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RESEARCH ARTICLE

The PLAUR signaling promotes chronic pruritus

Weiwei Chen[1] | **Yanqing Li**[1] | **Martin Steinhoff**[2,3,4,5,6,7] | **Wenhao Zhang**[1] | **Joerg Buddenkotte**[2,3,4] | **Timo Buhl**[8] | **Renkai Zhu**[1] | **Xinrong Yan**[1] | **Zhiping Lu**[1] | **Song Xiao**[1] | **Jiafu Wang**[9] | **Jianghui Meng**[10]

[1]School of Life Sciences, Henan University, Kaifeng, China

[2]Department of Dermatology and Venereology, Hamad Medical Corporation, Doha, Qatar

[3]Translational Research Institute, Academic Health System, Hamad Medical Corporation, Doha, Qatar

[4]Dermatology Institute, Academic Health System, Hamad Medical Corporation, Doha, Qatar

[5]Department of Dermatology, Weill Cornell Medicine-Qatar, Doha, Qatar

[6]College of Medicine, Qatar University, Doha, Qatar

[7]Department of Dermatology, Weill Cornell Medicine, New York, New York, USA

[8]Department of Dermatology, Venereology and Allergology, University Medical Centre Göttingen, Göttingen, Germany

[9]Faculty of Science and Health, School of Biotechnology, Dublin City University, Dublin 9, Ireland

[10]Faculty of Science and Health, National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland

Correspondence
Jianghui Meng, National Institute for Cellular Biotechnology, Faculty of Science and Health, Dublin City University, Glasnevin, Dublin 9, Ireland.
Email: Jianghui.meng@dcu.ie

Jiafu Wang, Faculty of Science and Health, School of Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland.
Email: jiafu.wang@dcu.iess

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Abstract

Chronic itch is a complex sensation of the skin frequently associated with skin diseases, such as atopic dermatitis (AD) and psoriasis. Although Serpin E1 is implicated in chronic itch, its receptor and signaling pathways involved in itch are not known. In this study, the clinical relevance of a putative Serpin E1 receptor PLAUR to chronic itch, and the neuro-cutaneous Serpin E1-PLAUR signaling are explored. We found that PLAUR is overexpressed in skin specimens of human lesional AD and lesional psoriasis, and sensory neurons innervating MC903-induced AD-like murine skin. Murine PLAUR$^+$ sensory neurons responded to Serpin E1, resulting in enrichment of numerous itch- and inflammation-related genes and their protein release. PLAUR resides in TLR2$^+$ neurons and Serpin E1 stimulus led to transcriptional upregulation of TLR2 and its co-signaling proteins. Agonists of TLR2 propagated itch-related gene transcription including BNP, OSM, and PAR2. OSM induced acute itch in mice and promoted G-CSF and IL-8 release from human keratinocytes. Serpin E1 inhibitor reduced MC903-induced itch, epidermal

Abbreviations: AD, atopic dermatitis; BNP, natriuretic neuropeptide B; DRGs, dorsal root ganglion neurons; Dusp, specificity phosphatase; Fos, fos proto-oncogene; HC, healthy individuals; ITGAV, Integrin Subunit Alpha V; Junb, JunB Proto-Oncogene; LAD, lesional AD; LPS, lesional psoriasis; Mapk, mitogen-activated protein kinase; mDRGs, mouse dorsal root ganglia neurons; mTGNs, mouse trigeminal neurons; Nfkb, NF-κB signaling protein; NLAD, non-lesional AD; NLPS, non-lesional psoriasis; OSM, Oncostatin M; Peli1, Pellino E3 Ubiquitin Protein Ligase 1; phKCs, primary human keratinocytes; PLAUR, plasminogen activator/urokinase receptor; PS, psoriasis; uPA, urokinase plasminogen activator.

Jiafu Wang and Jianghui Meng joint senior author.

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hyperplasia, immunocyte infiltration, and resulted in lower transcription/expression levels of Serpin E1 and OSM. Taken together, the PLAUR-TLR2-OSM signaling promotes skin-nerve communication, cutaneous inflammation, and itch, all feeding into an aggravation of AD and exaggerated itch circuits.

KEYWORDS

atopic dermatitis, OSM, OSMR, PLAUR, Serpin E1, toll-like receptor

1 | INTRODUCTION

Chronic itch is a persistent, debilitating condition, that can result from various systemic disorders and skin diseases.[1–3] Yet, no FDA-approved therapeutic agent is available for its treatment due to our lack of understanding of the central drivers of chronic itch.[4] Neuro-immunocutaneous communication networks have recently been identified to partake in the initiation and aggravation of chronic skin diseases, suggesting that a dysregulated communication between neurons, immune, and skin cells exists in the center of chronic itch pathology.

Atopic dermatitis (AD) and psoriasis (PS) are commonly associated with severe acute and chronic itch.[5,6] The precise origin of the chronicity of itch and its connection to the neuro-cutaneous interplay is still unestablished.[7–9] Our recent work identified Serpin E1 as a dermally overexpressed mediator in lesional skin from AD patients. Interestingly, intradermal injection of Serpin E1 induced an itch-like behavior in mice, and its antagonist ameliorated itch in AD mice, implicating it in the dysregulated neuro-cutaneous communication observed in AD patients with chronic itch.[10]

Serpin E1 is released from primary human keratinocytes (phKCs) in response to TRPV3 activation. Its release can be potentiated by pre-stimulation with natriuretic neuropeptide B (BNP, encoded by Nppb gene),[10] a pruritic neuropeptide on the skin and spinal cord level.[11,12] As the major physiologic regulator of the urokinase plasminogen activator (uPA)-dependent pericellular plasmin-generating cascade, Serpin E1 interacts with uPAR (encoded by the PLAUR gene) and Integrin Subunit Alpha V (encoded by the ITGAV gene).[13–15] However, the receptors in Serpin E1-mediated itch have not been clarified.

Neuro-immune communication could play an essential role in chronic itch through the alternation of neuronal excitability and skin inflammation.[4,16] One of the environmental trigger factors is microbiota (e.g., *Staphylococcus aureus*, microbiome composition of AD skin), which influences immune homeostasis and neuronal signaling in the skin.[3,17–19] TLR2 is an important innate immune receptor during skin defense, but aberrant activation can trigger itch, exaggerate T cell-mediated autoimmune responses, and unwanted inflammation, resulting in the development of chronic itch.[18,20–22] However, how TLR2 could modulate epidermal neuronal communication is not elucidated. Serpin E1 has been implicated as a downstream target of TLR2 activation in other diseases,[23] but nothing is known in pruritus. Thus, it is worthwhile to investigate the possible regulation of TLR2 in Serpin E1 pathways in AD.

Another critical itch mediator linked to TLR2 is oncostatin M (OSM), a growth and differentiation factor regulating neurogenesis and itch.[24] Its cognate receptor OSM receptor ß (gene *OSMR*), is expressed in various cell types including sensory neurons and keratinocytes in the human body[24–27] and was found upregulated together with TLR2 in AD.[28,29] Further understanding the possible signaling network between Serpin E1/TLR2/OSM will give a clue to improve itch treatment.

Here, we investigate the mechanism of Serpin E1-induced itch on a neuro-epidermal level with a particular focus on the mechanistic relationship between Serpin E1, PLAUR, TLR2, and OSM in acute and chronic itch in vivo, ex vivo, in vitro models, and evaluate its translational clinical impact. Overall, our findings revealed a novel Serpin E1-PLAUR axis-mediated itch circuit in chronic itch and highlights the importance of Serpin E1 as a potential drug target for the treatment of AD and probably other pruritic skin diseases.

2 | MATERIALS AND METHODS

2.1 | Ethic

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

2.2 | RNA-seq

RNA-seq dataset of human skin samples was reanalyzed from our previous published paper,[10] wherein

the skin punch biopsies were taken from patients with LAD ($n = 5$), NLAD ($n = 5$), LPS ($n = 5$), and HC ($n = 5$) after obtaining written informed consent, according to the Declaration of Helsinki principles and processed as previously described.[10] MC903 (2 nmol/20 μl in ethanol, Sigma) or vehicle (ethanol) was topically applied to the left ear of C57BL/6 female mice for a consecutive 12 days. RNA-seq of mouse tissue and cells was performed by BGI (Beijing Genomics institution) as previously described.[12,30]

2.3 | Culture of phKCs and primary murine sensory neurons

phKCs (Lonza) were cultured in KBM-Gold medium supplemented with KBM-Gold SingleQuot KC (Lonza) for 3 days before use.[10] Murine neurons were isolated from postnatal d5 C57BL/6 mice after deep anesthetization. The ganglia were digested and cultured in DMEM supplemented with 5% (v/v) FBS (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 × B27, and 50 ng/ml nerve growth factor (Sigma). Seven days of culture in the presence of 10 μM cytosine β-d-arabinofuranoside (Sigma) enriched >95% neurons characterized by NeuN staining. For RNA-seq, neurons were incubated with a medium with either Pam3CSK4 (1 μg/ml, Novus), FSL-1 (1 μg/ml, Sigma), Serpin E1 (20 μg/ml, Raybiotech), or the appropriate vehicle for 6 h.

2.4 | Knockdown of PLAUR and ITGAV

Sensory neurons were challenged with a medium containing shRNA lentiviral particles specifically targeting PLAUR or ITGAV (HanBio Technology Co. Ltd.), or non-targeted scrambled lentiviral particles, and incubated for 9 days.[31] Cells were either lysed for Western blotting using specific antibodies against PLAUR (1:1000, ab103791, Abcam) or ITGAV (1:1000, ab179475, Abcam) for confirmation of gene knockdown efficiency or stimulated with Serpin E1 (20 μg/ml, Raybiotech) for intracellular calcium imaging.

2.5 | Cytokine release antibody array

Sensory neurons or phKCs were incubated with Serpin E1 (20 μg/ml, Raybiotech) or OSM (500 ng/ml, R&D) or the appropriate vehicle for 24 h. Cell culture supernatants were collected and subjected to a cytokine array following the manufacturer's protocol (R&D).

2.6 | Animal models

The MC903-induced mouse ear model was performed as before[10] using the C57BL/6 strain. An oral Serpin E1 inhibitor TM5275 (57 mg/kg, MedChemExpress) or vehicle (0.5% carboxymethyl cellulose) was administered orally using a gavage tube (gauge 21) once daily throughout the model. Ears or sensory ganglia were collected on day 10 and processed for immunohistochemical analysis. The OSM-induced acute itch was established by intradermal injection of 100 ng/4 μl OSM (R&D) or vehicle to the right cheek of mice. Only hind paw movement toward the injected right cheek was considered scratching.

2.7 | Histology

Paraffin sections of human skin and normal human DRG sections were purchased from Tissue Solutions (Glasgow, UK) and AMSBIO, respectively. Mice were deeply anesthetized and transcardially perfused using PBS containing 4% PFA before specimens were processed for dehydration, wax leaching, embedding, and cutting into 4-μm slices for HE staining and ICH. Histological assessments of immune cell infiltrations in the epidermis and dermis were performed in a blinded manner and scored based on the consensus of two independent observers. We randomly selected a single keratinocyte as a region of interest under each skin condition and analyzed the fluorescence intensity. The pooled intensity was plotted in Figure 3D,E. Each keratinocyte was represented as a single spot in the graphs.

2.8 | Intracellular Ca^{2+} measurement

mTGNs (>95% purity) were probed with Fluo-4 AM before adding Serpin E1 (20 μg/ml, Raybiotech) or vehicle. Images were captured at 2-sec intervals using MateXpress 6 software by ImageXpress Micro 4 Automated Cell Imaging System (Molecular Devices). phKCs were treated by OSM (20, 100, and 500 ng/ml) for calcium imaging, and analyzed as before.[10]

2.9 | Immunofluorescence staining

Paraffin sections were de-paraffinized, rehydrated, permeabilized, followed by incubation with rabbit antibody to TLR2 (1:300, Abcam ab213676), Serpin E1 (1:300, Abcam ab66705), PLAUR (1:300, Abcam ab103791),

BNP (1:100, Abcam ab236101), OSM (1:75, Thermo PA576861), mouse antibody to PGP9.5 (1:300, Abcam ab8189), NeuN (1:500, Novus NBP1-92693), TLR2 (1:300, Abcam ab16894), or Antigen Affinity-purified polyclonal goat IgG against PLAUR (10ug/ml, AF534, R&D) in blocking solution (4°C, overnight). The samples were washed in PBS and incubated with donkey anti-rabbit Alexa 594 (1:500, Abcam ab150064) or anti-mouse Alexa 488 (1:500, Abcam ab150109). Subsequent to a final wash, specimens were mounted onto slides using prolonged anti-fade reagents containing DAPI (ThermoFisher Scientific) and captured using an IX73 Olympus microscope. Fluorescence intensity was analyzed using CellSens Dimension Imaging software and Image J.

2.10 | Data statistical analysis

For RNA-seq analysis, differentially expressed gene (DEG) are defined based on thresholds: (log2FCH ≥ 1.0 and the adjusted p-value FDR (False Discovery Rate); ***FDR < 0.001, 0.001 < **FDR < 0.01, 0.01 < *FDR < 0.05, ^{ns}FDR > 0.05. For genes not reaching these thresholds, these are not defined as DEGs. Behavior data are presented as means ± SEMs ($n = 8$ or 9 mice/group). Others are presented as the mean ± SEM from at least three independent experiments. *P*-values were calculated using the two-tailed unpaired student *t* test; *p*-values <.05 were considered to be significant. Data analysis was performed using Prism software (GraphPad).

3 | RESULTS

3.1 | Serpin E1 selectively interacts with PLAUR in sensory neurons

To determine the involvement of PLAUR or ITGAV in mediating skin sensation, each expression was silenced in cultured murine trigeminal ganglionic neurons (mTGNs) using lentiviral mediated shRNA, and Serpin E1-dependent calcium transients were measured. Western blotting confirmed a reduction of PLAUR protein by 60% by shRNA *vs.* scrambled control (Figure 1A). This resulted in a decreased average level of calcium transients following Serpin E1 treatment (Figure 1B). Moreover, in comparison to the control, PLAUR knockdown (KD) resulted in a reduced number of mTGNs responding to Serpin E1 (Figure 1B), and the level of calcium influx (red, control; black, KD) as well as the area under the curve (AUC, representing changes with 100s-200s) of calcium spikes. PLAUR KD slowed recovery of basal Ca^{2+} concentration suggests it may play role in maintaining the neuronal baseline excitability. In contrast, lentivirally delivered shRNA achieved an 80% decrease in ITGAV expression in mTGNs (Figure 1D), however, Serpin E1-stimulated calcium influx, the number of responders, and AUC of calcium spikes were increased (Figure 1D). These findings suggest that PLAUR, but not IGTAV, is functionally linked to Serpin E1 signaling in sensory neurons. Although the exact reason for the elevation of calcium influx in Serpin E1 stimulated ITGAV KD mTGNs vs. control is unknown, it has been reported that Serpin E1 initiates endocytosis of uPA–uPAR–αV-integrin complexes, leading to the inactivation of integrins (i.e., αVβ3, αVβ5) and their endocytic clearance,[32] whereas KD prevented inactivation of integrins and their endocytic clearance, thus, resulting in higher calcium influx than control cells.

3.2 | PLAUR is upregulated in lesional skin from AD and psoriasis patients

Subsequently, the clinical significance of PLAUR in lesional (L) and non-lesional (NL) skin samples from patients with AD (LAD, NLAD), and lesional psoriasis (LPS), as well as healthy control (HC) was analyzed. Whole-skin RNA-seq revealed that PLAUR transcription was increased in LAD, but not in NLAD, when compared to HC (Figure 2A), and also increased in LAD vs. NLAD (Figure 2B). Interestingly, PLAUR transcripts were also increased in LPS vs. HC (Figure 2C). In contrast, ITGAV expression was not significantly changed in LAD and NLAD, compared to HC (Figure 2D), LAD vs. NLAD (Figure 2E), or LPS vs. HC (Figure 2F). This finding suggests a correlation between PLAUR expression and chronically inflamed or pruritic skin.

3.3 | Serpin E1 induces neurogenic inflammatory factors

We then focused on the consequence of Serpin E-PLAUR activation in sensory neurons. cytokine antibody array revealed that 24 h stimulation of mDRGs with Serpin E1 induced the release of various inflammatory mediators from mDRGs, including TNF-α, CCL20, PCSK9, CXCL1, CCL5, LIF, IL12p40, CD14, MMP-3, CXCL2, CXCL10, CXCL5, G-CSF, angiopoietin, VCAM-1, and CXCL16, compared to the vehicle-treated cells (Figure 3A). Consistently, RNA-seq of Serpin E1(6h)-treated mDRGs also revealed transcriptional upregulation of similar pro-inflammatory cytokines (Figure 3B). Among these, CCL20, CCL5, CXCL10, and G-CSF were

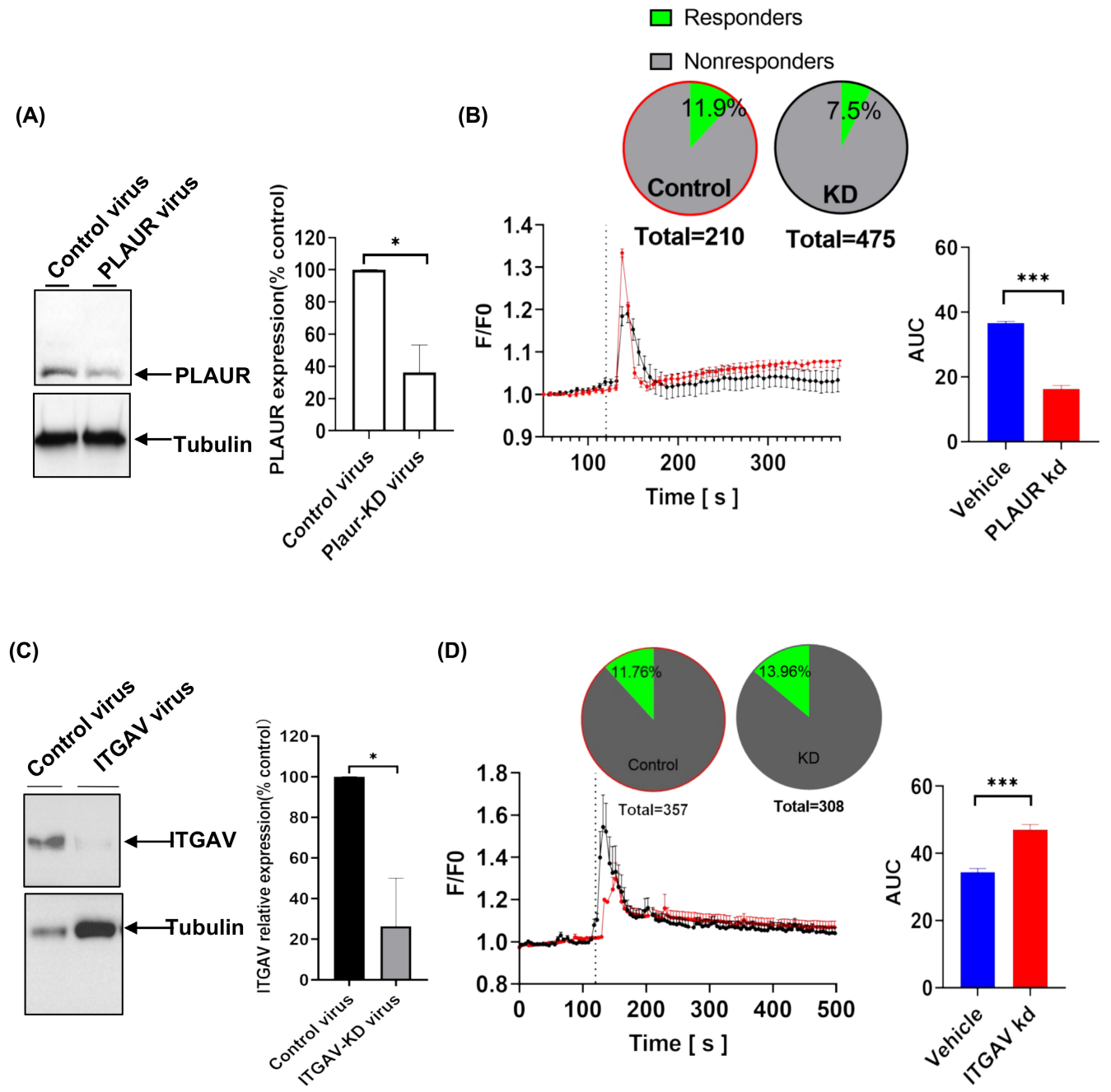


FIGURE 1 PLAUR contributes to Serpin E1-elicited response in cultured mTGNs. (A) Knockdown (KD) of PLAUR is revealed by Western blotting analysis. In mTGNs, PLAUR KD resulted in reduced calcium spikes elicited by Serpin E1, the number of responders to Serpin E1, and AUC of Serpin E1-induced calcium transients (B). (C) KD of ITGAV is confirmed by Western blotting analysis in mTGNs. (D) ITGAV KD failed to reduce Serpin E1-induced calcium spikes, the number of responders to Serpin E1, and AUC of calcium transients elicited by Serpin E1in mTGNs. Each cell intracellular calcium increases were normalized to F/F0, with F denoting the fluorescence and F0 the baseline fluorescence, and graphed relative to time. Responding cells were analyzed and presented as % total neurons. For all the data, means ± SEMs ($n \geq 3$); $^{ns}P > 0.05$, and $^{***}p < .001$, student *t*-test, $n = 3$. Scales = 5 μm.

found significantly upregulated in LAD skin tissue.[33-35] Enhanced production of keratinocyte-derived G-CSF contributes to the establishment and chronicity of AD lesions.[36] These highlights the importance of Serpin E1-PLAUR as a potent regulator of neurogenic inflammation.

3.4 | Serpin E1 induces TLR2 transcription in sensory neurons

Our subsequent RNA-seq analysis of Serpin E1-treatment of mDRG demonstrated a marked upregulation of TLR2 transcripts, along with an enhanced transcription of its

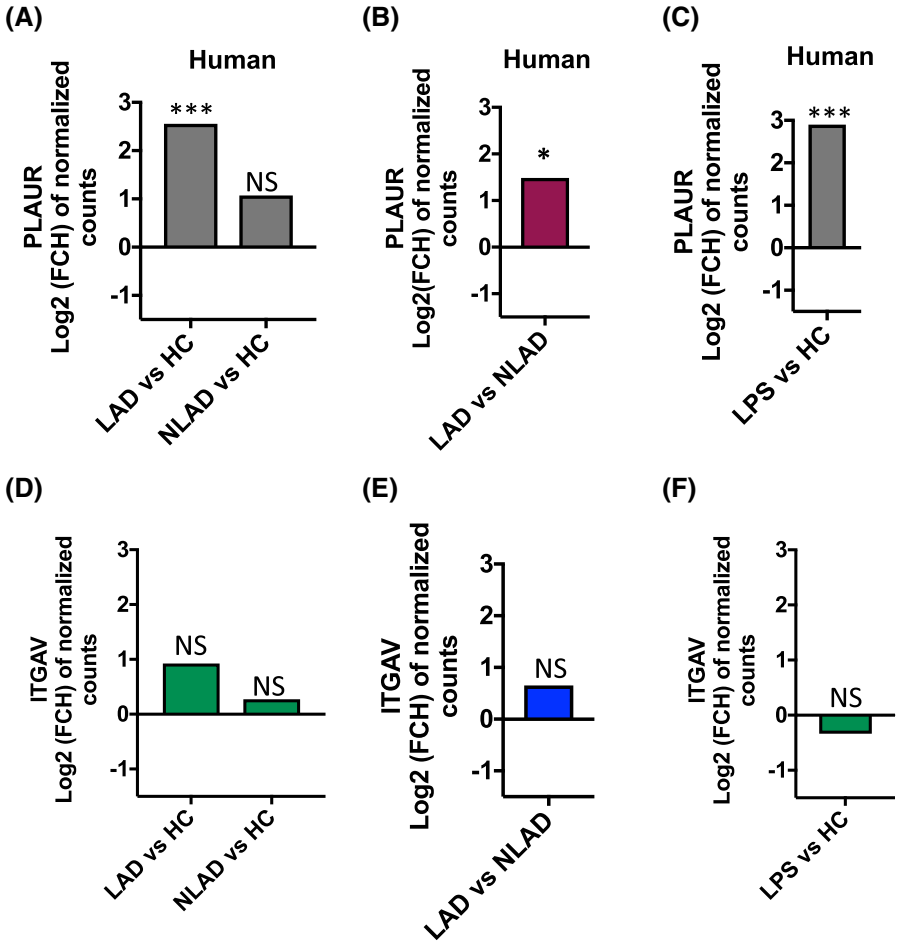


FIGURE 2 Transcription levels of PLAUR correlate with skin lesions of AD and psoriasis. Transcripts of PLAUR in human LAD and NLAD versus HC (A), and in LAD versus NLAD (B). Transcripts of PLAUR in LPS versus HC (C). Transcripts of ITGAV in LAD and NLAD versus HC (D), in LAD versus NLAD (E), and in LPS versus HC (F). Significances were defined with transcripts showing greater than a 2-fold change and an FDR value <0.05. Scales = 100 μm. For (A–C) ***FDR < .001, **0.001 < FDR < 0.01, *0.01 < FDR < 0.05, ^{ns}FDR > 0.05. For E, means ± SEMs ($n \geq 3$); N.S., ***$p < .001$, student *t*-test.

co-signaling proteins belonging to pro-inflammatory signaling pathways including NF-κB signaling proteins (Nfkbia, Nfkb1, Nfkb2), Dual Specificity Phosphatase (Dusp4, 6), JunB Proto-Oncogene (Junb), Fos Proto-Oncogene (Fos), Mitogen-Activated Protein Kinase Kinase Kinase (Map3k8, 1), and Pellino E3 Ubiquitin Protein Ligase 1 (Peli1) (Figure 3C). Together, these highlight TLR2 is a downstream target of Serpin E1-PLAUR.

3.5 | PLAUR resides in TLR2$^+$ human DRG neurons

To investigate the relation between TLR2 and PLAUR in human sensory neurons, we performed a co-expression analysis of PLAUR and TLR2 in human DRG (hDRG) tissue sections. PLAUR was found in a small subset (4.12%) of neurons labeled by the PGP9.5 antibody. PLAUR resided in 13.87% of TLR2$^+$ neurons (Figure 3D). Thus, both PLAUR and TLR2 are expressed in hDRG and partly overlapped in some neurons.

3.6 | PLAUR$^+$/TLR2$^+$ sensory subset was increased in murine chronic AD-like model

To understand whether cutaneous inflammation and skin dermatitis impact the expression/distribution of TLR2 and PLAUR in the sensory peripheral nervous system, ipsilateral and contralateral trigeminal sensory ganglia (TG) at the site of MC903-treated mouse ear were subjected to immunohistochemical analysis. In fact, higher levels of TLR2 immunosignal were detected in ipsilateral TG than in contralateral (Figure 3E). Furthermore, in ipsilateral TGs there was higher co-occurrence of TLR2$^+$/PLAUR$^+$ immunosignals in small-to-medium-sized neurons (Figure 3F), suggesting disease-driven upregulation

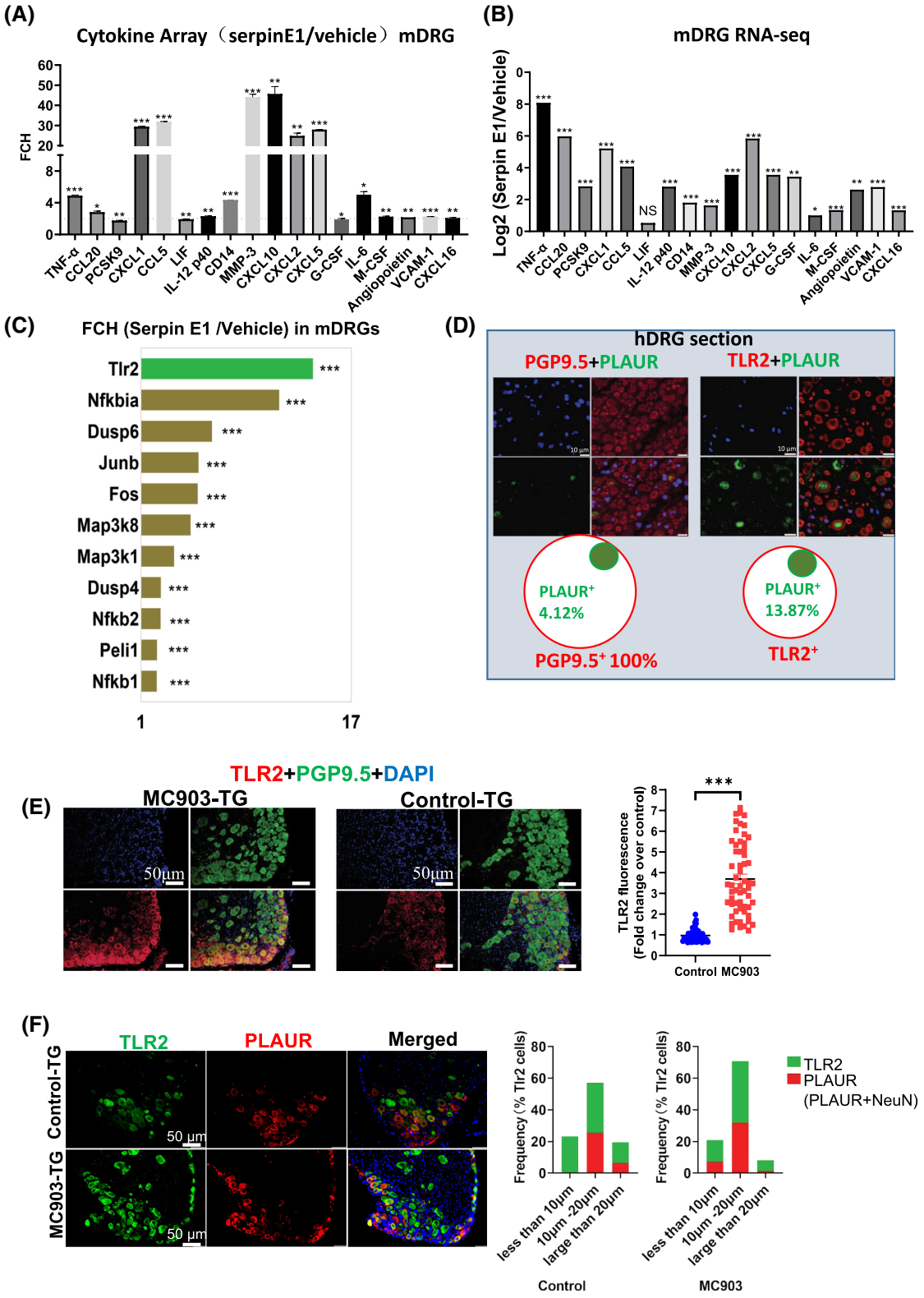


FIGURE 3 Serpin E1 induces itch-related gene transcription and itch-related cytokine release in mDRGs. Cytokine array for Serpin E1-stimulated release (A), and RNA-seq for Serpin E1-induced gene transcription (B). (C) RNA-seq revealing Serpin E1 upregulated TLR2-related gene network. (D) Localization of PLAUR and TLR2 in hDRG sections. Venn diagrams in D depict the relation of $PLAUR^+$ and $TLR2^+$ in hDRGs. Over 300 neurons were analyzed. Representative images for co-staining of TLR2 and PGP9.5 (E) or TLR2 and PLAUR (F) in TG innervating MC903-ear versus EtOH-ear. Scatter plot showing the fluorescence intensity of TLR2 against PGP9.5 in neurons within ipsilateral versus contralateral TGs. Bar chart showing the size distribution of $PLAUR^+$ against $TLR2^+$ neurons within ipsilateral versus contralateral TGs. Data are means ± SEMs ($n \geq 3$). For A, F, $^{ns}p > 0.05$, $^{**}p < 0.01$, and $^{***}p < .001$, student *t*-test. (B–C) ***FDR < 0.001, **0.001 < FDR < 0.01, *0.01 < FDR < 0.05, ^{ns}FDR > 0.05. Scales = 10 μm.

of TLR2 and PLAUR in sensory neurons. However, the mediators and cascades downstream of the Serpin E1-PLAUR-TLR2 axis remain unknown.

3.7 | The PLAUR-TLR2-associated signaling network contributes to BNP and PAR2 synthesis and OSM-involved itch

TLR2 is known to trigger inflammatory cascades that convert transient T_H2 cell-mediated dermatitis into persistent inflammation, as seen in chronic human AD.[17,18,20] To confirm that TLR2 transcription is dependent on AD activity, we assessed TLR2 transcription levels in LAD, NLAD, and HC. Our RNA-seq revealed that TLR2 transcripts are increased in LAD, but not in NLAD, when compared to HC (Figure 4A), indicating that TLR2 is increased in inflamed skin of AD patients.

We next investigated the consequence of TLR2 activation in mTGNs using Pam3CSK4 (TLR1/2 agonist), FSL-1 (TLR2/6 agonist), or vehicle. RNA-seq revealed that Pam3CSK4 treatment led to an upregulation of PAR2 (gene F2RL), a critical itch receptor,[37] TLR1, 2, and 6,[17,19] Serpin E1, and NPPB (Figure 4B). Among these transcripts, TLR2 was found upregulated strongest by both TLR2 agonists suggesting a self-enhancing feedback loop of TLR2 activation. Our findings further suggest that AD-associated Serpin E1 and NPPB are downstream signaling molecules of TLR2. Serpin E1 has been reported to be expressed in sensory neurons.[38] The agonistic specificity of the substances indicates that PAR2 upregulation is probably mediated via activation of TLR1/2, not TLR2/6. NPPB (BNP) has been implicated in various diseases associated with chronic itch, including AD, PS, and prurigo nodularis.[12,31,39] Indeed, the co-expression of both PLAUR and BNP proteins in TGNs was observed (Supporting Information Figure S1). In addition, we also detected that PLAUR and OSM co-resided in sensory neurons (Supporting Information Figure S1). Collectively, Serpin E1 might be involved in the pathology of chronic itch by activating PLAUR in $TLR2^+$ sensory neurons which results in upregulation of neuronal TLR2, in turn facilitating the upregulation of BNP in sensory neurons. In contrast to the effect of Serpin E1 in sensory neurons, calcium influx could also be detected in cultured phKCs, however, only a minimum increment of IL-8, among the 105 cytokines in the cytokine array profile, was observed (Supporting Information Figure S2). IL-8 levels in the stratum corneum are a biomarker for the severity of inflammation in the lesions of AD. It is closely related to the severity of local skin inflammation in AD. Improvements in skin symptoms in patients with AD after therapy are associated with a lowered level of IL-8.[40]

Our RNA-seq data further revealed that Pam3CSK4-activated mTGNs contains enhanced levels of OSM transcript, but not OSMR (Figure 4C), suggesting that OSM is a downstream target of the TLR2 signaling pathway. OSM was found upregulated in LAD and to a lesser extent in NLAD when compared to HC (Figure 4D). Subsequently, our intradermal check injections of OSM (100 ng/4 μl) into mice ($n = 9$/group), proved that OSM evoked site-directed scratching bouts than vehicle control (Figure 4E), peaking at 20 min (Figure 4F). Moreover, we observed that OSM, like IL-31, can activate phKCs. Consistently, the existence of a functional type II OSMR comprising gp130 and OSMRβ, but not the type I OSMR, in normal human keratinocytes, and upregulation in skin lesions from psoriatic and AD patients was reported.[29] We indeed recorded an OSM concentration-dependent intracellular calcium influx in keratinocytes (Figure 4H), and furthermore observed an OSM-initiated release of G-CSF, IL8, GM-CSF, and IL17E (Figure 4G). Together, the PLAUR-TLR2-OSM axis might contribute to chronic itch by promoting the transcription/release of a cocktail of pruritogens that comprises OSM, Serpin E1, and NPPB and by upregulation of the pruriceptors PAR2.

3.8 | Inhibition of Serpin E1 signaling attenuates MC903-induced itch and reduces epidermal hyperplasia and expression of Serpin E1 and OSM

To determine further whether the PLAUR-TLR2-OSM axis is implicated in cutaneous inflammation and chronic itch, we deployed pharmacological loss-of-function studies. We utilized the MC903-mouse model of chronic itch in combination with (or without) daily inhibition of Serpin E1 by oral TM5275 (a Serpin E1 inhibitor) or vehicle. Serpin E1 inhibition resulted in a reduction of cutaneous inflammation reflected by the reduced immune cell infiltration in the dermis (Figure 5A) but not the epidermis, based on HE histology analysis (Figure 5B). The analysis of HE staining in mice (TM5275+MC903) treatment with TM5275 revealed reduced epidermal thickness when compared to control mice (Vehicle+MC903) (Figure 5C). Furthermore, consistent with our previous study,[10] TM5275 attenuated itch behavior on days 5 and 9 post model when compared to control mice (Figure 5D). RNA-seq of ear skin obtained from untreated MC903 model revealed an upregulation pattern PLAUR, Serpin E1, OSMR, and OSM expression on the MC903-ear skin vs. ethanol-treated (Figure 5E), which was in line with our findings in human AD skin (c.f. Figure 4A,D). We thus analyzed if Serpin E1 inhibition in chronic itch mice is associated with the reduction of Serpin E1 and

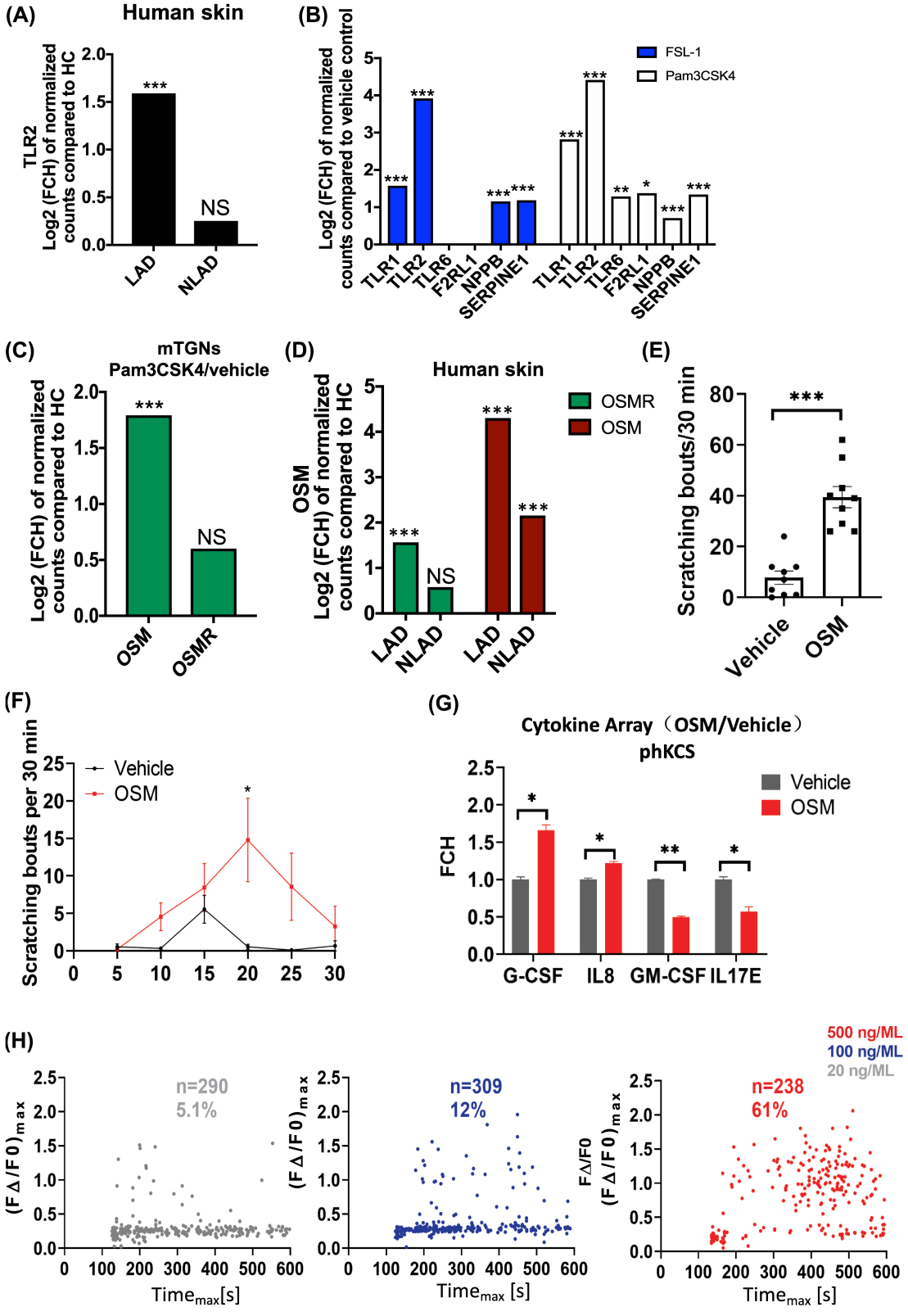
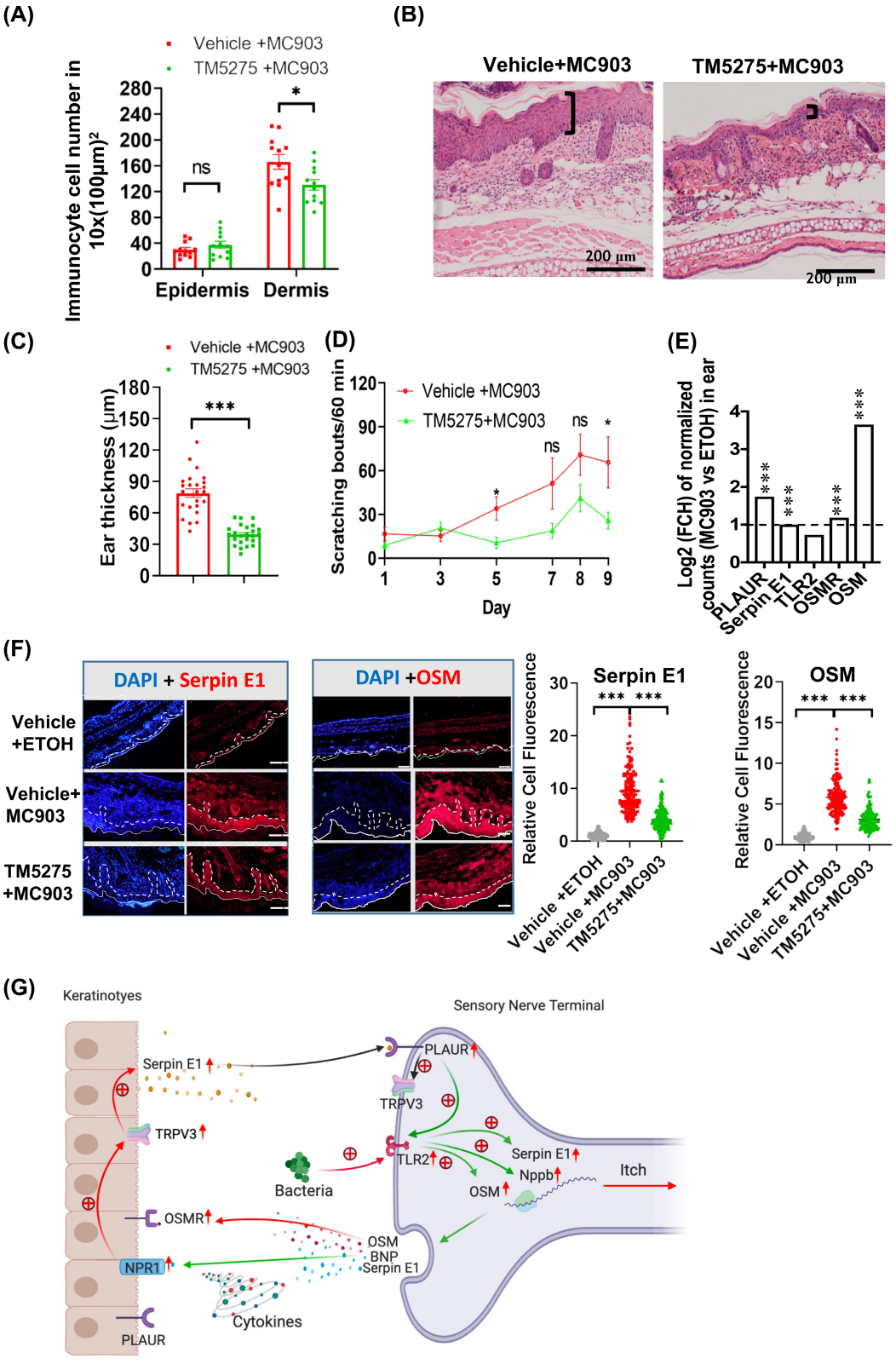


FIGURE 4 TLR2 is linked to AD disease severity; sensory TLR2 is implicated in itch-related gene transcription and OSM itch. (A) TLR2 transcription in human LAD and NLAD versus HC skin. FSL-1 and Pam3CSK4 induce itch-related transcripts in mTGNs (B). (C) Pam3CSK4 induces OSM transcription, but not OSMR. (D) OSMR and OSM transcription in human LAD, NLAD versus HC skin. Scratching bouts (E) after intradermal injection of OSM versus vehicle in mouse cheek models and time course (F) ($n = 9$ mice/group). OSM induces AD-related cytokine release (G), and dose-dependently triggered calcium influx (H) in cultured phKCs. FΔ, the change of stimulated fluorescence; $(F\Delta/F0)_{max,}$ maximum intensity of fluorescence over basal; $Time_{max}$, time at which maximum fluorescence was achieved. Only cells showing a 20% increase over baseline were deemed as the responders. For (A–E), ***FDR < 0.001, **0.001 < FDR < 0.01, *0.01 < FDR < 0.05, ^{ns}FDR > 0.05. For (E–G), means ± SEMs ($n \geq 3$); N.S., *$p > 0.05$, and ***$p < .001$, student t-test.

OSM content in the skin. Indeed, TM5275-treated mice were associated with a reduced abundance of Serpin E1 and OSM immunosignal in MC903-ear (Figure 5F). At the transcription level, downregulation of Serpin E1, and OSM in the MC903-ear following treatment with oral administration of TM5275 (Supporting Information Figure S3) was also detected by RT-qPCR. Altogether, these results indicate that the Serpin E1-PLAUR in connection with its downstream targets is vitally involved in chronic itch in mice.



Overall, this study provides a functional link between sensory PLAUR and AD and reveals a novel disease-relevant neuro-epidermal pathway for chronic itch and inflammation amplification, highlighting a presumable role of the Serpin E1-PLAUR axis in human AD and chronic pruritus (Figure 5F). Based on our findings, we believe that targeting the sensory PLAUR receptor will prove beneficial in the treatment of chronic itch conditions.

4 | DISCUSSION

We have demonstrated in this study that epidermal Serpin E1 initiates a cascade of cellular events that lead to the promotion of acute itch, chronic itch, or cutaneous inflammation in mice, by facilitating communication between epidermal keratinocytes and sensory neurons. Serpin E1 signals through PLAUR to modulate the expression of the pruriceptor TLR2 and the pruritogens BNP and OSM in sensory neurons, thus acting as a positive regulator of acute and chronic itch. Our results have therefore defined a new molecular network of these critical itch mediators that engages in the modulation of neuronal excitability and peripheral neuroimmune responses during chronic itch and AD in mice.

Our group described in a previous study, that epidermal Serpin E1 is upregulated in human AD skin, and this regulation is dependent on TRPV3 activation and potentiated by BNP.[10] In this report, we demonstrate that Serpin E1 acts through PLAUR which resides in a proportion of $TLR2^+$ neurons in murine and human sensory neurons. Like its agonist, PLAUR was found upregulated in lesional human AD skin. Serpin E1 activation of $PLAUR^+$ sensory neurons resulted in TLR2 upregulation, which, in turn, upregulated BNP, TLR2, and PAR2 transcripts, thereby dysregulating epidermal neuronal communication in the skin, and ultimately worsening Serpin E1-dependent itch behavior in mouse models of acute and chronic itch. In fact, PLAUR, Serpin E1, OSM, and BNP expressions are all upregulated in chronic AD skin of MC903-treated mice. Our findings, therefore, confirm earlier results that Serpin E1, PAR2, and BNP are critically involved in itch[10,12,41] and that PLAUR, OSM, and probably the OSM type II receptor act as a pruritogenic axis. Recently, OSMR is confirmed in BNP^+ sensory neurons (90% overlap), and OSM potentiates sensory neurons to other pruritogens,[42] confirming crosstalk between OSM and BNP. Similar to the OSM-elicited scratching bouts in the neck model showing a late-onset profile,[42] our OSM cheek model showed 20 min to peak, indicating a requirement of regulatory mechanism. In that study, OSM (1 μg/μl, 10 μl), higher than tested herein, failed to induce significant scratching bouts within 30 min post-injection, despite being significant between 30 and 60 min.[42] Considering scratching response induced by the agonists of GPCR has an optimal concentration range, that fits the typical bell-shaped concentration-dependent curve due to the endocytosis and desensitization of GPCR receptors in vivo, that is IL-31 and LTC4,[43] we assume OSM induces itch mainly through indirect activation of sensory neurons, by cutaneous signaling and intermediate mediators that fall into the category of GPCR antagonist. PLAUR-TLR2-OSM might play a role to amplify this signaling, however, other regulatory possibilities could not be excluded.

In our cultured mTGNs, TLR2 agonist Pam3CSK4 enhanced OSM expression, which seemed to be unexpected as OSM is predominantly expressed in immune cells.[42] In fact, single-cell RNA-seq revealed both TLR2 and OSM did reside in NF3 murine normal DRG neurons, albeit at a low level.[27]

Our results further implicate a PLAUR-dependent role for neuronal TLR2 in chronic itch and probably human AD. In AD patients, functional TLR2 expression appears to be impaired in keratinocytes,[44] and macrophages resulting in an attenuated innate immune response which might "contribute to an enhanced susceptibility to skin infections with *S. aureus* in AD".[18] Whether bacteria, viruses, or their products also directly interact with itch-relaying

FIGURE 5 TM5275 attenuates MC903-mediated itch, epidermal hyperplasia, and reduces the expression of Serpin E1, and OSM in the skin of MC903-mice. (A) Immunocyte cell number in MC903-mice treated with or without TM5275. Representative HE staining (B) and ear skin epidermal thickness analysis (C) in MC903-mice with or without TM5275 treatment. (D) TM5275 attenuated itch-like behavior in MC903-mice. (A) RNA-seq showing upregulation of transcripts of PLAUR, Serpin E1, OSMR, and OSM in ear skin from MC903-mice. (F) Representative images showing T5275 reduced expression of Serpin E1 and OSM in ear skin from MC903-mice. Data in A–C are means ± SEMs; All data in D,E are from $n = 6$–8 mice/group. For A, C and F, data are from ($n \geq 4$ mice/group). For (E), ***FDR < 0.001. For A and C, N.S., $p > 0.05$, *$p < 0.05$, and ***$p < .001$, student t test; for (F), ***$p < .001$, one-way ANOVA. (F) Schematic showing signaling pathways for PLAUR-TLR2 in chronic itch. Serpin E1 receptor PLAUR and TLR2 are upregulated in AD skin from patients and mouse models. Serpin E1 activated PLAUR in TLR^+-neurons, leading to transcriptional upregulation of TLR2 and its co-signaling proteins. TLR2 in turn promotes gene transcription of critical itch mediators including BNP and OSM, as well as AD-associated PAR2. OSM induces acute itch-like behaviors in mice and promotes inflammatory mediator release from human keratinocytes. Serpin E1 facilitates transcription/expression levels of Serpin E1 and OSM. This study uncovered that PLAUR is a new pruritogenic receptor in promoting AD itch. We propose targeting PLAUR represents a novel target for the therapeutic development of treating AD itch and skin inflammation

neurons is not known. Our finding of an AD-associated upregulation of neuronal TLR2, however, provides a direct link between the innate immune system and sensory neurons suggesting that microbes could directly activate pruriceptors by engaging in TLR2 to initiate itch and neurogenic inflammation in AD. In a positive feedback loop, TLR2 signaling results in an increase in neuronal Serpin E1 and TLR2, enhancing the capacity of the PLAUR-TLR2 axis to perpetuate AD-associated chronic itch and inflammation. Indeed, although an earlier study reported that TLR2 is absent from murine sensory neurons,[45] a number of recent reports revealed the role of TLR2 in murine DRG and TGN.[27,46,47] For example, sensory neuronal TLR2 mediates different itch and pain behaviors through activating TLR1/TLR2 or TLR6/TLR2 heterodimers.[47] In our study, TLR2 expression was detected in murine and human sensory neurons in vitro and in situ, and the TG neuronal TLR2 was upregulated remotely by skin lesion in MC903-mice. The mechanism for intra-axonal upregulation may represent an important disease-driven sensitization mechanism. For example, house dust mite chronically induced ipsilateral TG neuronal IL-4RA and IL-31RA upregulation in the PAR-2 overexpressing mice.[48] Also, in a peripheral pain model, the initial activation of the TLR2 complex induced TLR2 upregulation at both the site of the original injury and in the DRG cell bodies.[46] This intra-axonal sensation mechanism should be further explored.

Our data also suggests that TLR2 regulates neuronal inflammatory processes via three distinct canonical inflammatory signaling pathways (NFkB, MAP kinase, JunB) and is probably involved in neuro-epidermal-driven exacerbation of cutaneous inflammation and eczema in AD skin. Hence, we hypothesize that neuronal TLR2 drives cutaneous inflammation in human AD through a neurogenic mechanism.

Interestingly, Serpin E1, BNP, and OSM are co-regulated in sensory neurons in response to TLR2 innate immune activation, sustaining the importance of TLR2 as an itch-promoting receptor and suggesting that these critical pruritogens probably resume a role in defense mechanisms in the skin and the neurodermatitis feature of AD. However, the subtype of PLAUR$^+$ sensory neurons in AD-affected patients and the status of TLR2 expression in human AD still await confirmation. It has been shown that PLAUR resides in NP1-3 neurons with the rank order of NP1 > NP2 > NP3,[27] of cause, the isolation method, location, and age of tissue used may also influence neuronal signature.

In the MC903-mice, inhibition of the Serpin E1 signaling pathway leads to a reduction of immunocyte infiltration and epidermal hyperplasia as well as epidermal Serpin E1 and OSM expression sustaining the existence of a Serpin E1-PLAUR-OSM axis on the epidermal level. In conjunction with our earlier finding that Serpin E1 is a downstream pruritogen of TRPV3, that is upregulated by BNP, an itch peptide stimulated by IL-31, it is predicated that the PLAUR-TLR2-OSM axis could be regulated by IL-31, to infer a novel itch process.

Taken together, we here present findings of a functional PLAUR takes a pivotal role in the induction of chronic itch and an AD-like phenotype in mice and propose a neuro-epidermal-innate immune communication network, that might represent a promising target for the treatment of AD in humans.

AUTHOR CONTRIBUTIONS STATEMENT

Supervision: Jianghui Meng, Jiafu Wang; Conceptualization: Jianghui Meng, Jiafu Wang, Martin Steinhoff, Weiwei Chen; Data Curation: Weiwei Chen, Yanqing Li, Martin Steinhoff, Joerg Buddenkotte, Wenhao Zhang, Renkai Zhu, Xinrong Yan, Zhiping Lu, Song Xiao; Funding Acquisition: Jianghui Meng, Jiafu Wang; Formal Analysis: Jianghui Meng, Jiafu Wang, Martin Steinhoff, Joerg Buddenkotte, TB, Weiwei Chen; Resources: Weiwei Chen, Joerg Buddenkotte, Timo Buhl; Writing—Original Draft Preparation: Weiwei Chen, Yanqing Li, Martin Steinhoff, Wenhao Zhang, Joerg Buddenkotte, Timo Buhl, Renkai Zhu, Xinrong Yan, Zhiping Lu, Song Xiao, Jiafu Wang, Jianghui Meng; Writing—Review and Editing: Weiwei Chen, Yanqing Li, Martin Steinhoff, Wenhao Zhang, Joerg Buddenkotte, Timo Buhl, Renkai Zhu, Xinrong Yan, Zhiping Lu, Song Xiao, Jiafu Wang, Jianghui Meng.

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DISCLOSURES

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available at GSE189333 which will be immediately released upon publication. The rest of the data will be available from the corresponding author on reasonable request.

ORCID

Weiwei Chen https://orcid.org/0000-0002-1965-7606
Yanqing Li https://orcid.org/0000-0002-4406-7878
Martin Steinhoff https://orcid.org/0000-0002-7090-2187
Wenhao Zhang https://orcid.org/0000-0003-2797-1125

Joerg Buddenkotte https://orcid.org/0000-0002-2394-3269
Timo Buhl https://orcid.org/0000-0002-3139-129X
Renkai Zhu https://orcid.org/0000-0002-0171-158X
Xinrong Yan https://orcid.org/0000-0001-5177-1583
Zhiping Lu https://orcid.org/0000-0001-9927-0120
Song Xiao https://orcid.org/0000-0003-2254-9486
Jiafu Wang https://orcid.org/0000-0002-3654-4400
Jianghui Meng https://orcid.org/0000-0002-3107-4200

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SUPPORTING INFORMATION

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