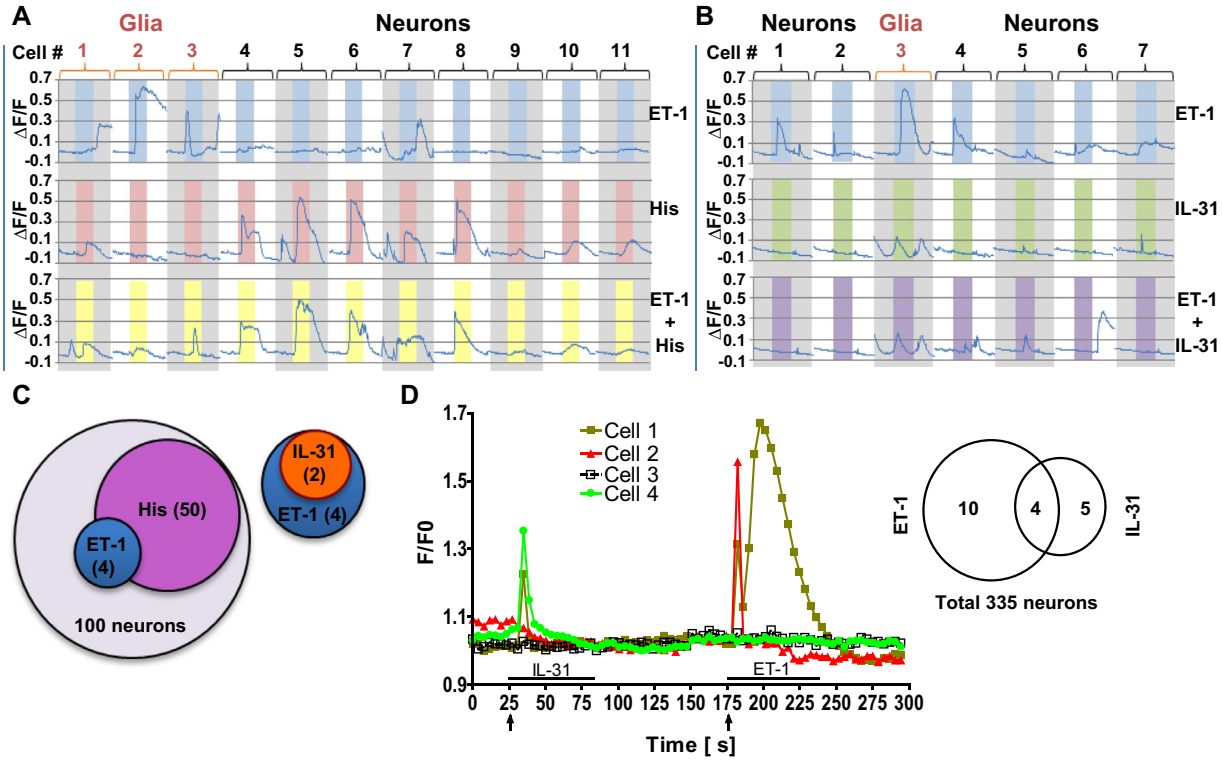

FIG 2. Pruritogen-induced calcium mobilization and characterization of IL-31–responsive cells in hDRGs and mDRGs. **A,** Representative traces for calcium measurement after sequential pruritogen mediator application. Traces show individual cells responding to ET-1 only *(top)*, histamine only *(middle)*, and ET-1 plus histamine *(bottom)*. Notably, there are some ET-1–responsive neurons that also responded to histamine. **B,** Neurons responding to ET-1 only *(top)*, IL-31 only *(middle)*, and ET-1 plus IL-31 *(bottom)*. **C,** Venn diagrams for percentages of neurons that responded to pruritogenic compounds. The total number of hDRGs recorded was 100. **D,** Four representative traces of mDRG cells responsive to IL-31, ET-1, or both. Note that cell 1 responded to both IL-31 and ET-1 and cell 2 responded only to ET-1, whereas cell 3 did not respond to either IL-31 or ET-1; cell 4 is an IL-31–responsive cell that did not respond to ET-1. The Venn diagram shows relative proportions of mDRGs responsive to IL-31, ET-1, or both. A total of 335 mDRGs were recorded. *His*, Histamine.

(both lesional and nonlesional) and skin from age-matched WT nontransgenic mice. Higher levels of *Nppb* mRNA were detected in DRGs isolated from both lesional and nonlesional IL-31Tg mice (Fig 3, *D*).

Collectively, our data indicate that BNP is implicated in patients with AD and might be associated with the severity of AD-like skin conditions in mice.

IL-31 augments release and upregulates synthesis of BNP but not CGRP or SP from cultured mDRGs

IL-31 directly activates peripheral sensory neurons to induce pruritus[8]; however, its effect on BNP has not yet been reported. To explore this possibility, we cultured mDRGs and incubated neurons with 300 nmol/L IL-31 for 30 minutes for BNP release. IL-31–induced release was compared with the response to histamine and high-potassium (60 mmol/L) and low-potassium (3.5 mmol/L) chloride buffers (Fig 4, *A*). Notably, IL-31 elicited an approximately 2.7-fold increase in BNP release over the basal level (low potassium). Histamine and potassium depolarization (high potassium) also elicited BNP release (Fig 4, *A*). Moreover, IL-31–induced BNP release was concentration (Fig 4, *B*) and time (Fig 4, *C*) dependent. No significant difference in BNP release (Fig 4, *B*) and mRNA synthesis over 30 minutes (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org) was detected at IL-31 concentrations of between 300 nmol/L and 1 μmol/L. RT-PCR results revealed that 300 nmol/L IL-31–induced *Nppb* mRNA synthesis peaked at 4 hours and decreased at 8 hours (see Fig E2, *B*). In contrast, neither CGRP nor SP release was affected by IL-31 or histamine, although both were elicited by high potassium (Fig 4, *D*). For the first time, this finding demonstrates that IL-31 directly activates sensory neurons to release BNP rather than CGRP or SP. Consistently, incubation of mDRGs with IL-31 for 6 hours did not induce significant changes in mRNA levels of *Calca* (CGRP gene) or *Tac1* (SP gene; Fig 4, *E*). In contrast, IL-31 increased *Nppb* mRNA levels by approximately 2-fold. Altogether, these findings confirm that IL-31 directly induces BNP release and upregulates its synthesis, indicating BNP might contribute to IL-31-mediated itch signaling.

Subsequently, we performed immunofluorescence studies using antibodies against BNP or CGRP with their specificities verified (see Fig E3 in this article's Online Repository at www.jacionline.org) to investigate their expression and distribution pattern in mDRGs. Notably, BNP was expressed in

A

Nppb	FPKM		ipsi/contra	NPR1	FPKM		ipsi/contra	NPR2	FPKM		ipsi/contra
Clinial Score	ipsi	contra	Fold Change	Clinial Score	ipsi	contra	Fold Change	Clinial Score	ipsi	contra	Fold Change
8	21.1	8.7	2.4	8	0.5	0.7	0.6	8	15.2	13.4	1.1
7	17.6	3.9	4.5	7	0.6	0.3	2.4	7	14.6	14.9	1.0
7	29.4	7.7	3.8	7	0.8	1.2	0.7	7	15.9	14.0	1.1
5	19.7	8.1	2.4	5	1.0	0.7	1.3	5	11.8	14.5	0.8
4	10.2	8.8	1.2	4	1.1	0.7	1.5	4	16.1	15.0	1.1
3	20.0	12.2	1.6	3	0.4	0.3	1.2	3	15.7	14.5	1.1
3	12.5	6.8	1.8	3	0.7	0.3	2.2	3	21.1	15.5	1.4
2	5.4	13.4	0.4	2	1.4	0.6	2.5	2	19.1	18.8	1.0
correlation coefficient			0.799	correlation coefficient			-0.556	correlation coefficient			-0.126

IL-31	FPKM		ipsi/contra	IL-31RA	FPKM		ipsi/contra	OSMR	FPKM		ipsi/contra
Clinial Score	ipsi	contra	Fold Change	Clinial Score	ipsi	contra	Fold Change	Clinial Score	ipsi	contra	Fold Change
8	0.0	0.0	-	8	3.6	4.4	0.8	8	17.5	20.0	0.9
7	0.0	0.0	-	7	1.6	2.2	0.7	7	15.0	10.1	1.5
7	0.0	0.0	-	7	3.5	4.4	0.8	7	16.3	13.3	1.2
5	0.0	0.0	-	5	2.5	2.2	1.1	5	13.8	14.3	1.0
4	0.0	0.0	-	4	2.6	2.0	1.3	4	10.3	11.0	0.9
3	0.0	0.0	-	3	2.9	1.4	2.0	3	15.0	11.4	1.3
3	0.0	0.0	-	3	6.2	2.0	3.2	3	17.5	10.0	1.7
2	0.0	0.0	-	2	2.1	3.3	0.6	2	10.1	14.9	0.7
correlation coefficient			-	correlation coefficient			-0.500	correlation coefficient			0.020

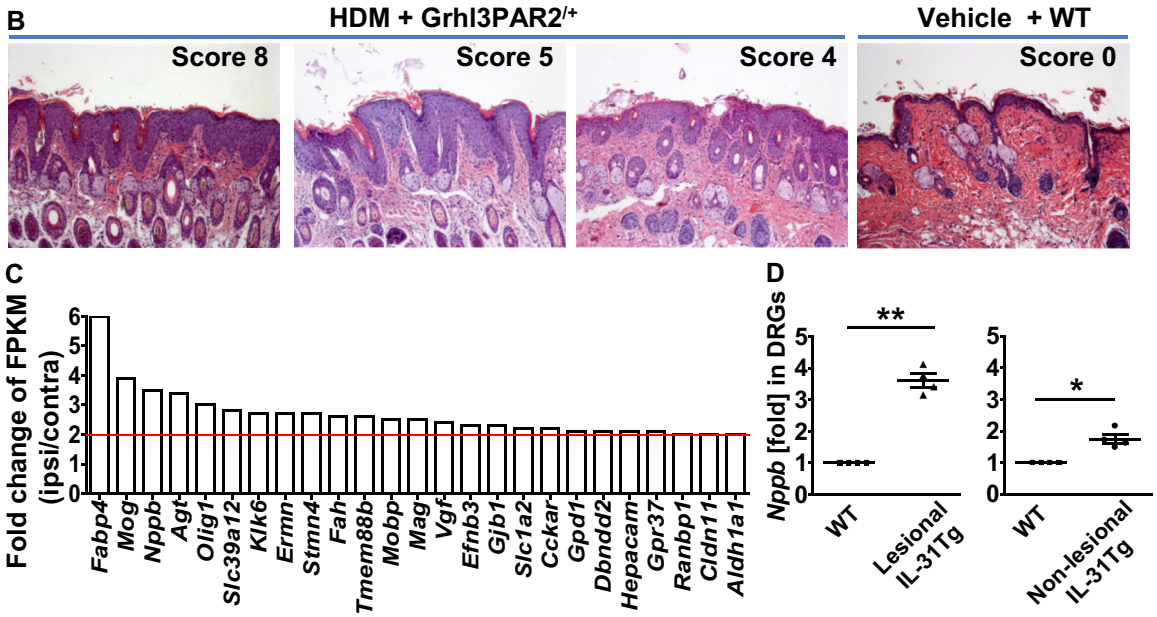

FIG 3. *Nppb* is upregulated in sensory ganglia from HDM-treated GrhI3PAR2$^{/+}$ mice and IL-31Tg mice. **A,** FPKM values for *Nppb*, *NPR1*, *NPR2*, IL-31, *IL31RA*, and *OSMR* genes from TGs of ipsilateral *(ipsi)* versus contralateral *(contra)* were analyzed by using RNA-Seq and compared. Eight mice were treated with HDM on the right cheek and nothing on the left cheek, and the cheek biopsy specimens were scored clinically by using H&E staining. The correlation coefficient between clinical score and fold change of *Nppb* is about 0.8, whereas for other genes analyzed, values are not significant. For these 8 mice, the average fold change of *Nppb* is 2.3. **B,** Representative H&E staining images show the severity of skin lesions from HDM-treated mice compared with vehicle-treated WT control mice. Images were taken by using a ×10 magnification objective. **C,** FPKM values analyzed by using RNA-Seq of the top 3 high clinical scores (scores were 8, 7, and 7) show the *Nppb* gene was upregulated by 3.5 times (ipsilateral vs contralateral). Average fold change for genes upregulated and reaching significance were plotted. A 2-fold change in FPKM value was deemed significant. **D,** RT-PCR analysis of *Nppb* mRNA levels in DRGs isolated from lesional and non-lesional IL-31Tg or WT mice. Values were normalized to the housekeeping gene *GAPDH*. Results (means ± SEMs) are pooled data from multiple animals. Significant differences are indicated as follows: $^{*}P < .05$ and $^{**}P < .01$.

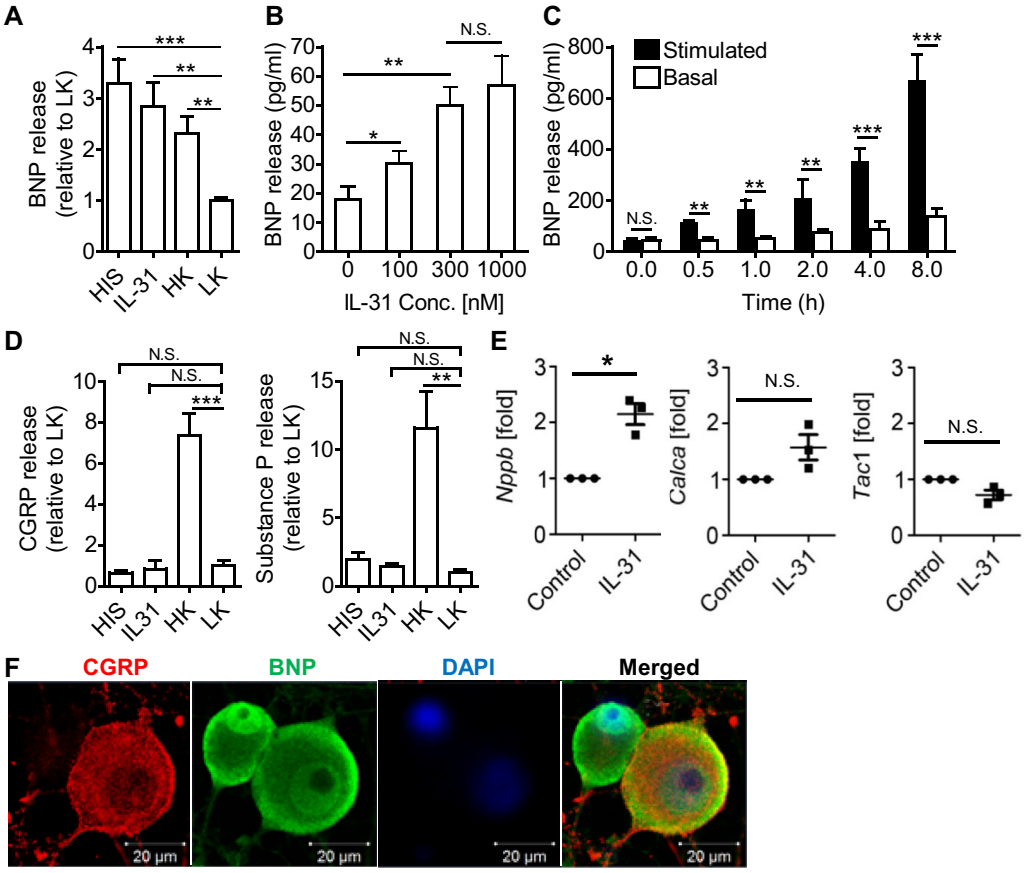

FIG 4. IL-31 induces release and mRNA synthesis of BNP (but not CGRP or SP) from cultured mDRGs. **A** and **D,** Release of BNP (Fig 4, *A*), CGRP, and SP (Fig 4, *D*) over 30 minutes was measured based on specific ELISAs or EIAs, and the increment of release after each treatment was plotted relative to the basal level. The amount in picograms per milliliter in basal (LK) release is 15.3 ± 1.2 for BNP, 3.9 ± 0.9 for SP, and 49.3 ± 11.8 for CGRP. *HIS*, Histamine; *HK*, high-potassium stimulation buffer; *LK*, low-potassium basal buffer. **B,** BNP release induced by various doses of IL-31 over 30 minutes. **C,** Time dependence of BNP release induced by 300 nmol/L IL-31. **E,** RT-PCR for mRNA of *Nppb*, *Calca*, and *Tac1* in cultured mDRGs after treatment with 100 ng/mL IL-31 for 6 hours. Data in Fig 4, *A-E*, are presented as means ± SEMs (n ≥ 3 independent experiments). Significant differences are indicated as follows: *N.S.*, $P > .05$; $*P < .05$; $**P < .01$; and $***P < .001$. *HIS*, Histamine. **F,** Immunofluorescence study for cellular localization of CGRP *(red)* and BNP *(green)* after dual labeling of DRGs with each specific antibody and counterstained with 4′,6-diamidino-2-phenylindole (*DAPI*; *blue*). Scale bars are as indicated.

both CGRP$^+$ and CGRP$^-$ neurons (Fig 4, *F*). This distinct but overlapping distribution suggests that BNP and CGRP can have discrete functional roles in transduction of itch signaling.

IL-31 induces BNP release from cultured mDRGs through SNARE-mediated vesicle fusion

BNP release from sensory neurons in response to itch stimuli might be regulated by selective exocytotic machinery. In terms of IL-31–induced BNP release, its mechanism is of particular importance because of its relevance in patients with AD. SNAREs are critical membrane fusion proteins and serve as therapeutic targets for many neurological diseases.[26] To explore their role in BNP release, synaptosomal-associated protein 25k (SNAP-25) and 2 other vesicular SNARE proteins, vesicle-associated membrane protein (VAMP) isoforms 1 (V1) and 7 (V7), were selectively knocked down by using lentiviral shRNA particles. Notably, expression levels of each protein were reduced substantially compared with nontargeted control levels (Fig 5, *A*). Knockdown of SNAP-25 and V1, but not V7, resulted in complete blockade of IL-31–elicited BNP release (Fig 5, *B*). For the first time, these findings demonstrated that selective SNARE proteins control IL-31–induced BNP release.

Expression of BNP and its receptors is increased in the skin of patients with AD

To understand BNP-induced signaling and identify the functional consequence of BNP in human skin, we examined the expression of BNP and its receptors, NPR1 and NPR2, in cultured hKCs. Using immunostaining, we readily detected expression of NPR1 and NPR2 in cultured hKCs (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). Both receptors showed punctate distribution. In great contrast, the BNP immune signal was less than the detection limit in these cultured cells (see Fig E4, *A*).

Next, similar experiments were performed on human skin sections from patients with AD and healthy control subjects. We

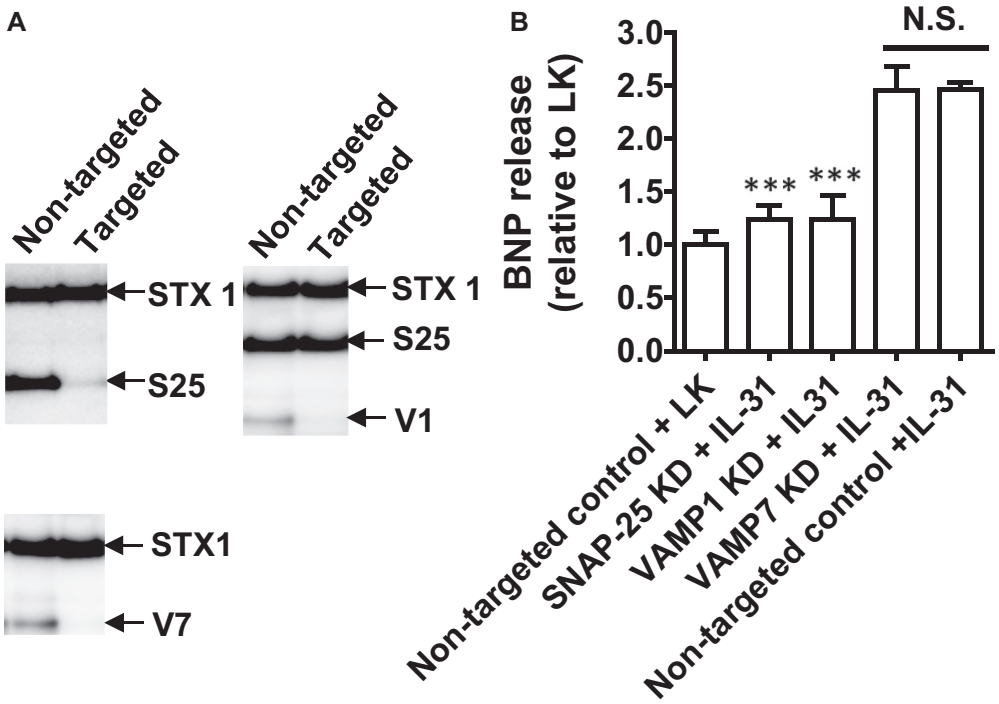

FIG 5. IL-31–induced BNP release from cultured mDRGs requires SNARE proteins. **A,** Representative immunoblots show knockdown of SNAP-25 (S25), VAMP1 (V1), and VAMP7 (V7) protein expression in cultured DRGs by using lentiviral shRNA particles. Syntaxin 1 *(STX 1)* serves as an internal control in each blot. Nontargeted virus-treated samples were loaded to the gels for comparison. **B,** The quantified plot demonstrates that 300 nmol/L IL-31–elicited BNP release over 30 minutes is inhibited after knockdown of S25 and V1 but not V7. Data are presented as means ± SEMs (n ≥ 3 independent experiments). Significance between specific shRNA-treated cells and nontargeted control cells is indicated as follows: not significant *(N.S.)*, $P > .05$; $***P < .001$.

found that in the epidermis no BNP staining was discernible in normal skin, similar to the observation in cultured keratinocytes, but it was readily detected in the skin of patients with AD (Fig 6, *A*), indicating a disease-related upregulation of BNP expression. We also detected immune signals of NPR1 and NPR2 in the epidermal keratinocytes of healthy skin (Fig 6, *A*), and both signals were enhanced in AD skin (Fig 6, *A*). We further analyzed the detailed distribution of these receptors by taking high-resolution confocal images in epidermal keratinocytes. Distinct localization of NPR1 and NPR2 was revealed in epidermal keratinocytes of AD skin sections. NPR1 resided predominantly along the plasma membrane of keratinocytes, whereas NPR2 was detected in both the plasmalemma and cytoplasm (Fig 6, *A*). The enhanced labeling of NPR1 and NPR2 might not be simply due to the acanthosis because we did observe increased immunoreactivity at the single-KC level by using immunofluorescence staining.

We further investigated BNP, NPR1, and NPR2 expression in the dermis of human skin. BNP expression was detected in the dermis of healthy skin, and its level appeared to be increased in dermal structures, including the secretory portion of sweat glands and blood vessels of AD skin (Fig 6, *B*). Interestingly, NPR2 (Fig 6, *C*, left panel) and NPR1 (data not shown) did not seem to colocalize with PGP9.5 in these structures. NPR1 and NPR2 co-occurred on the dermal structures, including the secretory portion of sweat glands and blood vessels (Fig 6, *C*, middle and right panels). There was no obvious incremental labeling of NPR1 or NPR2 in the dermal structures (Fig 6, *C*) and immunoreactive cells in AD skin compared with healthy skin (see Fig E5 in this article's Online Repository at www.jacionline.org).

Consistently, using RT-PCR, we also found *Nppb* transcripts were upregulated in lesional and nonlesional skin compared with that in WT skin (see Fig E6, *A*, in this article's Online Repository at www.jacionline.org). Moreover, BNP receptor *NPR1* mRNA levels were also increased in the lesional skin of IL-31Tg mice (see Fig E6, *B*).

Taken together, these collective data suggest that BNP might be involved in promoting the dermatitis response through activated/upregulated receptor in patients with AD.

BNP induces release of IL-17A, CXCL10, and matrix metalloproteinase 9 from cultured human keratinocytes through activation of glycogen synthase kinase 3 > c-Jun N-terminal kinase > extracellular signal-regulated kinase 1/2 ≥ p38

Keratinocytes of patients with AD exhibit a propensity to produce exaggerated cytokines, chemokines, and proteases, which play major role in promoting and maintaining inflammation.[27,28] However, nothing is known about the influence of BNP on hKCs. We first investigated the possible BNP effect on hKCs. Cells in culture were exposed to BNP for 24 hours before

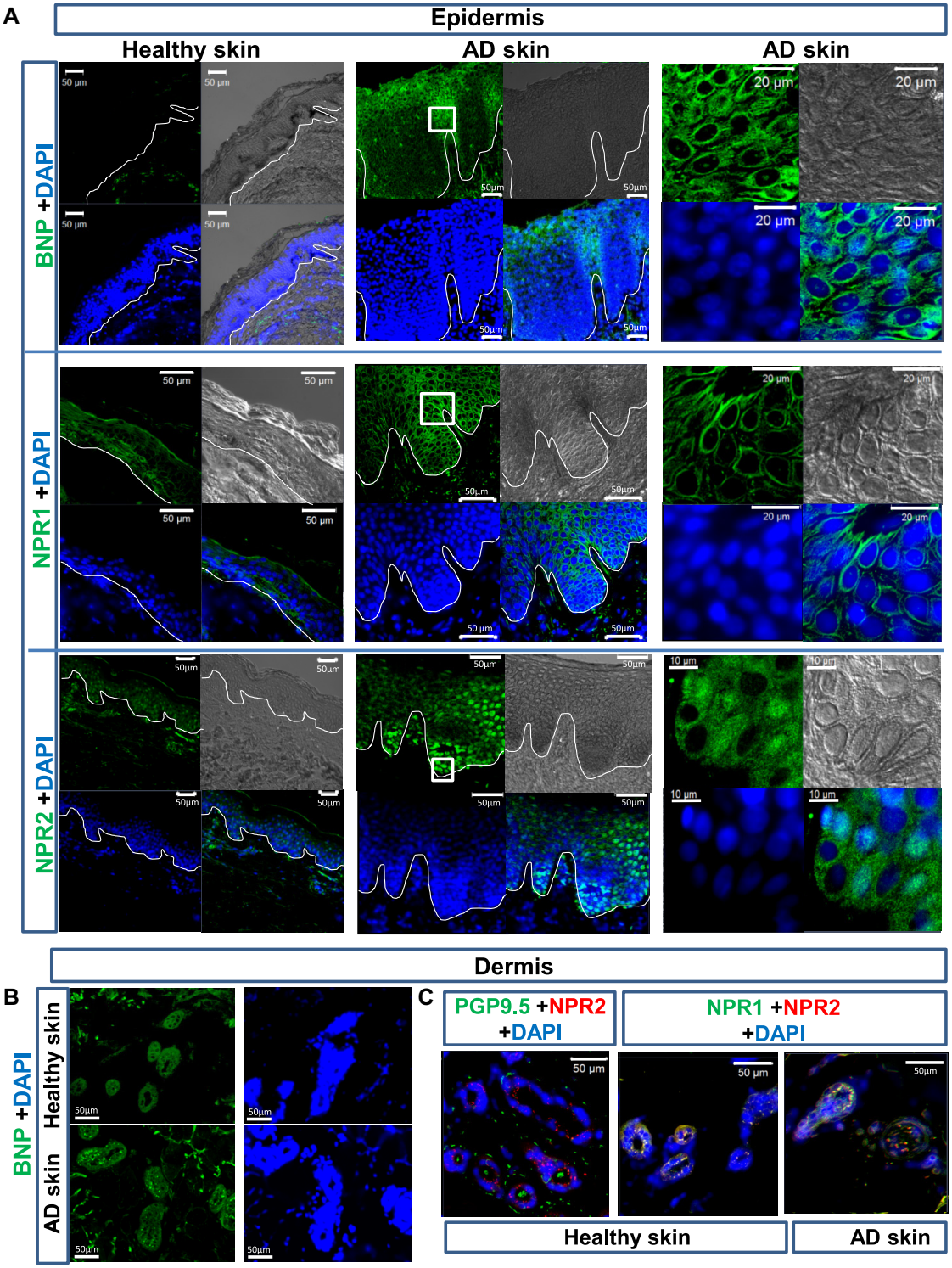

FIG 6. Immunohistochemical staining of BNP, NPR1, and NPR2 in the skin of human healthy control subjects and patients with AD. **A,** Representative fluorescent images show expression patterns of BNP, NPR1, and NPR2 in AD and healthy control skin. Note that the epidermal keratinocyte layer of AD skin showed enhanced staining of BNP, NPR1, and NPR2 compared with healthy control skin. Boxed areas in middle panels are shown at higher magnification in *right panels.* **B,** Images show increased BNP staining in dermal structures, including secretory portions of sweat glands and blood vessels in AD skin compared with healthy skin. **C,** NPR2 does not colocalize with the nerve marker PGP9.5 in dermal structures but shows high colocalization with NPR1. There is no obvious change in NPR1 and NPR2 expression in the dermis between skin of patients with AD and healthy control skin. Scale bars are as indicated. Paraffin-embedded human skin sections (n = 10) from each donor of patients with AD and healthy control subjects were stained for each condition. *White lines* denote epidermal-dermal junctions. *DAPI*, 4′,6-diamidino-2-phenylindole.

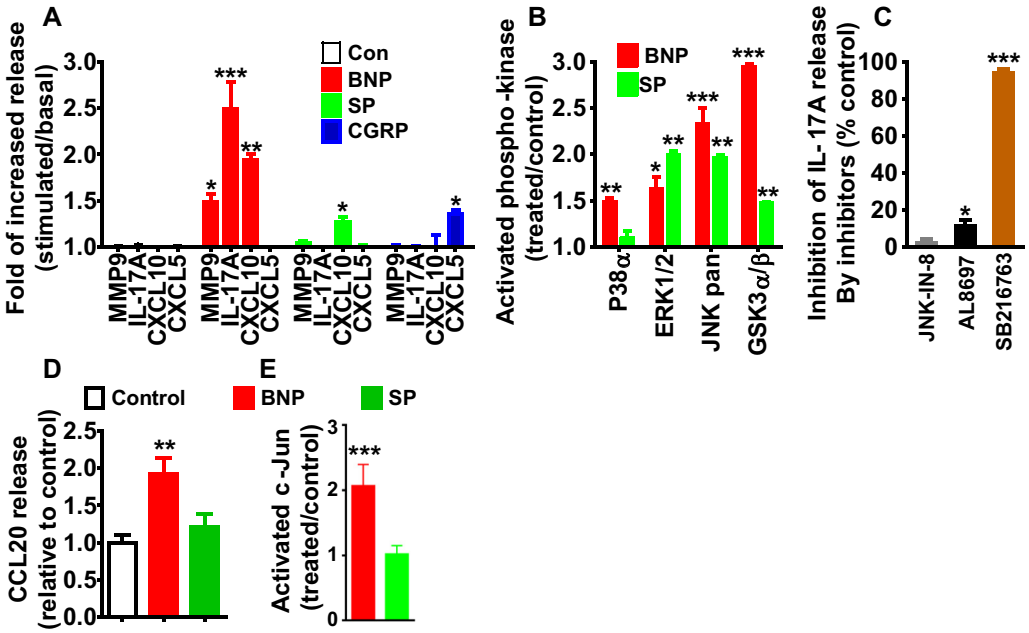

FIG 7. Effect of BNP on cytokine/chemokine release and activation of intracellular phosphokinase. **A,** Release profile of cytokines from cultured hKCs detected by using an antibody array. Data from BNP-, SP-, or CGRP-induced release were calculated relative to nontreated control (basal). **B,** Neuropeptide-induced activation of intracellular phosphokinases. Data plotted are the ratio of phosphorylated signal obtained from neuropeptide-treated cells relative to nontreated control cells. **C,** Percentages of inhibition by selective kinase inhibitors of BNP-induced IL-17A release from hKCs. **D,** CCL20 release from cultured hDCs induced by BNP or SP. **E,** Phosphorylation of intracellular c-Jun from hDCs after incubation with BNP or SP. Data are presented as means ± SEMs (n ≥ 3 independent experiments). Significance between stimulated and basal values is indicated as follows: $*P < .05$, $**P < .01$, $***P < .001$.

supernatants were collected and used for the proteome profiler human XL cytokine array, which allowed detection of 102 different cytokines simultaneously. An increase in IL-17A (approximately 2.5-fold), CXCL10 (approximately 2-fold), and, to a lesser degree, matrix metalloproteinase 9 (MMP9) levels was detected in culture supernatants (Fig 7, *A*). In contrast, significantly lower levels of cytokine release were triggered by the same dose of SP. In comparison, incubation of hKCs with 1 μmol/L human CGRP peptide for 24 hours induced only minimal CXCL5 release (Fig 7, *A*). IL-17A is a critical cytokine involved in the pathogenesis of AD.[29,30] Its expression in keratinocytes was reported[31-33] and detected in our primary culture (see Fig E4, *B*). CXCL10 mediates cell adhesion, migration, and inflammatory infiltrates in patients with AD.[34] Increased levels of IL-17A and CXCL10 are implicated in patients with AD.[28,29,35] Thus peripheral BNP might promote release of itch-related cytokines by acting on keratinocytes to propagate itch signals in patients with AD.

Subsequently, we investigated the signaling pathways involved in keratinocyte activation by using phosphokinase arrays on cell lysates of hKCs harvested after exposure to 1 μmol/L human BNP for 8 minutes. Glycogen synthase kinase 3 (GSK3), c-Jun N-terminal kinases (JNK; JNK kinase family includes 3 proteins named JNK1, JNK2, and JNK3), extracellular signal-regulated kinase 1/2, and p38 were significantly phosphorylated/activated by application of BNP. In contrast, extracellular signal-regulated kinase 1/2, JNK, and, to a lesser extent, GSK3 were activated by SP (Fig 7, *B*).

IL-17A is considered a potent stimulator of further inflammatory mediator production, amplifying the inflammatory response. Therefore, by using selective phosphokinase inhibitors, we investigated which intracellular kinase was responsible for the BNP-mediated IL-17A release from hKCs. The p38α inhibitor AL8697, GSK3 inhibitor SB216763 (inhibiting the activity of α and β isozymes of GSK3), or JNK inhibitor JNK-IN-8 (all at 1 μmol/L) were incubated with cultured hKCs for 1 hour before and during 24 hours of incubation with BNP before measuring IL-17A release with the IL-17A ELISA kit. Inhibition of GSK3 by SB216763 abolished IL-17A release, whereas AL8697 produced only minor blockade (Fig 7, *C*). Because IL-17A is regarded as a key cytokine involved in the pathogenesis of AD,[36,37] targeting GSK3 might prove effective for inhibition of itch transmission.

BNP stimulates release of CCL20 through c-Jun activation in monocyte-derived hDCs

We then assessed a possible functional consequence of BNP on hDCs, skin immune cells known to express BNP receptors.[38] To do this, monocyte-derived hDCs were maintained in the presence of IL-4 and GM-CSF to allow their differentiation before incubating with 1 μmol/L human BNP for 24 hours. We found CCL20 release was increased significantly by BNP, and this was not observed when incubated with 1 μmol/L SP (Fig 7, *D*). In the phosphokinase array BNP also activated c-Jun through phosphorylation, unlike SP, which did not show any effect on c-Jun activation (Fig 7, *E*). CCL20 levels are strongly increased in lesional skin tissue of patients with AD, and this increase stimulates migration of various immune cells.[39-41] Thus BNP induction of CCL20 release might

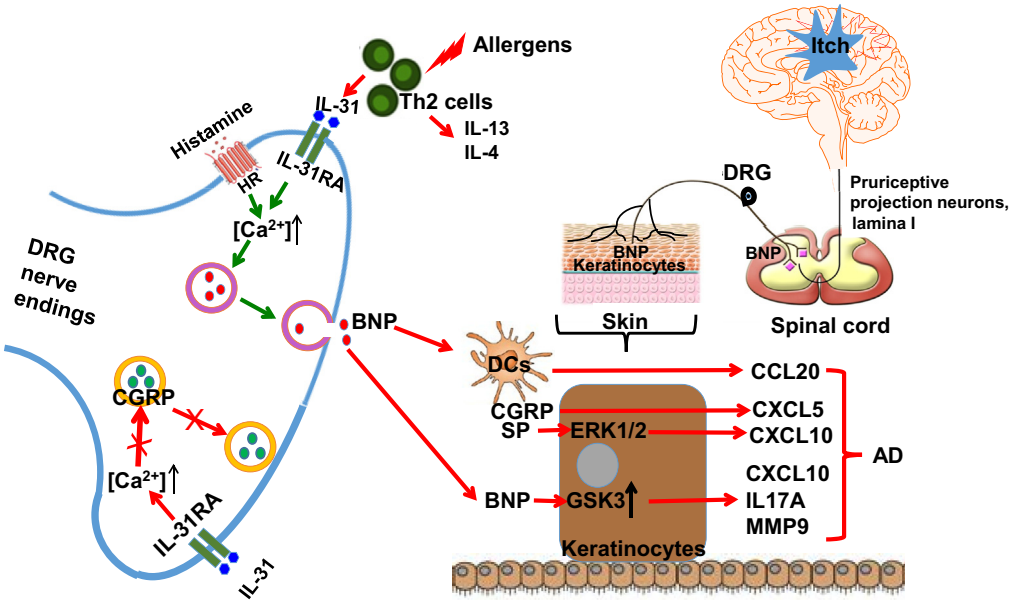

FIG 8. Schematic diagram showing an important communication link between IL-31 and BNP, both of which are key players in the signaling pathways implicated in pruritus. Itch inducers, such as IL-31 and histamine, elicit BNP release through SNARE-controlled mechanisms. Augmented release and synthesis by IL-31 of BNP might contribute to central and peripheral itch signaling. In contrast, CGRP and SP are not elicited by these pruritogens. Released BNP subsequently increases IL-17A, CXCL10, and MMP9 release from keratinocytes; however, SP and CGRP elicit only minimal release of CXCL10 and CXCL5, respectively. BNP also mediates CCL20 from dendritic cells *(DC)*. Taken together, it is postulated that these released cytokines/chemokines modulate itch transmission and pathogenesis of AD.

constitute a novel neuron immunomodulatory mechanism that results in activation of immature dendritic cells, which could contribute to the persistent itch.

DISCUSSION

Patients with AD experience a "vicious cycle of itching and scratching."[42] However, the molecular basis for this phenomenon is still unknown. Although it has been revealed recently that cytokines such as IL-31 and IL-4 or IL-13 play a role in immunity and itch transmission,[8,43,44] it is currently unknown whether cytokines like IL-31 also regulate neuropeptide release from peripheral nerve endings, thereby modulating neurogenic inflammation in patients with AD (Fig 8). Understanding the importance of neuropeptide- and cytokine-mediated intercellular communication would further explain not only the interplay between inflammation and itch but also how this debilitating symptomatology of inflammation and itch can be therapeutically interrupted. Here, for the first time, we show that peripherally released BNP is implicated in AD pathophysiology through IL-31 stimulation and subsequent regulation of cytokine, chemokine, and MMP9 release. Moreover, we show that BNP also activates several major cell types in skin that are pivotal in AD itch transmission, such as keratinocytes and dendritic cells (Fig 8). Thus we have revealed another mechanism of how T_H2 cells simultaneously affect itch and neuroinflammation.

BNP was originally identified as an important contributor of central itch because it is released from central primary afferents to the dorsal horn of the spinal cord.[19] Here, for the first time, we show that BNP is released from peripheral nerve endings on stimulation by pruritogens, such as IL-31 and histamine. These findings provide a new link between T_H2 cells, mast cells, and peripheral sensory nerves. Notably, this is unique to BNP as to other neuropeptides, such as CGRP and SP, because neither are synthesized or released under the same conditions, despite both being linked to itch.[45,46] In addition, BNP and its receptor, NPR1, are expressed in $CGRP^+$ small sensory neurons.[47] In fact, although traditionally viewed primarily as a pain mediator,[48] SP has also been implicated as an itch mediator in patients with AD, with increased numbers of SP^+ nerve fibers found concomitantly with a decrease in cutaneous SP levels in lesional skin of patients with AD[49,50] and responsiveness to the neurokinin 1 receptor antagonist aprepitant.[45,51] In particular, BNP-expressing sensory neurons are found not to be involved in acute, inflammatory, or neuropathic pain,[19,52] unlike SP and CGRP. Therefore our findings further differentiate BNP from CGRP and SP and highlight its importance in the neuropeptide-mediated pathogenesis of AD-associated itch.

Here, for the first time, we show that IL-31RA and OSMRβ highly overlap in hDRGs, similar to the results observed previously in mDRGs.[53] Moreover, in hDRGs IL-31 elicited the Ca^{2+} transient in approximately 50% of ET-1–responsive neurons and approximately 2% of total neurons. Half of ET-1–responsive neurons also responded to histamine. To our knowledge, this is the first detailed characterization of human sensory subpopulations according to their response to sequential and combined pruritogens. In contrast, IL-31 and ET-1 excited a partly overlapping small subset of mDRG neurons. In response to IL-31, *Nppb* transcripts in cultured mDRGs were specifically upregulated, but this was not found for *Tac1* or *Calca*. IL-31 was capable of eliciting an approximately 2.7-fold increase in BNP release over the basal level. The elicited release was completely dependent on the particular SNARE proteins, SNAP-25 and VAMP1, which are

also important for several other inflammatory diseases.[54] In contrast to the minimal BNP release at resting conditions, IL-31–induced augmentation of peripheral BNP release can link to excessive itch in patients with AD induced by a positive feedback loop of amplifying itch mediators. This might also explain the difficulty of treating AD-associated itch.

Using deep sequencing, we detected upregulation of *Nppb* transcripts in isolated TGs from HDM-treated PAR2Tg mice, which showed AD-like skin conditions. Furthermore, our data confirm that the *Nppb* gene is implicated in severe AD conditions because mice with high clinical scores exhibit high levels of *Nppb* transcripts.

Our findings provide the first evidence showing a functional association of BNP with the severity of AD. Consistently, we established that *Nppb* transcripts in DRGs and skin of lesional IL-31Tg mice were also greatly upregulated. NPR1 transcripts were increased in lesional IL-31Tg skin. In fact, upregulation of BNP in murine AD-like skin has also been reported by a recent finding using the global transcriptome, which identified a significant *Nppb* upregulation (>3-fold) in skin of oxazolone-challenged mice with AD-like dermatitis.[55]

In a translational approach we also provide evidence for the importance of BNP in patients with AD by using skin from patients with AD and healthy control subjects. We detected increased protein levels of NPR1 and NPR2 in the epidermis, particularly in keratinocytes. Moreover, enhanced expression of BNP in both the epidermis and dermis of patients with AD was revealed. In fact, immunostaining signal of BNP, NPR1, and NPR2 protein seemed to be increased in single-cell levels of keratinocytes; however, we could not exclude that the possibility that acanthosis might also contribute to the increment. Upregulated expression levels of BNP and its receptors in patients with AD suggest a role for the IL-31/BNP/BNP receptor axis in peripheral itch amplification in patients with AD.

Although IL-31 rapidly induces itch in mice[7,8] and is regarded as one of the major "drivers" of itch in patients with AD, it did not induce immediate itch responses in human subjects, as shown in a recent study including both patients with AD and healthy subjects monitored by using skin prick testing.[56] Although there is a possibility of insufficient amounts of IL-31 being administered that did not induce an immediate itch response, late onset of IL-31–induced mild itch sensations can also be attributed to this cytokine exerting its pruritic effect indirectly through keratinocytes and secondary mediators rather than through a sole action on its receptors on cutaneous sensory nerves.[56,57] In addition, similar to IL-4,[44] IL-31 might be a sensitizer in some patients and not a direct itch inducer. However, variable expression levels of IL-31 and IL-31RA in patients with AD might explain that some patients will respond more to IL-31 stimulation. Consequently, some patients can profit better from anti–IL-31 therapy than others.[11,12] It will be interesting to learn whether IL-4 and IL-13 are also linked to BNP release.

We demonstrate that BNP acts as the downstream skin-derived effector of IL-31. In addition to its contribution to spinal processing of itch,[19] excessive release of BNP from peripheral sensory nerves stimulates NPR1 receptor to activate multiple intracellular signaling pathways in the skin, thereby contributing to skin inflammation in patients with AD. We found that BNP activates the GSK3 pathway and leads to secretion of several important inflammatory or itch modulators, such as IL-17A, CXCL10, or MMP9, from skin keratinocytes. BNP activates c-Jun to release CCL20 from DCs. Levels of all of these cytokines are known to be increased in patients with AD and represent an important potential component of the pathology of AD.[58,59] Upregulation of BNP receptors, in turn, could further augment secretion of the above important itch modulators from these cells. Thus our results support a new pathway in which BNP is a peripheral contributor of IL-31–mediated neurogenic inflammation in patients with AD and establishes the missing link between T_H2 nerve-mediated inflammation and pruritus.

In conclusion, we describe a novel functional link between T_H2 cells and sensory nerves through IL-31 and BNP and provide the first evidence between a functional association of peripheral BNP in AD using human and rodent DRGs, animal models, and patient skin in a translational fashion. Indeed, T_H2 cells, sensory neurons, keratinocytes, and dendritic cells release a complex network of cytokines and chemokines establishing a local milieu and environment that favors AD skin inflammation. In this context BNP seems to act as an important "relay center" for peripheral and central itch circuits to facilitate both itch and neuroinflammation in the pathogenesis of AD. We demonstrate that BNP acts through multiple mechanisms: (1) IL-31 modulates pruriceptive neurons to rapidly induce SNARE-dependent release of BNP; (2) IL-31 increases *Nppb* synthesis; (3) BNP receptors are upregulated in patients with AD; and (4) BNP signals through its upregulated receptors to directly activate multiple intracellular kinases in skin cells to elicit the release of itch-related proinflammatory cytokines (Fig 8). Our findings provide new insights about BNP as an important regulator of neuroinflammation, as well as itch, in the skin of patients with AD, and close a missing link about why the itch-related inflammatory skin disease AD has been also defined as neurodermatitis. In addition, peripheral BNP signaling provides a new basis for the development of more effective therapies for AD and probably other skin diseases.

Key messages

- **IL-31 induces SNARE-dependent BNP release and its synthesis in sensory nerves.**
- **BNP increases AD-related cytokine release from keratinocytes and dendritic cells through GSK3-dependent and c-Jun activation pathways, respectively.**
- **AD is associated with upregulation of BNP and its receptor.**

REFERENCES

1. Bieber T. Atopic dermatitis. N Engl J Med 2008;358:1483-94.
2. Weidinger S, Novak N. Atopic dermatitis. Lancet 2016;387:1109-22.
3. Steinhoff M, Bienenstock J, Schmelz M, Maurer M, Wei E, Biro T. Neurophysiological, neuroimmunological, and neuroendocrine basis of pruritus. J Invest Dermatol 2006;126:1705-18.
4. Roosterman D, Goerge T, Schneider SW, Bunnett NW, Steinhoff M. Neuronal control of skin function: the skin as a neuroimmunoendocrine organ. Physiol Rev 2006;86:1309-79.
5. Buddenkotte J, Steinhoff M. Pathophysiology and therapy of pruritus in allergic and atopic diseases. Allergy 2010;65:805-21.
6. 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;117:411-7.
7. 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;5:752-60.
8. 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;133:448-60.

9. Feld M, Garcia R, Buddenkotte J, Katayama S, Lewis K, Muirhead G, et al. The pruritus- and TH2-associated cytokine IL-31 promotes growth of sensory nerves. J Allergy Clin Immunol 2016;138:500-8.e24.
10. Raap U, Weissmantel S, Gehring M, Eisenberg AM, Kapp A, Folster-Holst R. IL-31 significantly correlates with disease activity and Th2 cytokine levels in children with atopic dermatitis. Pediatr Allergy Immunol 2012;23:285-8.
11. Ruzicka T, Hanifin JM, Furue M, Pulka G, Mlynarczyk I, Wollenberg A, et al. Anti-interleukin-31 receptor A antibody for atopic dermatitis. N Engl J Med 2017;376:826-35.
12. Ruzicka T, Mihara R. Anti-interleukin-31 receptor A antibody for atopic dermatitis. N Engl J Med 2017;376:2093.
13. 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;27:505.e136.
14. Michels GM, Ramsey DS, Walsh KF, Martinon OM, Mahabir SP, Hoevers JD, et al. A blinded, randomized, placebo-controlled, dose determination trial of lokivetmab (ZTS-00103289), a caninized, anti-canine IL-31 monoclonal antibody in client owned dogs with atopic dermatitis. Vet Dermatol 2016;27:478.e129.
15. 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;171:5049-58.
16. Grimstad O, Sawanobori Y, Vestergaard C, Bilsborough J, Olsen UB, Gronhoj-Larsen C, et al. Anti-interleukin-31-antibodies ameliorate scratching behaviour in NC/Nga mice: a model of atopic dermatitis. Exp Dermatol 2009;18:35-43.
17. Bilsborough J, Mudri S, Chadwick E, Harder B, Dillon SR. IL-31 receptor (IL-31RA) knockout mice exhibit elevated responsiveness to oncostatin M. J Immunol 2010;185:6023-30.
18. Bautista DM, Wilson SR, Hoon MA. Why we scratch an itch: the molecules, cells and circuits of itch. Nat Neurosci 2014;17:175-82.
19. Mishra SK, Hoon MA. The cells and circuitry for itch responses in mice. Science 2013;340:968-71.
20. Liu XY, Wan L, Huo FQ, Barry DM, Li H, Zhao ZQ, et al. B-type natriuretic peptide is neither itch-specific nor functions upstream of the GRP-GRPR signaling pathway. Mol Pain 2014;10:4.
21. Matsukawa N, Grzesik WJ, Takahashi N, Pandey KN, Pang S, Yamauchi M, et al. The natriuretic peptide clearance receptor locally modulates the physiological effects of the natriuretic peptide system. Proc Natl Acad Sci U S A 1999;96:7403-8.
22. Zhang FX, Liu XJ, Gong LQ, Yao JR, Li KC, Li ZY, et al. Inhibition of inflammatory pain by activating B-type natriuretic peptide signal pathway in nociceptive sensory neurons. J Neurosci 2010;30:10927-38.
23. Li CL, Li KC, Wu D, Chen Y, Luo H, Zhao JR, et al. Somatosensory neuron types identified by high-coverage single-cell RNA-sequencing and functional heterogeneity. Cell Res 2016;26:83-102.
24. Davidson S, Copits BA, Zhang J, Page G, Ghetti A, Gereau RW. Human sensory neurons: membrane properties and sensitization by inflammatory mediators. Pain 2014;155:1861-70.
25. Frateschi S, Camerer E, Crisante G, Rieser S, Membrez M, Charles RP, et al. PAR2 absence completely rescues inflammation and ichthyosis caused by altered CAP1/Prss8 expression in mouse skin. Nat Commun 2011;2:161.
26. Meng J, Wang J. Role of SNARE proteins in tumourigenesis and their potential as targets for novel anti-cancer therapeutics. Biochim Biophys Acta 2015;1856:1-12.
27. Albanesi C, Scarponi C, Giustizieri ML, Girolomoni G. Keratinocytes in inflammatory skin diseases. Curr Drug Targets Inflamm Allergy 2005;4:329-34.
28. Giustizieri ML, Mascia F, Frezzolini A, De Pita O, Chinni LM, Giannetti A, et al. Keratinocytes from patients with atopic dermatitis and psoriasis show a distinct chemokine production profile in response to T cell-derived cytokines. J Allergy Clin Immunol 2001;107:871-7.
29. Koga C, Kabashima K, Shiraishi N, Kobayashi M, Tokura Y. Possible pathogenic role of Th17 cells for atopic dermatitis. J Invest Dermatol 2008;128:2625-30.
30. Toda M, Leung DY, Molet S, Boguniewicz M, Taha R, Christodoulopoulos P, et al. Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. J Allergy Clin Immunol 2003;111:875-81.
31. Li ZJ, Choi DK, Sohn KC, Lim SK, Im M, Lee Y, et al. Induction of interleukin-22 (IL-22) production in CD4+ T cells by IL-17A secreted from CpG-stimulated keratinocytes. Ann Dermatol 2016;28:579-85.
32. Yu J, Xiao Z, Zhao R, Lu C, Zhang Y. Paeoniflorin suppressed IL-22 via p38 MAPK pathway and exerts anti-psoriatic effect. Life Sci 2017;180:17-22.
33. An J, Li T, Dong Y, Li Z, Huo J. Terminalia Chebulanin attenuates psoriatic skin lesion via regulation of heme oxygenase-1. Cell Physiol Biochem 2016;39:531-43.
34. Flier J, Boorsma DM, van Beek PJ, Nieboer C, Stoof TJ, Willemze R, et al. Differential expression of CXCR3 targeting chemokines CXCL10, CXCL9, and CXCL11 in different types of skin inflammation. J Pathol 2001;194:398-405.
35. Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity 2004;21:467-76.
36. Kirkham BW, Kavanaugh A, Reich K. Interleukin-17A: a unique pathway in immune-mediated diseases: psoriasis, psoriatic arthritis and rheumatoid arthritis. Immunology 2014;141:133-42.
37. Dhingra N, Guttman-Yassky E. A possible role for IL-17A in establishing Th2 inflammation in murine models of atopic dermatitis. J Invest Dermatol 2014;134:2071-4.
38. Morita R, Ukyo N, Furuya M, Uchiyama T, Hori T. Atrial natriuretic peptide polarizes human dendritic cells toward a Th2-promoting phenotype through its receptor guanylyl cyclase-coupled receptor A. J Immunol 2003;170:5869-75.
39. Campbell JJ, Hedrick J, Zlotnik A, Siani MA, Thompson DA, Butcher EC. Chemokines and the arrest of lymphocytes rolling under flow conditions. Science 1998;279:381-4.
40. Dieu MC, Vanbervliet B, Vicari A, Bridon JM, Oldham E, Ait-Yahia S, et al. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. J Exp Med 1998;188:373-86.
41. Krzysiek R, Lefevre EA, Bernard J, Foussat A, Galanaud P, Louache F, et al. Regulation of CCR6 chemokine receptor expression and responsiveness to macrophage inflammatory protein-3alpha/CCL20 in human B cells. Blood 2000;96:2338-45.
42. Steinhoff M, Cevikbas F, Yeh I, Chong K, Buddenkotte J, Ikoma A. Evaluation and management of a patient with chronic pruritus. J Allergy Clin Immunol 2012;130:1015-6.e7.
43. Oh MH, Oh SY, Lu J, Lou H, Myers AC, Zhu Z, et al. TRPA1-dependent pruritus in IL-13-induced chronic atopic dermatitis. J Immunol 2013;191:5371-82.
44. Oetjen LK, Mack MR, Feng J, Whelan TM, Niu H, Guo CJ, et al. Sensory neurons co-opt classical immune signaling pathways to mediate chronic itch. Cell 2017;171:217-28.e13.
45. Azimi E, Reddy VB, Pereira PJ, Talbot S, Woolf CJ, Lerner EA. Substance P activates Mas-related G protein-coupled receptors to induce itch. J Allergy Clin Immunol 2017;140:447-53.e3.
46. 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;286:1140-5.
47. Marchenkova A, Vilotti S, Fabbretti E, Nistri A. Brain natriuretic peptide constitutively downregulates P2X3 receptors by controlling their phosphorylation state and membrane localization. Mol Pain 2015;11:71.
48. Neumann S, Doubell TP, Leslie T, Woolf CJ. Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature 1996;384:360-4.
49. Fantini F, Pincelli C, Romualdi P, Donatini A, Giannetti A. Substance P levels are decreased in lesional skin of atopic dermatitis. Exp Dermatol 1992;1:127-8.
50. Giannetti A, Fantini F, Cimitan A, Pincelli C. Vasoactive intestinal polypeptide and substance P in the pathogenesis of atopic dermatitis. Acta Dermatovenereol Suppl 1992;176:90-2.
51. Stander S, Siepmann D, Herrgott I, Sunderkotter C, Luger TA. Targeting the neurokinin receptor 1 with aprepitant: a novel antipruritic strategy. PLoS One 2010;5:e10968.
52. Pitake S, Debrecht J, Mishra SK. [EXPRESS] Brain natriuretic peptide (BNP) expressing sensory neurons are not involved in acute, inflammatory or neuropathic pain. Mol Pain 2017;1317448069177369 93.
53. Bando T, Morikawa Y, Komori T, Senba E. Complete overlap of interleukin-31 receptor A and oncostatin M receptor beta in the adult dorsal root ganglia with distinct developmental expression patterns. Neuroscience 2006;142:1263-71.
54. Meng J, Wang J, Lawrence G, Dolly JO. Synaptobrevin I mediates exocytosis of CGRP from sensory neurons and inhibition by botulinum toxins reflects their anti-nociceptive potential. J Cell Sci 2007;120:2864-74.
55. Ewald DA, Noda S, Oliva M, Litman T, Nakajima S, Li X, et al. Major differences between human atopic dermatitis and murine models, as determined by using global transcriptomic profiling. J Allergy Clin Immunol 2017;139:562-71.
56. 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;69:113-7.
57. Cheung PF, Wong CK, Ho AW, Hu S, Chen DP, Lam CW. Activation of human eosinophils and epidermal keratinocytes by Th2 cytokine IL-31: implication for the immunopathogenesis of atopic dermatitis. Int Immunol 2010;22:453-67.
58. Harper JI, Godwin H, Green A, Wilkes LE, Holden NJ, Moffatt M, et al. A study of matrix metalloproteinase expression and activity in atopic dermatitis using a novel skin wash sampling assay for functional biomarker analysis. Br J Dermatol 2010;162:397-403.
59. Kanda N, Shimizu T, Tada Y, Watanabe S. IL-18 enhances IFN-gamma-induced production of CXCL9, CXCL10, and CXCL11 in human keratinocytes. Eur J Immunol 2007;37:338-50.

METHODS

Materials

Skin samples from patients with AD and healthy control subjects were bought from Tissue Solutions. EIA kits for CGRP and SP were bought from Bioquote (York, United Kingdom); rabbit anti–syntaxin 1, anti-VAMP1, and anti-VAMP7 were bought from Synaptic Systems GmbH (Göttingen, Germany). SP, BNP, CGRP, histamine, and ET-1; mAb specific for CGRP (4901); rabbit anti-PGP9.5; ELISA kit for BNP; and shRNA lentiviral particles were bought from Sigma-Aldrich (St Louis, Mo). Donkey anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 594, donkey anti-goat Alexa Fluor 594, and anti-mouse Alexa Fluor 488 were supplied by Jackson ImmunoResearch, (West Grove, Pa). Ibidi GmbH (Planegg, Germany) provided culture chambers. Rabbit anti-BNP antibody (G-011-23) was purchased from Phoenix Pharmaceuticals (Burlingame, Calif), and monoclonal anti-SNAP-25 (SMI 81) was bought from Sternberger Monoclonals (Baltimore, Md). Mouse monoclonal anti-PGP9.5 (Ab8189), anti-NPR2 (Ab55724), rabbit anti-NPR1 (Ab14356), anti-BNP (Ab19645), and anti-NeuN (Ab177487) antibodies and an IL-17A ELISA kit were provided by Abcam (Cambridge, United Kingdom). AL8697, goat anti–IL-31RA, mouse anti-OSMRβ, CCL20 ELISA kit, proteome profiler human XL cytokine array, human phosphokinase array kits, and IL-17A antibody (AF-317-NA) were bought from R&D Systems (Minneapolis, Minn). SB216763 was purchased from Tocris Bioscience (Bristol, United Kingdom). JNK-IN-8 was obtained from Axon Medchem (Reston, Va). Rabbit anti-NPR1 antibody (NBP1-31333) was purchased from Novus Biologicals (Littleton, Colo). Adult normal hKCs and hDCs and their culture medium were bought from Lonza. hDRG paraffin sections were bought from Zyagen. Alexa Fluor 594–conjugated mouse anti-human vimentin antibody, Alexa Fluor 488–conjugated mouse anti-human CD4, and Alexa Fluor 488–conjugated mouse anti-human CD80 were purchased from BioLegend (San Diego, Calif). Mouse C57 DRG sections were purchased from Amsbio.

HDM application in the Grhl3PAR2$^{/+}$ mouse model

Grhl3PAR2$^{/+}$ mice were maintained in the C57BL6/J-129X1/SvJ mixed strain and used for experiments as an AD model. We used mite extract ointment/HDM (BiostirAD catalog no. 303-34131) on the right cheek to induce severe skin lesions. For HDM treatment, 8 mice were shaved on the cheek 1 day before application. On the application day, 4% SDS was rubbed on the shaved cheek area 2 hours before HDM application. HDM was applied on the right cheek twice a week with at least a 3-day resting period before the next application for 6 weeks. At the end of the treatment, we killed the mice and harvested lesional, perilesional, and nonlesional skin for pathologic diagnosis using H&E staining to measure the clinical score, and TGs were harvested for gene expression experiments.

RNA-Seq

For gene expression experiments, TGs were harvested from Grhl3PAR2$^{/+}$ mice. Total RNA was isolated from the right (ipsilateral) and left (contralateral) TGs with TRIzol Reagent (catalog no. 15596; Life Technologies, Darmstadt, Germany). Then the mRNA library was made with the Ovation Universal RNA-Seq System (NuGEN catalog no. 0343). Samples were subsequently sequenced on the HiSeq2500 (Illumina, San Diego, Calif).

Quantitative real-time PCR

DRGs were isolated from adult C57Bl6 mice (6-8 weeks old) and treated with 3 mg/mL collagenase (Sigma-Aldrich) and 0.25 mg/mL trypsin (PAA) for 30 minutes. DRGs were triturated for dissociation and plated onto cell-culture dishes coated with poly-L-lysine (100 mg/mL; Sigma-Aldrich) and laminin (5 mg/mL; Sigma-Aldrich) in MEM supplemented with 10% horse serum, 1% penicillin/streptomycin, 1% vitamins, 1% N2 supplement, and 2% B27 supplement. After an overnight rest, DRGs were treated with 100 ng/mL murine IL-31 (ZymoGenetics, Seattle, Wash) for 6 hours. Quantitation of mRNA levels was performed by using real-time fluorescence detection with Absolute SYBR Green ROX mix (ABI).

Primers used for both DRGs and skin were as follows: m*Nppb* forward, 5′-gtcagtcgtttgggctgtaac-3′; m*Nppb* reverse, 5′-agacccaggcagagtcagaa-3′; m*Calca* forward, 5′-agcaggaggaagagcagga-3′; m*Calca* reverse, 5′- cagattcccacaccgcttag-3′; m*Tac1* forward, 5′- agcctcagcagttctttgga-3′; and m*Tac1* reverse, 5′- tctggccatgtccataaagag-3′. Primers and probes specific for 18S RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Life Technologies and Eurofins Genomics (Ebersberg, Germany). Target gene expression was analyzed on an ABI Prism 7000 supplemented with SDS 1.2.3 software, and the expression profile was normalized with 18S or GAPDH expression. In the case of mouse tissue, primers used were as follows: *mNPR1* forward, 5′- ttccacactggaggttctggct-3′; *mNPR1* reverse, 5′- ctctgagaccagctccttttcc-3′.

BNP, CGRP, and SP release assays

DRGs were isolated from postnatal d5 C57BL/6 mice and dissociated by collagenase I, as previously described, to investigate BNP, SP, and CGRP release.[E1] Neurons were cultured in the presence of cytosine β-d-arabinofuranoside (Sigma) and nerve growth factor 100 ng/mL for 7 days *in vitro*. Basal low-potassium release buffer (22.5 mmol/L HEPES, 135 mmol/L NaCl, 3.5 mmol/L KCl, 1 mmol/L $MgCl_2$, 2.5 mmol/L $CaCl_2$, 3.3 mmol/L glucose, and 0.1% BSA, pH 7.4) was added into each well, followed by a 30-minute incubation at 37 °C.[E2] Cells were then stimulated for 30 minutes or as indicated in figures by high potassium (60 mmol/L K^+ isotonically balanced with NaCl), 1 μmol/L histamine, or 300 nmol/L IL-31 (ZymoGenetics). BNP, CGRP, or SP release was quantified by using ELISA or EIA kits.

Measurement of intracellular Ca^{2+}

For hDRG calcium measurement, cells in culture were loaded with 5 μmol/L Fluo-8 AM (AAT Bioquest 21081) containing 0.1% Pluronic F-127 (Sigma P2443) for 20 minutes. The extracellular solution contained the following: 145 mmol/L NaCl, 3 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, and 10 mmol/L glucose adjusted to pH 7.4 with NaOH. Fluo-8–loaded cells were excited at 480 nm, and emission was collected at 520 nm with a pcoEDGE sCMOS camera (PCO) mounted on an inverted microscope (IX71; Olympus, Center Valley, Pa). After a baseline period of 60 seconds, ET-1 (30 nmol/L), histamine (10 μmol/L), and IL-31 (300 nmol/L) were applied sequentially for 60 seconds each, followed by washout for 5 minutes. Images were acquired at 0.2 Hz. In the case of mDRGs, 3 μmol/L Fluo-4 AM was used, and images were acquired with a Zeiss LSM710 confocal microscope (Carl Zeiss MicroImaging) with an argon laser at 2-second intervals.

Immunofluorescence staining

For cellular localization of CGRP and BNP in cultured mDRGs, cells were processed by means of dual labeling overnight at 4°C with 1:100 rabbit anti-BNP antibody (G-011-23; Phoenix Pharmaceuticals) and 1:200 mouse anti-CGRP antibody (clone 4901; Sigma). Secondary donkey anti-rabbit Alexa Fluor 488 (1:2000) and anti-mouse Alexa Fluor 594 (1:2000) were added for 1 hour at room temperature.

hDRGs and skin paraffin sections (15 μm) were deparaffinized, rehydrated before permeabilization in PBS with 0.2% Triton X-100, and then incubated in PBS containing 5% normal donkey serum (blocking solution) at room temperature for 1 hour. Specimens were then incubated with primary antibodies in blocking solution (at 4°C overnight). For hDRG sections, primary antibodies were used at the following dilutions: 1:100 goat anti–IL-31RA (R&D Systems), 1:100 mouse anti-OSMRβ (R&D Systems), 1:40 mouse PGP9.5 mAb (Abcam) or 1:1000 rabbit anti-PGP9.5 (Sigma), and 1:500 rabbit anti-NeuN antibody (Abcam). Sections were washed in PBS and incubated in donkey anti-goat Alexa Fluor 594 and donkey anti-mouse Alexa Fluor 488 (Jackson ImmunoResearch) or donkey anti-rabbit Alexa Flour 488 diluted 1:2000 with blocking solution (at room temperature 1 hour). For human skin sections (15 μm), we used rabbit polyclonal antibody to BNP

(1:1000; Abcam), rabbit polyclonal antibody to NPR1 (1:500; Novus Bio), or mouse mAb to NPR2 (1:80; Abcam) or rabbit anti-PGP9.5 (Sigma).

For hKC staining, cultured cells were fixed in 3.7% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, and then blocked in 5% normal donkey serum (blocking solution) at room temperature for 1 hour before samples were incubated with the primary antibodies overnight at 4°C. Antibodies used were as follows: 1:500 rabbit anti-NPR1 (Abcam), 1:1000 rabbit anti-BNP (Abcam), 1:80 mouse mAb to NPR2 (Abcam), 1:200 mouse mAb to K14 (cytokeratin 14; Ab7800), 1:100 goat anti–IL-17A (R&D Systems), 1:200 Alexa Fluor 594–conjugated mouse anti-human vimentin antibody, 1:200 Alexa Fluor 488–conjugated mouse anti-human CD4, and 1:200 Alexa Fluor 488–conjugated mouse anti-human CD80. The specimens were washed in PBS and incubated in donkey anti-goat Alexa Fluor 594 or donkey anti-goat Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 488 or donkey anti-rabbit Alexa Fluor 488 (Jackson ImmunoResearch) diluted 1:2000 with blocking solution (at room temperature for 1 hour).

Rabbit anti-BNP antibody (G-011-23; Phoenix Pharmaceuticals) was preincubated with or without mouse BNP-45 peptide (Phoenix Europe) at a 1:10 molar ratio to check the specificity of BNP and CGRP antibodies. Likewise, mouse anti-CGRP antibody (clone 4901; Sigma) was preincubated with or without rat α-CGRP peptide (Sigma) at a 1:10 molar ratio. Antibodies alone or with their antigen mixtures were incubated overnight at 4°C. Mouse C57 DRG sections purchased from Amsbio were formalin fixed, permeabilized in PBS with 0.1% Triton X-100, and blocked in PBS containing 5% normal donkey serum. Immunofluorescence labeling was performed overnight at 4°C with 1:100 rabbit anti-BNP antibody, 1:200 mouse anti-CGRP antibody alone, or antibody preincubated with its antigen (as specified in figure legends) in blocking solution. After extensive washing, secondary donkey anti-rabbit Alexa Fluor 488 (1:2000) and anti-mouse Alexa Fluor 594 (1:2000) diluted in blocking solution were added for 1 hour at room temperature.

After the final wash of the secondary antibody, specimens were mounted onto slides by using Prolong anti-fade reagents containing 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific, Waltham, Mass). Images were taken with a Zeiss LSM710 confocal microscope (Carl Zeiss MicroImaging) with argon and helium/neon lasers. Images were acquired with Zen software (Universal Imaging, Gottingen, Germany).

Lentivirus-mediated knockdown of SNARE proteins

At 7 days *in vitro*, mDRGs were incubated in medium containing shRNA lentiviral particles that specifically target SNAP-25, VAMP1, or VAMP7, or nontargeted PLK0.1-puro particles (400 transducing units per well) and were cultured as above. After 7 to 10 days in culture, cells were stimulated before being harvested in lithium dodecyl sulfate sample buffer and analyzed by means of Western blotting. Targeted gene/protein IDs and validated sequences for shRNA lentiviral transduction particles were as follows: NM_011428,SNAP-25, CCGGCATCAGGACTTTGGTTATGTTCTCGAGAACATAACCAAAGTCCTGATGTTTTTG; NM_009496,VAMP1, GTACCGGCATCGTGGTAGTGATTGTAATCTCGAGATTACAATCACTACCACGATGTTTTTTG; and NM_011515,VAMP7, CCGGGCACAAGTGGATGAACTGAAACTCGAGTTTCAGTTCATCCACTTGTGCTTTTTG.

Culture of hKCs and monocyte-derived hDCs, cytokine antibody arrays, and phosphokinase arrays

Human primary KCs were cultured in the KBM-Gold medium with KBM-Gold SingleQuot keratinocytes supplement (Lonza). Release from hKCs was performed in hydrocortisone-free medium with or without stimulation compound. hDCs were cultured by means of *in vitro* differentiation of $CD14^+$ monocytes (subpopulation of PBMCs) with LGM-3 Lymphocyte Growth Medium plus 50 ng/mL GM-CSF plus 50 ng/mL IL-4 (Lonza). Cells were maintained in the above medium for 3 days before use. Release was performed in plain LGM-3 Lymphocyte Growth Medium in the presence of neuropeptides. After release, cell-culture supernatants were collected for proteome profiler human cytokine antibody array, according to the manufacturer's protocol. Briefly, array membranes were blocked and then incubated with the sample overnight at 4°C. The membranes were washed 3 times and then incubated in diluted primary antibodies for 1 hour at room temperature. After washing, membranes were incubated with diluted horseradish peroxidase–conjugated streptavidin for 1 hour at room temperature, and membranes were developed by using enhanced chemiluminescence reagent. Images were captured and densitometrically scanned. Each cytokine spot was analyzed with ImageJ software (National Institutes of Health, Bethesda, Md), the average of positive controls of each treatment was set to 100, and all cytokines of the treatment were compared with that value. Resultant values from the treatment were calculated relative to the nontreated control value to determine fold increase.

For the kinase array, cells grown in T175 flasks were treated with or without neuropeptides for 8 minutes, and cells were lysed for intracellular phosphokinase array, according to the manufacturer's protocol.

Phosphokinase inhibitor treatment

The selective p38 mitogen-activated protein kinase inhibitor AL8697, the selective inhibitor of the α and β isozymes of GSK-3 SB216763, or the selective JNK1/2/3 inhibitor JNK-IN-8 (1 μmol/L) was applied to cultured hKCs 1 hour before and during 24 hours of incubation with 1 μmol/L BNP or 1 μmol/L SP in serum-free and hydrocortisone-free medium to release the cytokines. The supernatant was collected for assay of cytokines by using an antibody array or ELISA, as detailed above.

REFERENCES

E1. Malin SA, Davis BM, Molliver DC. Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. Nat Protoc 2007;2:152-60.

E2. Meng J, Ovsepian SV, Wang J, Pickering M, Sasse A, Aoki KR, et al. Activation of TRPV1 mediates calcitonin gene-related peptide release, which excites trigeminal sensory neurons and is attenuated by a retargeted botulinum toxin with anti-nociceptive potential. J Neurosci 2009;29:4981-92.

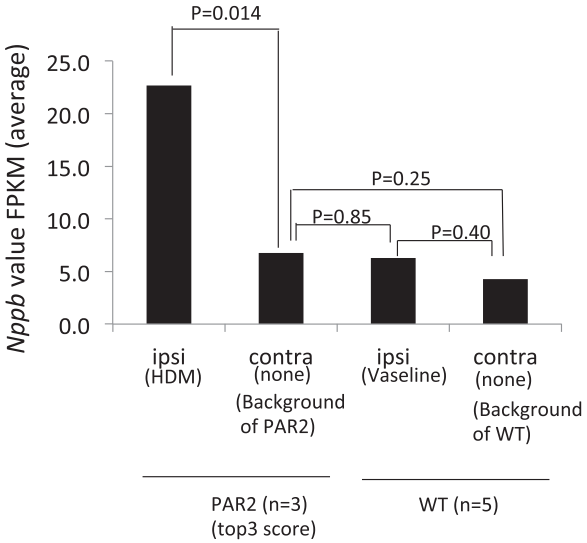

FIG E1. Average *Nppb* FPKM value from the top 3 clinically scored Grhl3PAR2$^{/+}$ mice and WT mice. Statistical analysis demonstrated that *Nppb* FPKM values from the nontreated contralateral cheeks of Grhl3PAR2$^{/+}$ mice did not differ significantly from values in baseline-treated ipsilateral cheeks or nontreated contralateral cheeks in WT mice. However, HDM induced significantly greater *Nppb* FPKM values in ipsilateral cheeks compared with contralateral cheeks in Grhl3PAR2$^{/+}$ mice. Average FPKM values are plotted. *P* values of less than .05 indicate significance. *ipsi,* Ipsilateral; *contra,* contralateral.

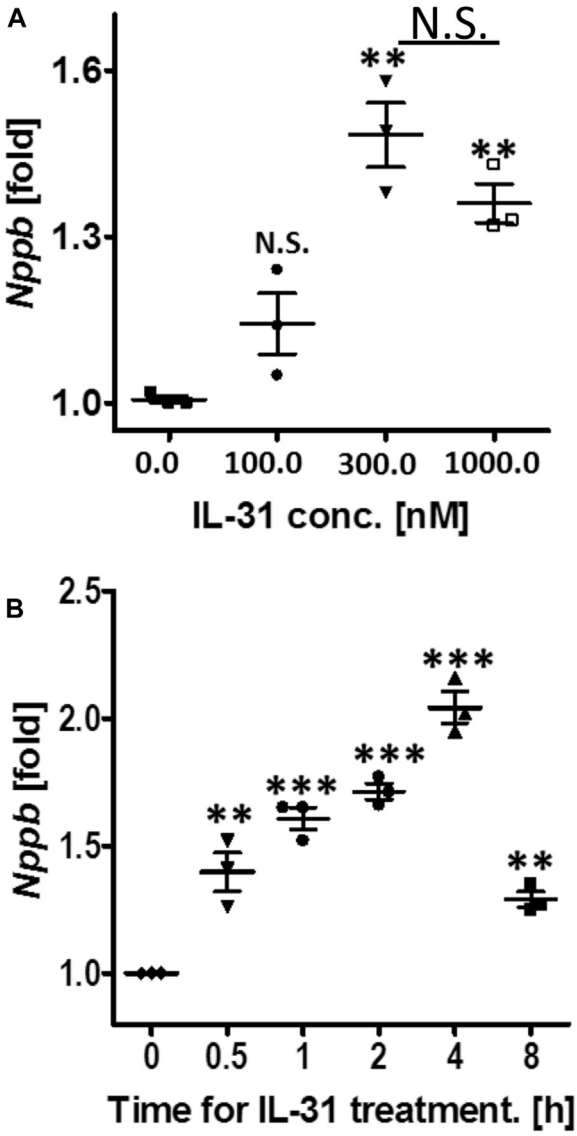

FIG E2. IL-31–induced *Nppb* mRNA synthesis in cultured mDRGs is time and concentration dependent. **A,** RT-PCR analysis of *Nppb* mRNA synthesis in mDRGs on treatment with various concentrations of IL-31 for 30 minutes. **B,** Time course of *Nppb* mRNA synthesis analyzed by using RT-PCR after treatment with 300 nmol/L IL-31. Data are expressed relative to GAPDH and presented as means ± SEMs (n = 3 independent experiments). Not significant *(N.S.)*, $P > .05$. $^{**}P < .01$ and $^{***}P < .001$.

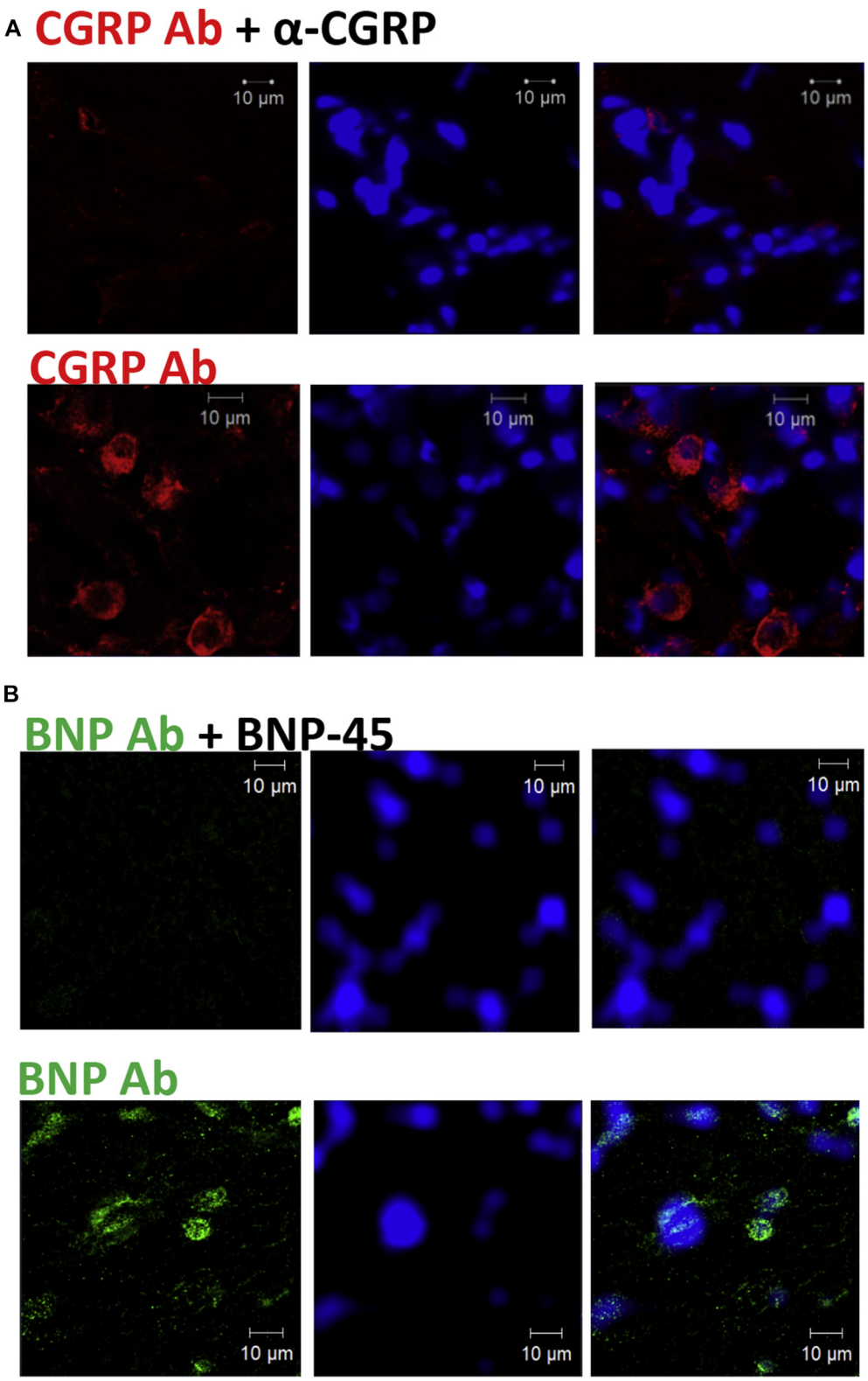

FIG E3. Antibody *(Ab)* absorption test confirmed BNP and CGRP antibody specificities. **A,** Confocal images show that the fluorescence signal of CGRP in mDRG sections was greatly reduced by preabsorption of CGRP antibody with α-CGRP peptide. **B,** Immunofluorescence images showing the staining pattern in mDRG sections using BNP antibody in comparison with its antigen-absorbed control.

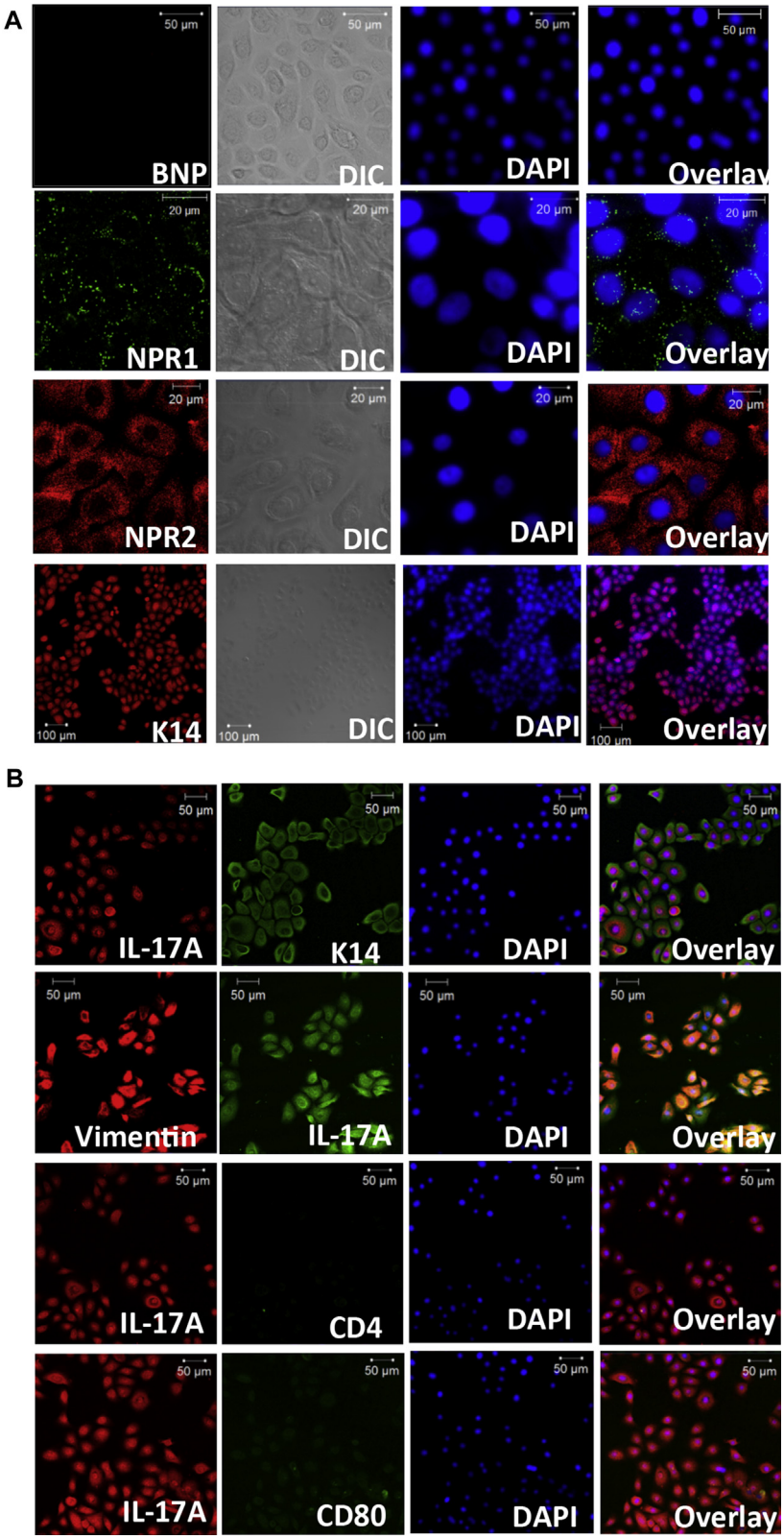

FIG E4. Characterization of cultured hKCs using immunocytochemical staining. **A,** Immunofluorescence staining of BNP, NPR1, and NPR2, as well as the proliferative keratinocyte marker keratin 14 *(K14)*, in hKCs. BNP was undetectable. NPR1 and NPR2 showed distinct distribution patterns. **B,** IL-17A immune signal is detected in cultured hKCs. K14 and vimentin were expressed in all cultured cells. Immune cell markers CD4 and CD80 antibody staining signal are either absent or negligible. *DAPI*, 4′,6-diamidino-2-phenylindole; *DIC*, differential interference contrast.

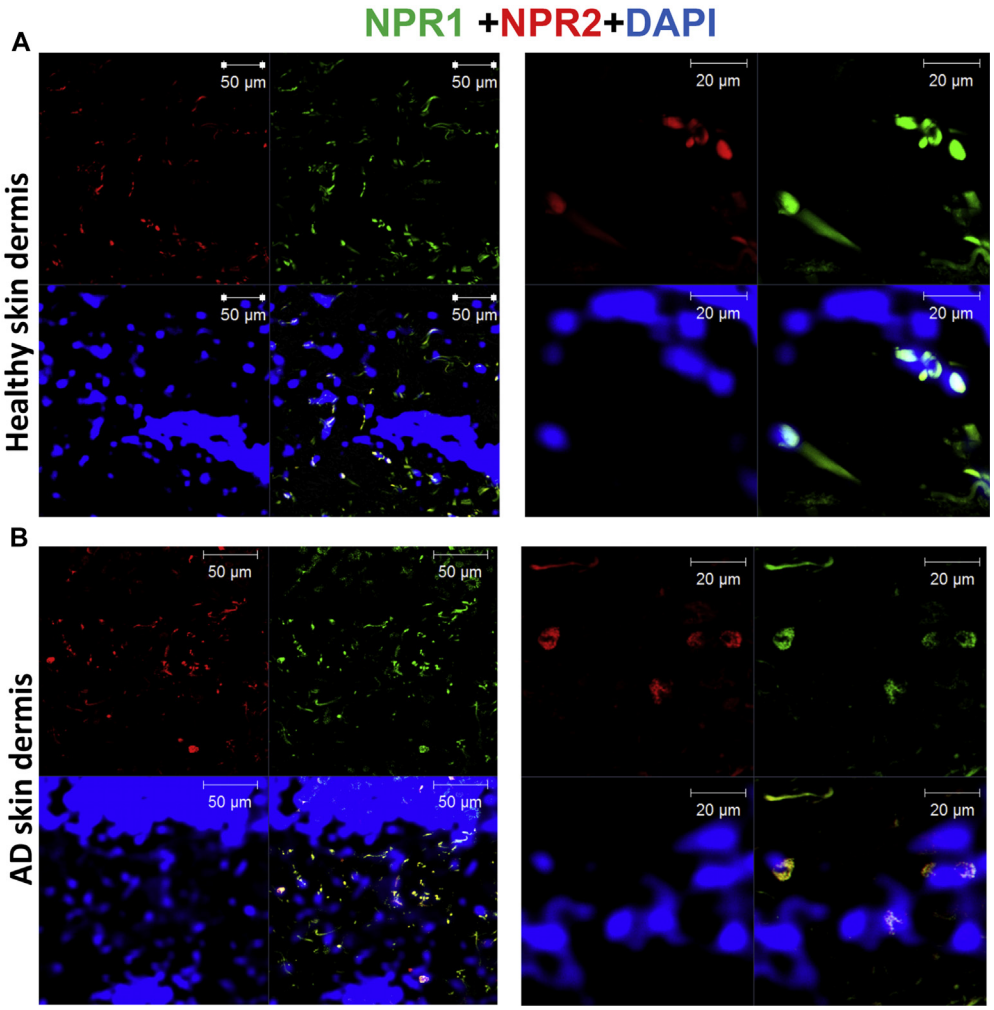

FIG E5. Immunohistochemical dual staining of NPR1 and NPR2 in skin of a human healthy control subject and a patient with AD. Representative fluorescent images at low *(left column panels)* and high *(right column panels)* magnification of NPR1 and NPR2 in skin of a healthy control subject **(A)** and a patient with AD **(B)**. NPR1 and NPR2 are highly localized in some cells in the dermis. Specimens were counter-stained with 4′,6-diamidino-2-phenylindole *(DAPI)*.

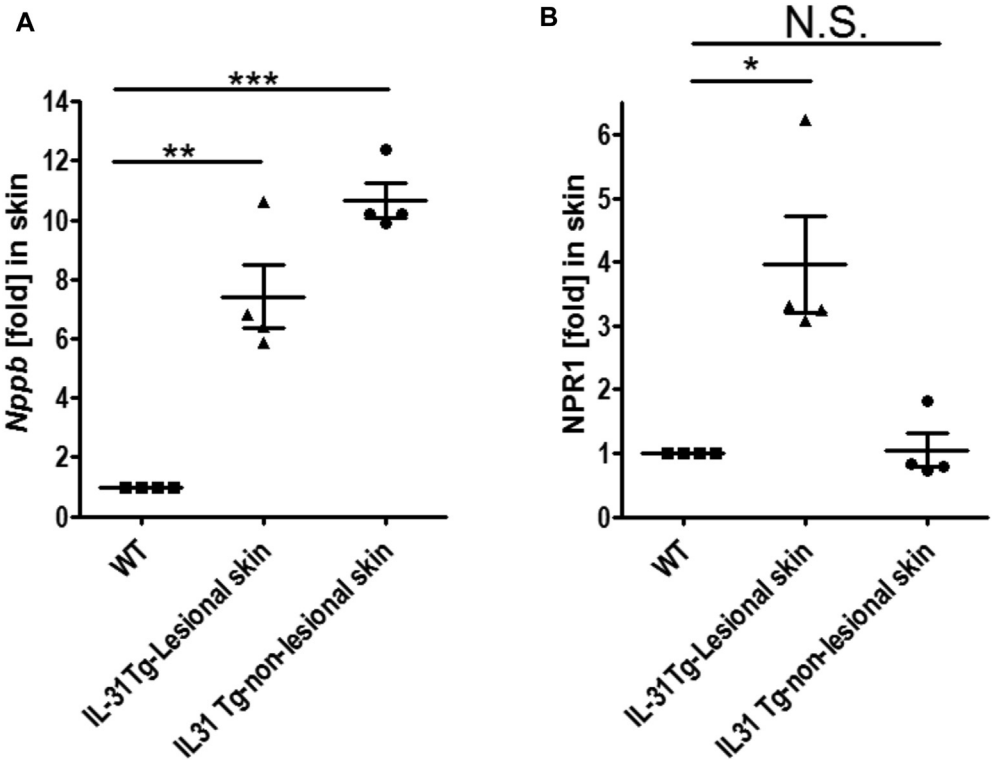

FIG E6. RT-PCR analysis of *Nppb* and *NPR1* mRNA levels in skin of IL-31Tg or WT mice. RT-PCR analysis of *Nppb* mRNA **(A)** or *NPR1* mRNA **(B)** expression in skin isolated from lesional or nonlesional IL-31Tg or WT mice. Values were normalized to the housekeeping gene *GAPDH*. Results (means ± SEMs) are pooled data from multiple animals. Significance is indicated as follows: not significant *(N.S.)*, $P > .05$; $^{*}P < .05$; $^{**}P < .01$; and $^{***}P < .001$.