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Novel insights into the TRPV3-mediated itch in atopic dermatitis

To the Editor:

Chronic pruritus (itch) is a widespread and debilitating condition associated with dermatologic, systemic, neuropathic, or psychogenic disorders. The pathophysiologic mechanisms underpinning the transduction and potentiation of this refractory pruritus remain unclear. Current therapeutics are largely ineffective.[1] Thus, we have aimed to address this gap in knowledge by specifically focusing on clinically relevant intercellular communication in human skin cells, murine models of acute and chronic itch, and samples from human atopic dermatitis (AD) and psoriasis.

In conditions of chronic dermatologic itch such as AD and psoriasis, certain members of the transient receptor potential (TRP) ion channel superfamily play an important role in the propagation of itch signaling. TRP vanilloid channel 3 (TRPV3) is a calcium-permeable cation channel that is abundantly expressed in epidermal keratinocytes. TRPV3 detects warm temperatures (>33°C), is gated by a wide range of chemical stimuli, and plays an essential role in skin homeostasis and repair. Heat-induced activation of TRPV3 stimulates the release of a potent itch inducer, thymic stromal lymphopoietin (TSLP), from cultured murine keratinocytes.[2] In mice, intradermal injection of carvacrol, a TRPV3 agonist, elicits scratching behaviors. Gain-of-function mutations in TRPV3 have been confirmed in Olmsted syndrome, a rare pruritic genodermatosis in humans[3] and associated with AD-like inflammation in rodents. TRPV3 is upregulated in the skin of patients with AD.[2] Despite this, much remains unknown about the clinical relevance of TRPV3-linked pathways in human dermatitis and pruritus.

Herein, real-time PCR was used to quantify TRPV3 expression in the skin of AD-like protease-activated receptor 2 (PAR2)-overexpressing mouse (Grhl3PAR2$^{/+}$ mice). The level of TRPV3 transcripts was significantly increased in lesional skin of Grhl3PAR2$^{/+}$ mice versus in age-matched wild-type controls (Fig 1, *A*). Moreover, relative TRPV3 levels were significantly higher in lesional skin of Grhl3PAR2$^{/+}$ mice than in nonlesional Grhl3PAR2/$^{+}$ mice (Fig 1, *A*), suggesting that TRPV3 expression is associated with the severity of dermatitis.

Human skin samples were then examined to evaluate the clinical relevance of these murine findings. Specimens were collected from patients with AD (both lesional AD [LAD] and nonlesional AD [NLAD]), from patients with psoriasis (both lesional psoriasis [LPS] and nonlesional psoriasis [NLPS]), and from healthy controls (HC). All were analyzed by RNA sequencing, with data indicating the mean change in transcript level relative to HC. In LAD samples, TRPV3 was the only member of the TRPV family to be upregulated, with transcripts showing greater than a 2-fold increase over the HC levels (Fig 1, *B*). Similar to our murine model, this upregulation was absent in NLAD skin (Fig 1, *C*). Levels of TRPV3 transcripts were also increased in LPS skin samples versus in HC skin samples, but not in NLPS samples versus in HC samples (Fig 1, *D*). No significant difference was detected in the TRPV3 transcription level in LAD samples versus in LPS samples (Fig 1, *E*). Furthermore, immunohistochemical evaluation of the LAD samples showed high levels of TRPV3 protein, revealing a striking increase when compared with the levels in the HC samples (Fig 1, *F*). Importantly, this upregulation was primarily localized to the keratinocyte layer. Similarly, a recent report described an enhanced TRPV3 immunosignal in LPS skin.[4] Together, these findings provide a clear link between TRPV3 expression and lesional AD and lesional psoriasis.

Previous reports from our group demonstrated the importance of the neuropeptide B-type natriuretic peptide (BNP) in IL-31–induced AD skin inflammation,[5,6] building on earlier evidence of its role in itch transmission.[7] As both NPR1 and NPR2 are expressed on human keratinocytes,[5,6] we postulated that BNP may modify TRPV3 expression or activity. First, cultured primary human keratinocytes (phKCs) were stimulated with BNP, substance P (SP), or calcitonin gene–related peptide (all at 1 μM). TRPV3 transcripts were measured by real-time PCR. Only BNP resulted in a significant increase in TRPV3 mRNA levels (Fig 1, *G*), suggesting that this AD-linked neuropeptide may be responsible for the upregulation of TRPV3 that is evident in our murine and human dermatitis samples.

Calcium imaging was then used to investigate whether BNP could affect the functional activity of the TRPV3 cation channel. Here, phKCs were stimulated with 1 of 2 concentrations of drofenine (250 μM or 500 μM), a specific and potent activator of TRPV3, after which the resulting calcium dynamics were recorded. In addition, a subset of phKCs were preincubated with BNP (1 μM for 4 hours) before stimulation with the lower concentration of drofenine (250 μM) (Fig 1, *H*). The BNP incubation time (4 hours) was chosen on the basis of a previous study showing that in keratinocytes the TRPV3 sensitivity was altered by TGFα treatment at 3 to 5 hours.[8] Both concentrations of drofenine resulted in notable calcium fluctuations (Fig 1, *I*; representative traces). BNP pretreatment significantly enhanced the drofenine-induced (250 μM) calcium flux, as indicated by the area under the curve (Fig 1, *J*). When expressed as peak calcium fluctuation versus the time at which this peak was reached ($Time_{max}$), drofenine-responsive phKCs were found to cluster into 2 distinct subpopulations (Fig 1, *K* and *L*) (rapid and slow, with rapid responders found in the gray shaded area in Fig 1, *L*). Importantly, preincubation with BNP resulted in a striking increase in the percentage of rapid responders following drofenine (250 μM) application (Fig 1, *K* and *L*). It is postulated that this subset of phKCs express high levels of TRPV3. A separate experiment found that stimulation with BNP alone resulted in calcium fluctuations in just a small population (0.8%) of phKCs, with responders displaying low-amplitude transients (Fig 1, *M*). Together, these data demonstrate that BNP can increase TRPV3-associated calcium responses, perhaps through sensitization or upregulation of the TRPV3 channel on the cell surface.

Although keratinocyte-derived mediators are known to facilitate dermatitis, little is known about TRPV3-induced intercellular communication. To address this, phKC-expressed TRPV3 was activated with drofenine (500 μM) and the supernatant was analyzed by cytokine array. Levels of both serpin E1 and TGFα were found to be significantly increased (~1.8-fold and ~2 -fold, respectively [Fig 2, *A*]). To confirm that drofenine-induced pharmacologic effects are mediated by TRPV3, we performed an experiment examining knockdown of TRPV3 by using short-

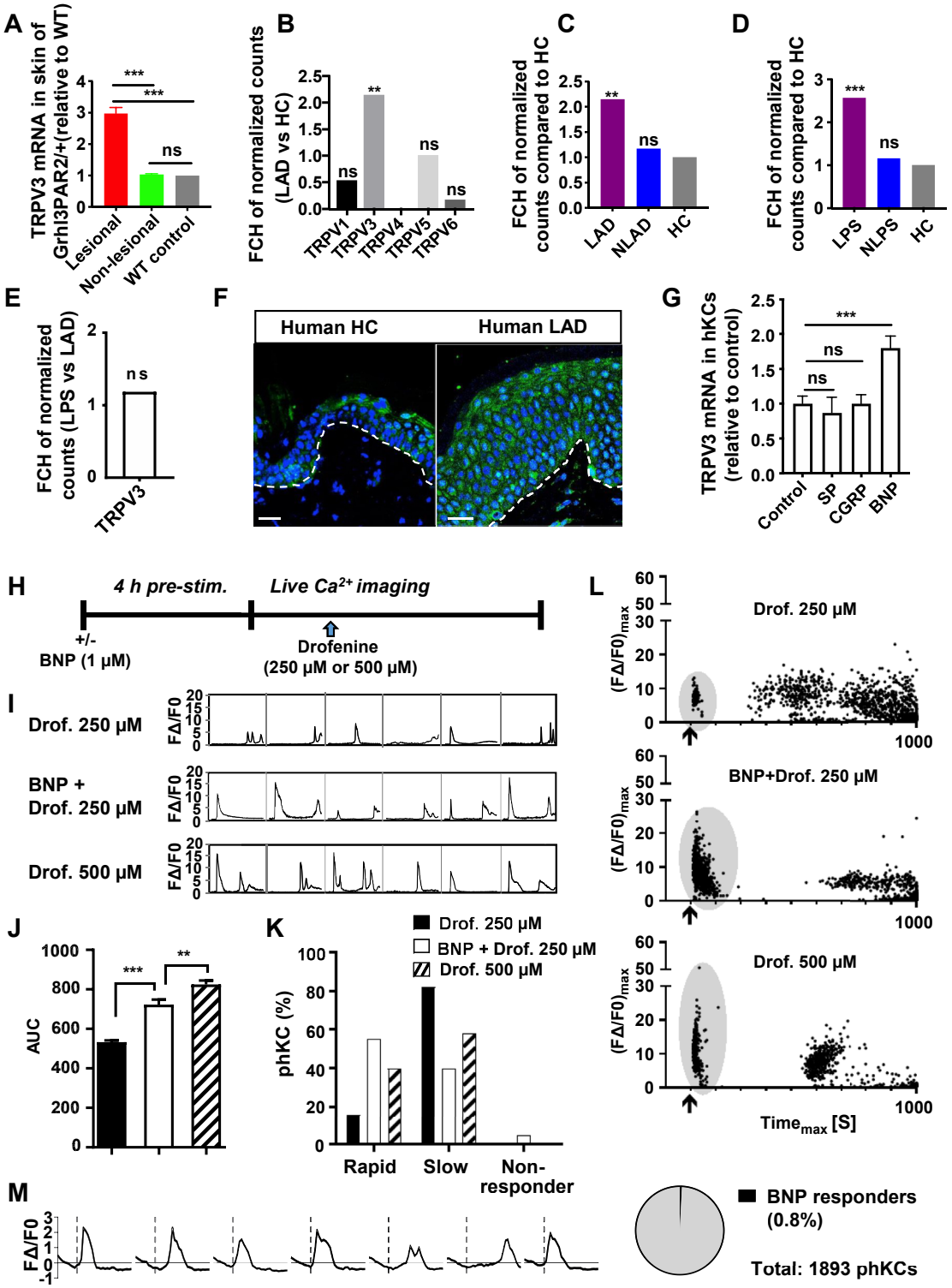

FIG 1. TRPV3 expression in skin is correlated with disease severity, and BNP enhances TRPV3 activity. **A,** Fold change (FCH) of TRPV3 mRNA expression in lesional and nonlesional skin from Grhl3PAR2/$^{+}$ mice versus in samples from WT mice. **B,** FCH of TRPV family expression in human LAD skin samples versus in HC skin. **C** and **D,** FCH of TRPV3 expression in human LAD and NLAD skin (**C**) and LPS and NLPS skin (**D**) (all relative to HC skin). **E,** Comparison of TRPV3 transcript level in LPS skin versus in LAD skin. **F,** Representative immunostaining of TRPV3 (*green*) in human LAD and HC skin. Scale = 20 µm. **G,** FCH of TRPV3 mRNA expression versus control in phKCs. **H-L,** Effect of BNP pretreatment on TRPV3 channel activity in phKCs; experimental design (**H**); representative traces (time versus FΔ/F0) following drofenine (Drof.) stimulation (± BNP pretreatment) (**I**); area under the curve (AUC) analysis (**J**); temporal subpopulation analysis (**K**); and $Time_{max}$ versus $(F\Delta/F0)_{max}$, with each dot representing the response profile of a single phKC (**L**). **M,** Representative traces following BNP stimulation and responder analysis. Data in (**A**), (**G**), (**J**) are means ± SEMs (n ≥ 3). **P* < .05; ***P* < .01; and ****P* < .001. Analysis performed with the Student *t* test (**A**, **G**) or ANOVA (**J**). *CGRP*, Calcitonin gene–related peptide; *hKC*, human keratinocyte; *ns*, not significant; *pre-stim.*, before stimulation; *SP*, substance P.

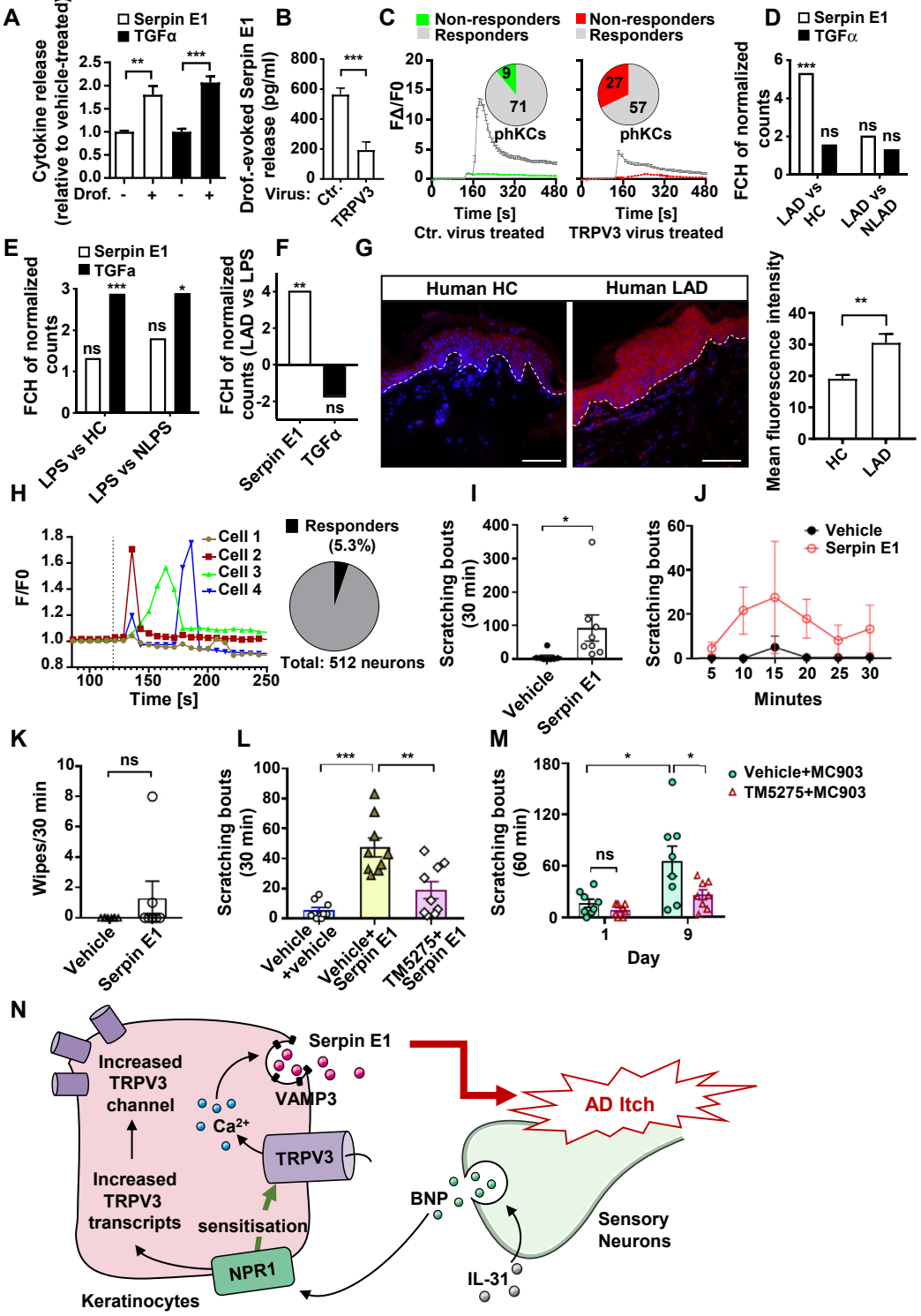

FIG 2. Serpin E1 is released from keratinocytes following TRPV3 activation, is upregulated in LAD, promotes acute itch-like behavior in mice, and is involved in chronic scratching in the MC903-induced AD model. **A,** Fold change (FCH) of mediator release following drofenine (Drof.) application (500 μM for 1 hour) versus in vehicle-treated phKCs. **B** and **C,** Effect of TRPV3 knockdown on Drof.-evoked serpin E1 release (**B**) and calcium transients (**C**). **D-F,** FCH of serpin E1 and TGF-α expression in human LAD skin versus in HC or NLAD skin (**D**), LPS skin versus HC or NLPS skin (**E**), and LAD skin versus LPS skin (**F**). **G,** Immunostaining with serpin E1 (*red*) in LAD and HC skin, and analysis of epidermal fluorescence intensity. Scale = 100 μm. **H,** Traces following serpin E1 stimulation of mouse dorsal root ganglionic neurons and responder analysis. **I-K,** Effect of intradermal injection of vehicle or serpin E1 in mice: scratching bouts (**I**), time course of itch-like response (**J**), and wiping behavior (**K**). **L** and **M,** Effect of vehicle or TM5275 on serpin E1–elicited scratching (**L**) and MC903-induced chronic itch (**M**). **N,** A model for TRPV3-mediated itch and sensitization in AD. Data in **A**, **B**, and **I-M** are means ± SEMs (n ≥ 3). **P* < .05; ***P* < .01; and ****P* < .001. Analysis performed with the Student *t* test. *Ctr.*, Control; *ns*, not significant.

hairpin RNA lentivirus. The latter reduced TRPV3 expression by approximately 75% in phKCs compared with the nontargeted control virus–treated cells (see Fig E1 in this article's Online Repository at www.jacionline.org). Knockdown of TRPV3 reduced levels of drofenine-elicited serpin E1 release (Fig 2, *B*) and the calcium transients, as well as the number of cells responding to drofenine (Fig 2, *C*), indicating that the drofenine-induced responses in phKCs were mediated by TRPV3. In addition, serpin E1 release was found to be facilitated by a specific v-SNARE protein, namely, vesicle associated membrane protein isoform 3 (VAMP3). Following targeted short-hairpin RNA–mediated knockdown of VAMP3 in phKCs (~90%) (see Fig E2, *A* in this article's Online Repository at www.jacionline.org), drofenine-induced serpin E1 release was reduced by approximately 62% (see Fig E2, *B* in this article's Online Repository). In contrast, TGFα release was not significantly altered (see Fig E2, *B* in this article's Online Repository), suggesting that serpin E1, but not TGFα, is released via exocytotic vesicles. These data reveal a previously unknown intercellular pathway, mediated in part by VAMP3-dependent exocytosis.

To investigate whether these TRPV3-linked mediators are involved in human dermatitis, we analyzed serpin E1 and TGF-α transcripts in AD and psoriasis skin by RNA sequencing. Interestingly, serpin E1 was found to be significantly upregulated in patients with LAD versus in HC, showing a striking 5.3-fold increase, whereas, TGF-α levels were not significantly different from those in HC (Fig 2, *D*). Conversely, the level of TGF-α, but not serpin E1, was significantly increased in patients with LPS compared with in either HCs or patients with NLPS (Fig 2, *E*). Notably, a 4-fold higher expression of serpin E1 was observed in patients with LAD versus in those with LPS (Fig 2, *F*). Immunohistochemical evaluation of HC and LAD skin revealed high levels of serpin E1 (*red*) protein in the samples of LAD skin, but not the HC skin samples (Fig 2, *G* [*left*]). The mean fluorescence intensity of serpin E1 in skin samples from patients with LAD was greater than that in samples from HCs, as revealed by analysis of regions of interest encompassing the epidermis of the skin (Fig 2, *G* [*right*]). Importantly, serpin E1 expression was primarily localized to the keratinocyte layer. Thus, TRPV3-linked mediators serpin E1 and TGF-α are differentially involved in human AD and psoriasis, representing important disease-specific pathways. Together, these results strengthen the notion that TRPV3 and its downstream mediators are involved in human dermatitis.

Serpin E1 is primarily classified as an inhibitor of fibrinolysis. The findings presented herein describe a correlation between serpin E1 expression and dermatitis. However, whether serpin E1 could act as a pruritogen (itch inducer) remained unknown. First, the effect of serpin E1 on mouse dorsal root ganglionic neurons was analyzed by using calcium imaging. In total, approximately 5.3% of mouse dorsal root ganglionic neurons were activated by serpin E1, showing that serpin E1 can directly activate a subpopulation of sensory neurons (Fig 2, *H*). In a mouse cheek model of itch, intradermal injection of serpin E1 elicited significant site-directed scratching behavior (Fig 2, *I*), with average scratching bouts peaking at 15 minutes (Fig 2, *J*). Urokinase plasminogen activator receptor (U-PAR), which is known to bind serpin E1,[9] is present in cultured mouse trigeminal ganglionic neurons (see Fig E3 in this article's Online Repository at www.jacionline.org). In contrast, serpin E1 did not induce wiping (Fig 2, *K*), a behavior that reflects pain-associated responses, suggesting that serpin E1 may represent a novel and specific itch inducer. Of note, a selective orally active inhibitor of serpin E1 (TM5275) attenuated serpin E1–elicited cheek scratching (Fig 2, *L*). Moreover, in a prolonged AD-like MC903-associated chronic pruritus mouse model, in which 7 days or longer is required for pruritus to be established, TM5275 also inhibited MC903-evoked itch behaviors at day 9 (Fig 2, *M*). Thus, serpin E1 represents a novel pruritogen in mice and may promote dermatitis-associated pruritus in humans.

Overall, this study provides a clear link between TRPV3 and dermatitis (Fig 2, *N*). In AD conditions, IL-31 induces BNP synthesis and release from sensory neurons.[5] BNP subsequently binds to NPR1 on keratinocytes to upregulate TRPV3 transcripts. This could increase the surface expression of TRPV3, resulting in heightened TRPV3 activity and increased serpin E1 release. Serpin E1 activates sensory fibers in the skin and promotes itch transduction. Our work has revealed a disease-relevant neuroepidermal pathway for TRPV3 upregulation and sensitization. It also highlights the role of TRPV3-linked mediators in human dermatitis and pruritus, with serpin E1 representing a novel itch inducer (Fig 2, *N*). On the basis of these results, we believe that targeting TRPV3 signaling will prove beneficial for the treatment of chronic itch conditions.

Ciara Larkin, BSc[a]*
Weiwei Chen, MSc[b]*
Imre Lőrinc Szabó, MD, PhD[c]
Chunxu Shan, PhD[a]
Zsolt Dajnoki, PhD[c]
Andrea Szegedi, MD, PhD[c]
Timo Buhl, MD[d]
Yuanyuan Fan, PhD[b]
Sandra O'Neill, PhD[a]
Dermot Walls, PhD[a]
Wenke Cheng, BSc[b]
Song Xiao, BSc[b]
Jiafu Wang, PhD[a,b]
Jianghui Meng, PhD[a,b]

From [a]the School of Biotechnology, Faculty of Science and Health, Dublin City University, Glasnevin, Dublin, Ireland; [b]the School of Life Sciences, Henan University, Henan, China; [c]the Department of Dermatology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary; and [d]the Department of Dermatology, Venereology and Allergology, University Medical Centre Göttingen, Göttingen, Germany. E-mail: jiafu.wang@dcu.ie. Or: jianghui.meng@dcu.ie.

*These authors share first authorship.

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METHODS

Assurance of human and animal rights and skin sample preparation

Skin punch biopsy samples were taken from LAD and NLAD skin and LPS and NLPS skin of patients with AD and from HCs after written informed consent had been obtained according to the Declaration of Helsinki principles. The study was institutionally approved by the Medical Research Council, National Scientific and Ethical Committee, Budapest, Hungary (ETT TUKEB, document identifiers: 50935/2012/EKU and 54256-1/2016/EKU). For RNA sequencing (RNA-seq), human samples of AD or psoriasis skin and HC skin samples were obtained from individuals over the age of 17 years. The LAD skin samples were obtained from 5 patients (1 female and 4 males), all with a SCORing Atopic Dermatitis score higher than 35. The LPS skin samples were obtained from 5 patients (3 females and 2 males) with a Psoriasis Area Severity Index severity higher than 11. Note that 2 NLPS samples were from the patients who also donated 2 LPS skin samples. One NLAD sample was derived from a patient who also donated 1 LAD sample.

In the case of the paraffin sections of human skin, skin biopsy samples from patients and HCs were obtained under local anesthesia after the patients gave their written consent. Permission for the human studies was given by the Ethical Committee of the University of Göttingen, Germany, in accordance with the ethical standards of the principles of the 1964 Declaration of Helsinki.

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Henan University and approved by the Animal Ethics Committee of Henan University.

Real-time PCR

cDNA from Grhl3PAR2$^{/+}$ mouse skin samples (lesional and nonlesional skin samples) were obtained previously[E1] and were used for real-time fluorescence detection with the SYBR Green ROX mix (Applied Biosystems Inc). The TRPV3 primers used were as follows: CGCTGGCCTCACTGATTGAGAA (forward) and CCCATGCGGAATCTGCTTCTCA (reverse).

RNA-seq

RNA-seq was performed by IMGM Laboratories (Martinsried, Germany). In brief, total RNA was isolated by using the RNeasy Fibrous Tissue Mini Kit (Qiagen) from up to 30 mg of human skin tissue from LAD skin (n = 5 patients), NLAD skin (n = 5), LPS skin (n = 5), NLPS skin (n = 2), and HC skin (n = 5) followed by on-column DNase digestion. All samples were analyzed on a 2100 Bioanalyzer (Agilent Technologies) by using RNA 6000 Nano/Pico LabChip kits (Agilent Technologies). Library preparation was performed with 300 ng of total RNA by using the TruSeq Stranded mRNA HT technology. The sequencing library generated by pooling was quantified by using the highly sensitive fluorescent dye–based Qubit ds DNA HS Assay Kit (Invitrogen). Sequencing of the library was performed at a final concentration of 1.8 pM and with a 1% PhiX version 3 control library spike-in (Illumina) on a NextSeq 500 sequencing system (Illumina). Sequencing was performed under control of NextSeq Control Software). Primary image processing was performed on a NextSeq 500 instrument by using Real-Time Analysis software, version 2.4.11. Primary data analysis was performed by using the bcl2fastq 2.15.04 software package. The Illumina Sequence Analysis Viewer, version 2.4.5, was used for imaging and evaluation of the sequencing run performance. The CLC Genomics Workbench (version 11.0.1, CLC bio [a Qiagen company]) was used for in-depth analysis of differential gene expression. The average fold changes for genes upregulated and reaching significance were plotted. A 2-fold change in the reads per kilobase (of transcript) per million mapped reads value was deemed significant.

Culture of phKCs

phKCs were seeded into 24-well plates and cultured in KBM-Gold medium with KBM-Gold SingleQuot KC supplement (Lonza, Belgium). Cells were maintained in the aforementioned medium for 2 days before use.

Short-hairpin RNA–mediated knockdown of VAMP3 and TRPV3

At 2 days *in vitro*, cultured phKCs were incubated in medium containing short-hairpin RNA lentiviral particles that specifically target VAMP3 (Sigma Aldrich, St Louis, Mo) or TRPV3 (OBiO Technology Corp Ltd) or nontargeted lentiviral particles, as described previously in Meng et al.[E2] After 2 days in culture, the cells were further cultured in medium containing 1 μg/mL of puromycin (Sigma) for 3 days. The cells were stimulated by drofenine for release of serpin E1 and TGFα or calcium imaging. Afterward, the cells were lysed in LDS sample buffer for Western blotting to confirm the knockdown.

Cytokine release assay

Before the signaling assays, phKCs were incubated with hydrocortisone-free medium for at least 24 hours. Following release, cell culture supernatants were collected, pooled, and analyzed by using the Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, Minneapolis, Minn), according to the manufacturer's protocol. Each cytokine spot was analyzed with ImageJ software. Resultant densitometry values from the treated samples were calculated relative to those for nontreated control values to determine the fold increase. In Fig 2, *B*, serpin E1 was measured by using an ELISA kit (Abcam, Cambridge, UK).

Intracellular Ca^{2+} measurement in phKCs and mouse dorsal root ganglionic neurons

Cultured phKCs on the IBIDI chambers were loaded with 3 μM Fluo-4 AM in hydrocortisone-free medium for 30 minutes (37°C). The cells were washed and allowed to equilibrate to room temperature. Fluo-4-AM–loaded cells were excited with an argon laser, and images were acquired at 10-second intervals with a Zeiss LSM710 confocal microscope (Carl Zeiss MicroImaging). After a baseline period of 100 seconds, drofenine (250 μM or 500 μM) or human BNP (1 μM) was applied. Raw fluorescence data were acquired and transformed to single-cell time course fluorescence by using Zen 2012 (blue edition). For each cell, intracellular calcium increases were normalized to FΔ/F0, with FΔ denoting the change in fluorescence and F0 denoting the baseline fluorescence, and graphed relative to time. Only cells showing a 30% increase over baseline were included in the analyses (FΔ/F0 ≥ 0.3). After drofenine stimulation, responding cells were evaluated in terms of 3 key properties: area under the curve (of FΔ/F0 versus time), maximum intensity of fluorescence ($(F\Delta/F0)_{max}$), and time at which maximum fluorescence was achieved ($Time_{max}$). On the basis of $Time_{max}$ data, the phKCs were grouped into 2 subpopulations, rapid and slow, with rapid responders being those with a $Time_{max}$ value of 300 seconds or less. The data represent 3 or more independent experiments, with at least 828 phKCs (drofenine stimulations [Fig 1, *H-L*]) or 1893 phKCs (BNP stimulations [Fig 1, *M*]).

For the serpin E1–induced calcium transient measurement, mouse dorsal root ganglionic neurons were isolated from postnatal d5 C57BL/6 mice and dissociated by collagenase I, as previously described.[E1] Cells were cultured in the presence of cytosine β-D-arabinofuranoside (Sigma) and nerve growth factor (100 ng/mL) for 7 days *in vitro*. Mouse dorsal root ganglionic neurons were loaded with Fluo-4 AM and stimulated with 20 μg/mL of Serpin E1 (RayBiotech) while images were captured by a ImageXpress Micro 4 Automated Cell Imaging System (Molecular Devices) using MateXpress6 software. Intracellular calcium level increases were normalized to F/F0, with F denoting the actual fluorescence value and F0 denoting the baseline fluorescence.

Immunofluorescence staining

Paraffin sections of human skin were deparaffinized, rehydrated before being permeabilized in PBS with 0.2% Triton X-100 (PBS-T), and then incubated in PBS containing 5% normal donkey serum (blocking solution) at room temperature for 1 hour. Specimens were then incubated with 1:500 mouse antibody to TRPV3 [N15/4] (Abcam) or rabbit polyclonal serpin E1 antibody (Abcam) in blocking solution (4°C overnight). The specimens were

washed in PBS and incubated with Alexa 488 goat anti-mouse IgG or Alexa 594 goat anti-rabbit IgG. After the final wash of the secondary antibody, specimens were mounted onto slides using ProLong Antifade reagent containing (4',6-diamidino-2-phenylindole) DAPI. Images were taken by Leica DMi8 confocal microscope using LASX software or IX73 Olympus inverted microscope using CellSens Dimension Imaging software.

Behavior experiments

C57BL/6 mice received intradermal injections of serpin E1 (0.42 μg/4 μL) or vehicle control into the right cheek; bouts of hind limb scratches directed to the injection site were considered indicative of pruritus. Wipes with the forepaw were also analyzed and considered pain behavior. An orally active serpin E1 inhibitor, TM5275 (57 mg/kg) or its vehicle was administered to the mice 1 hour before the cheek injection of serpin E1 (0.42 μg/4 μL) or its vehicle control to analyze the specificity of serpin E1-elicited itch response.

To establish a prolonged MC903-induced model of AD,[E3] MC903 (4 nmol/20 μL in ethanol [Sigma Aldrich]) was applied topically to the left ear of C57BL/6 mice. This was repeated daily for 9 consecutive days, resulting in the induction of an AD-like chronic itch model. Starting at day 1, these mice also received daily doses of TM5275 (57 mg/kg) or its vehicle. One hour later, MC903 was applied to the left ear. Thereafter, the mice were video recorded for 1 hour via time-lapse videography, and itch events were analyzed.

Data analysis

Data are presented as means plus or minus SEMs. For RNA-seq, false discovery rates were determined and classified as being less than 0.001, ranging from 0.001 to less than 0.01, ranging from 0.01 to less than 0.05, or equaling 0.05 or more. For the rest, *P* values were determined by using the Student 2-tailed *t* test or ANOVA; *P* values less than 0.05 were considered significant. Data analysis was performed with Prism software (GraphPad Software, La Jolla Calif).

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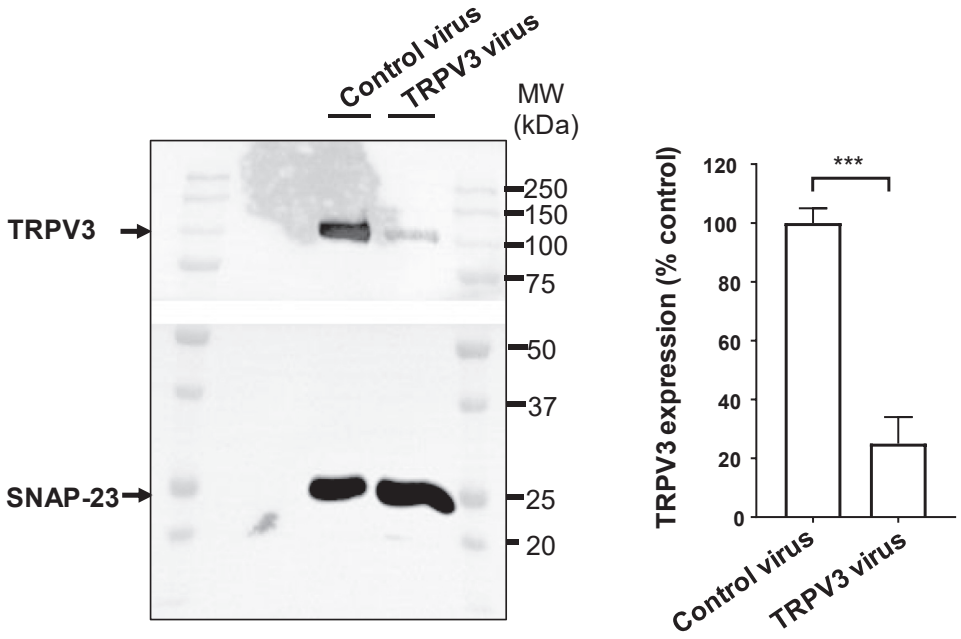

FIG E1. Knockdown TRPV3 expression in phKCs by short-hairpin RNA (shRNA) lentivirus. Cultured cells were treated with TRPV3-specific shRNA lentivirus or nontargeted control virus (see the Methods section). Western blot showing the expression of TRPV3 in phKCs was reduced by its specific shRNA when compared with the control virus–treated cells. SNAP-23 served as an internal control. TRPV3 expression in TRPV3 virus-treated cells was expressed as a percentage of that of control virus-treated cells. Data are represented as means ± SEMs (n = 3).

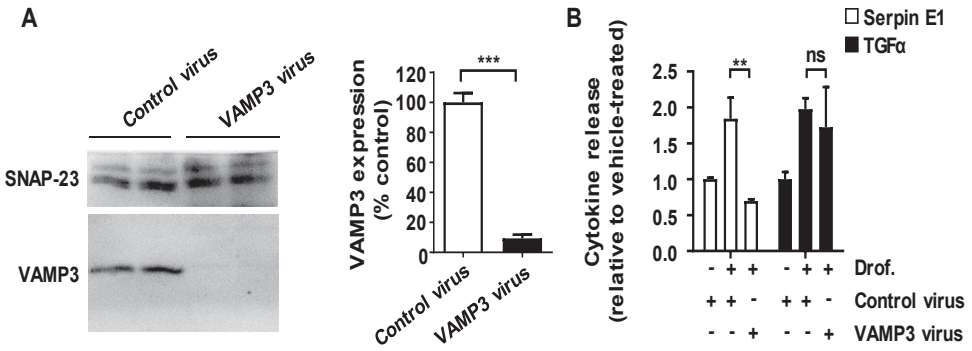

FIG E2. Knockdown (KD) VAMP3 expression in phKCs reduced drofenine (Drof.)-elicited serpin E1 but not TGFα release. **A,** KD of VAMP3 protein expression using short-hairpin RNA lentivirus. SNAP-23 serves as an internal control. Nontargeted control virus–treated samples were loaded to the gels for comparison. VAMP3 expression was first normalized to SNAP-23 and then expressed as a percentage of control expression. **B,** KD of VAMP3 attenuated drofenine-induced serpin E1 but not TGF-α release from phKCs.

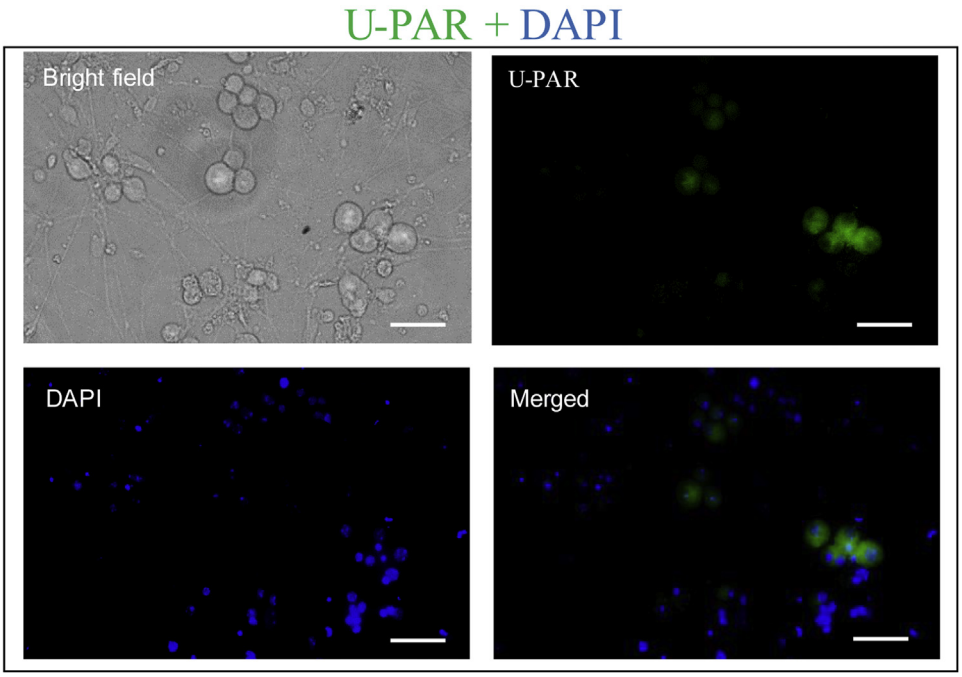

FIG E3. Immunofluorescence study of the cellular expression of the putative serpin E1 receptor in the cultured mouse trigeminal ganglionic neurons (TGNs). Urokinase plasminogen activator receptor (U-PAR) was detected by using rabbit polyclonal antibody (Abcam) followed by donkey anti-rabbit Alexa Fluor 488 antibody. Specimens were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bars = 50 μm.