

RESEARCH ARTICLE

IL-20 promotes cutaneous inflammation and peripheral itch sensation in atopic dermatitis

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Funding information

This work is supported by Henan University via the Double-First Class Project Program, and Science Foundation Ireland (15/SIRG/3508T and 13/CDA/2093) and a Type 2 innovation grant from Sanofi Genzyme to JM.

Abstract

Atopic dermatitis (AD) is a chronic skin disease, which is associated with intense itch, skin barrier dysfunction and eczematous lesions. Aberrant IL-20 expression has been implicated in numerous inflammatory diseases, including psoriasis. However, the role of IL-20 in AD remains unknown. Here, RNA-seq, Q-PCR, and immunocytochemistry were utilized to examine disease-driven changes of IL-20 and its cognate receptor subunits in skin from healthy human subjects, AD patients and murine AD-models. Calcium imaging, knockdown and cytokine array were used to investigate IL-20-evoked responses in keratinocytes and sensory neurons. The murine cheek model and behavioral scoring were employed to evaluate IL-20-elicited sensations in vivo. We found that transcripts and protein of IL-20 were upregulated in skin from human AD and murine AD-like models. Topical MC903 treatment in mice ear enhanced IL-20R1 expression in the trigeminal sensory ganglia, suggesting a lesion-associated and

Abbreviations: AD, atopic dermatitis; ALB, albumin; APOA1, apolipoprotein A-I; CCL2, C-C motif chemokine 2; CYP2A7, cytochrome P450; DAPI, 4',6-diamidino-2-phenylindole; DEGs, differentially expressed genes; DRGs, dorsal root ganglionic neurons; FDR, false discovery rate; FPKM, fragments per kilobase of transcript per million mapped reads; GSTA1, Glutathione S-Transferase Alpha 1; HC, healthy control; HGF, hepatocyte growth factor; HP, haptoglobin; IL-13R α , IL-13 receptor alpha; IL-31OE, IL-31 overexpressing; KD, knockdown; LAD, lesional atopic dermatitis; NLAD, non-lesional atopic dermatitis; NPY, neuropeptide Y; pHKCs, primary human keratinocytes; PLG, plasminogen; RBP4, retinol-binding protein; TDO2, tryptophan 2,3-dioxygenase; TGN, trigeminal ganglionic neurons; TLR, toll-like receptors; VIP, vasoactive intestinal peptide.

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epidermal-driven mechanism for sensitization of sensory IL-20 signaling. IL-20 triggered calcium influx in both keratinocytes and sensory neurons, and promoted their AD-related molecule release and transcription of itch-related genes. In sensory neurons, IL-20 application increased TLR2 transcripts, implicating a link between innate immune response and IL-20. In a murine cheek model of acute itch, intradermal injection IL-20 and IL-13 elicited significant itch-like behavior, though only when co-injected. Our findings provide novel insights into IL-20 function in peripheral (skin-derived) itch and clinically relevant intercellular neuron-epidermal communication, highlighting a role of IL-20 signaling in the pathophysiology of AD, thus forming a new basis for the development of a novel antipruritic strategy via interrupting IL-20 epidermal pathways.

KEYWORDS

atopic dermatitis, cytokine, IL-13, IL-13R α 1, IL-13R α 2, IL-20, IL-20R1, IL-20R2, toll-like receptor

1 | INTRODUCTION

Atopic dermatitis (AD), one of the most common chronic skin diseases world-wide, affects both children and adults, and is characterized by dysregulation of multiple immune pathways, pathological inflammation, and severe itch (pruritus).¹⁻³ Un-controlled communication between sensory nerves and the immune system plays an essential role in the pathophysiology of AD.⁴⁻⁶ Therefore, understanding the molecular mechanisms of “neuro-immune communication” and its dysregulation will lead to novel therapeutic strategies for AD.^{7,8}

The epidermal and dermal skin is highly innervated by itch-sensing primary sensory nerves which express receptors for inflammatory mediators (cytokines, chemokines, amines, proteases, peptides, toll-like receptors) and induce inflammation and itch in AD.⁹ IL-20 has been implicated in several diseases, including psoriasis, rheumatoid arthritis, atherosclerosis, stroke, autoimmune intestinal diseases. Functionally, IL-20 is involved in epidermal differentiation, angiogenesis, and chemotaxis. Thus, IL-20 has been proposed as a potential target for the treatment of inflammatory disease.¹⁰⁻¹⁴ Despite this, its role in the dysregulation of neuro-immune circuits and the pathophysiology of AD remains unexplored.

IL-20 belongs to the IL-10 family of cytokines (together with IL-19, -22, -24, and -26), and interacts with heterodimer receptor complexes, IL-20R1/IL-20R2 (type I) and IL-22R1/IL-20R2 (type II).¹⁵ IL-19 and IL-24 also bind IL-20R1/IL-22R2 and IL-24 binds IL-22R1/IL-20R2.^{16,17} Multiple studies have implicated epidermal cells as the primary producers of IL-20 in the skin and the drivers of IL-20-associated cutaneous inflammation.^{12,18} However, the role of IL-20 in itch and AD remains elusive.

IL-20 interrupts normal keratinocyte differentiation and is linked with epidermal hyperplasia. IL-20 and its receptor subunits, IL-20R1 and IL-20R2, are expressed in keratinocytes.^{10,12,19,20} IL-20 is partially responsible for IL-31-induced downregulation of filaggrin,^{21,22} an essential component of the epidermal differentiation complex and a key factor in the pathogenesis of AD.^{23,24} Despite this, the clinical relevance of IL-20 and its effect on epidermal and sensory neurons remain unclear. Moreover, the expression and distribution of IL-20 and its cognate receptor subunits are largely unknown.

Herein, primary human keratinocytes, primary murine sensory neurons, and samples from AD patients and AD-like mouse models were utilized to examine the molecular basis of IL-20-driven skin inflammation, downstream signaling pathways, and itch transduction in AD. This work aims to further our understanding of AD pathogenesis, ultimately identifying novel therapeutic targets for the treatment of AD and AD-like itch conditions.

2 | MATERIALS AND METHODS

2.1 | Animal rights

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.

2.2 | Human skin samples preparation

Skin punch biopsies were donated by 5 lesional and 5 non-lesional patients with atopic dermatitis (LAD and

NLAD) and 5 healthy individuals (HC). These samples were used in our previous work²⁵ and were analyzed by RNA-seq.²⁵ RNA-seq was performed by IMG Laboratory (Martinsried, Germany). This study was institutionally approved by the Medical Research Council, National Scientific and Ethical Committee, Budapest, Hungary (ETT TUKEB, document IDs: 50935/2012/EKU and 54256-1/2016/EKU), as detailed in Ref. [25]. 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, NLAD skin, and HC skin followed by on-column DNase digestion. All LAD are with a SCORing Atopic Dermatitis score higher than 35.

The paraffin sections of human skin biopsies from LAD patients and HC were purchased from Tissue Solutions (Glasgow, UK). Lesional severity score EASI for LAD is 21.1-50.0.

2.3 | RNA-seq

All the human skin 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, Germany). Sequencing of the library was performed on a NextSeq 500 sequencing system (Illumina) 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.

For cultured cells and mouse tissue, RNA isolation and RNA-seq were performed by BGI (Beijing Genomics institution, China). RNA was isolated from cultured mouse DRGs (mDRGs) by using the RNeasy kit (Qiagen, China). For mouse tissue, about 60 mg tissues were grinded with liquid nitrogen into powder and transferred into the 2 ml tube containing of 1.5 ml Trizol reagent for homogenizing 2 min and centrifuging at 12 000× g for 5 min at 4°C. The supernatant was transferred to a new 2.0 ml tube in the presence of 0.3 ml of chloroform/isoamyl alcohol v(24:1) per 1.5 ml of Trizol. After shaking the tubes vigorously for 15 s, samples were centrifuged at 12 000× g for 10 min at 4°C. The mixture was

separated into three layers: the lower phenol-chloroform phase, an interphase, and an upper aqueous phase. RNA remains in the aqueous phase. RNA pellet was washed with 1 ml 75% ethanol, and re-suspended and centrifuged at 13 600 rpm for 3 min at 4°C. Purified mRNA was fragmented into small pieces with fragment buffer. First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by a second-strand cDNA synthesis. The cDNA fragments obtained were amplified by PCR, and products were purified by Ampure XP beads, then dissolved in EB solution. The double stranded PCR products from previous step were denatured and circularized by the splint oligo sequence to get the final library. The single strand circle DNA was formatted as the final library. Sequencing data are called raw reads or raw data, and quality control (QC) is then performed on the raw reads to determine whether the sequencing data are suitable for subsequent analysis. Sequencing data filtering was performed using software SOAPnuke developed by BGI independently (Parameters -l 15 -q 0.2 -n 0.05).

A greater than 2-fold change ($\log_2\text{FCH} \geq 1$) in transcription was deemed significant in each comparison. FDRs were determined and classified as ***FDR < 0.001, **0.001 < FDR < 0.01, *0.01 < FDR < 0.05, ^{ns}FDR > 0.05.

2.4 | Culture of pHKCs and mTGNs and knockdown of IL-13 receptors in pHKCs

pHKCs (Catalog #: 00192627) were purchased from Lonza Inc. (Belgium), seeded into 24 well plates and cultured in medium containing KBM Gold Basal Medium (00192151) and KGM Gold SingleQuots supplements (00192152), both provided by Lonza Inc.

mTGNs were isolated from postnatal C57BL/6 mice and dissociated by collagenase I, as previously described.²⁶ Cells were cultured in the presence of cytosine β -d-arabinofuranoside (Sigma) and nerve growth factor 100 ng/ml for 7 days in vitro.

For knockdown of IL-13R α 1 and IL-13R α 2, cells were incubated in a culture medium containing shRNA lentiviral particles that specifically target IL-13R α 1, IL-13R α 2 (OBio Technology Corp. China), or non-targeted scrambled lentiviral particles, as before.²⁷ Cells were lysed in LDS sample buffer for Western blotting to confirm the knockdown effect. IL-13 or its vehicle was applied to the knockdown and control cells for 6 h before cells were harvested for RNA-seq. Rabbit anti-IL-13R α 1 (Abcam, [RRID:AB_1640587](#), AB79277, 1:1000) or goat anti-IL-13R α 2 (R&D Systems, [RRID:AB_354809](#), AF146, 1:1000) and rabbit anti-SNAP-23 (Synaptic

Systems, [RRID:AB_1263494](#), 111202, 1:1000) were used for immunoblot.

2.5 | Cytokine release assay (cytokine antibody array)

phKCs were incubated with hydrocortisone-free KBM Gold medium for ≥ 24 h and stimulated with IL-20 (500 ng/ml, 24 h). Following release, cell-culture supernatants were collected, pooled, and analyzed using the Proteome Profiler Human XL Cytokine Array Kit (R&D Systems), according to the manufacturer's protocol. Each cytokine spot was analyzed with the ImageJ software. Resultant densitometry values from treated samples were calculated relative to nontreated or vehicle-treated control values to determine the fold increase.²⁸

2.6 | Intracellular Ca^{2+} measurement in phKCs and mTGNs

Cultured phKCs and sensory neurons were loaded with 3 μM Fluo-4 AM in hydrocortisone-free medium for 30 min (37°C). After a baseline period of 200 s, IL-20 (500 ng/ml) or vehicle was applied. Images were captured by ImageXpress Micro 4 Automated Cell Imaging System (Molecular Devices, China) using MateXpress6 software. Intracellular calcium increases were normalized to F/F₀, with F denoting the actual fluorescence value and F₀ denoting the baseline fluorescence.²⁵

2.7 | Immunofluorescence staining

Paraffin sections of human skin were deparaffinized, rehydrated, and permeabilized in phosphate-buffered saline with 0.2% Triton X-100 (PBS-T), and pre-incubated in PBS containing 5% normal donkey serum (blocking solution) at room temperature for 1 h. Specimens were then incubated with antibody to IL-20 (Invitrogen, AB_2720694, PA5-76967, 1:100), IL-20R1 (Abcam, [RRID:AB_2750843](#), ab203196, 1:100), IL-20R2 (Abcam, [RRID:AB_10678407](#), ab95824, 1:200), or PGP9.5 (Abcam, [RRID:AB_1269733](#), ab72911, 1:500), or IL-13 (Abcam, [RRID:AB_10867235](#), ab106732, 1:500) in blocking solution (4°C, overnight). The specimens were washed with PBS and incubated with Alexa 488 or Alexa 594 conjugated IgG. Following removal of unbound secondary antibody, specimens were mounted using prolong anti-fade reagents containing (4',6-diamidino-2-phenylindole) DAPI. Images were taken by IX73 Olympus inverted microscope using the CellSens Dimension Imaging software.

2.8 | Real-time reverse transcription PCR

RNA was isolated from cultured mouse DRGs (mDRGs) by using the RNeasy kit (Qiagen, China). The TRIzol Reagent was used to isolate RNA as before.²⁵ mRNA quantitation was performed by using real-time fluorescence detection with the SYBR Green ROX mix (Applied Biosystems Inc, China). Primers were purchased from Origene (China).

Their sequences are as following:

mIL-20	F: TTCTGAGATTCGGGATAGTGTGC R: GATGACGAAGGAAGCAGCACCT
mIL-20R2	F: CTCTTGATGTGGAACCCAGTGAC R: CACACTCCAGACTTTTGGTCGG
mIL-20R1	F: CGAGACGGAACGCTTCTATCCT R: TTCTCTGGTGCTGTCAGGGCAA
mGAPDH	F: CATCACTGCCACCCAGAAGACTG R: ATGCCAGTGAGCTTCCC GTTCAG
mMMP3	F: CTCTGGAACCTGAGACATCACC R: AGGAGTCCTGAGAGATTTGCGC
mCXCL5	F: CCGCTGGCATTCTGTGTTGCTGT R: CAGGGATCACCTCCAAATTAGCC
mCXCL1	F: TCCAGAGCTTGAAGGTGTTGCC R: AACCAAGGGAGCTTCAGGGTCA
mCCL2	F: GCTACAAGAGGATCACCAGCAG R: GTCTGGACCCATTCTCTTCTGG
mTLR2	F: ACAGCAAGGTCTTCTCTGGTTCC R: GCTCCCTTACAGGCTGAGTTCT

2.9 | Animal models and behavioral analysis

To establish a prolonged MC903-induced model of AD, MC903 (2 nmol/20 μl in ethanol, Sigma Aldrich) was applied topically to the left ear of C57BL/6 mice. This was repeated daily for 7 consecutive days, resulting in the induction of an AD-like chronic itch model.²⁵

To analyze the influence of IL-20 on IL-13-induced itch-like behavior, C57 BL/6 mice ($n = 8$ mice/group) received a left cheek injection of vehicle, IL-20 (2.5 $\mu\text{g}/10 \mu\text{l}$) or IL-13 (1 $\mu\text{g}/10 \mu\text{l}$), or a co-injection of IL-20 (2.5 $\mu\text{g}/10 \mu\text{l}$) and IL-13 (1 $\mu\text{g}/10 \mu\text{l}$). Bouts of hind limb scratches were video recorded for 1 h and scratching bouts directed to the injection site were considered indicative of pruritus. All the mice were video-recorded for 1 h for the analysis of scratching bouts. Data presented as means \pm SEMs ($n = 8$ mice/group); * $p < .05$, ** $p < .01$, and *** $p < .001$, 2-way analysis of variance (ANOVA).

2.10 | Statistical analysis

For RNA-seq, average fold change for genes upregulated were plotted. Differentially expressed gene (DEG) are defined based on thresholds: ($\log_2\text{FCH} \geq 1.0$ and the adjusted p -value $\text{FDR} < 0.05$). FDRs were indicated as $***\text{FDR} < 0.001$, $**0.001 < \text{FDR} < 0.01$, $*0.01 < \text{FDR} < 0.05$, $^{\text{ns}}\text{FDR} > 0.05$. For genes not reaching these thresholds, although $\text{FDR} < 0.05$, these are not defined as DEGs.

Other data are presented as mean \pm SEM with dot plots or bar graphs from at least three independent experiments. We made two-group comparisons with a two-tailed Student's t -test followed by Welch's correction, except that datasets in Figure 5C was analyzed using 2-way ANOVA followed by Bonferroni's post hoc analysis to determine where those differences occurred among multiple groups. The criterion for statistical significance is $^{\text{ns}}p > .05$, $*p < .05$; $**p < .01$; $***p < .001$ (Prism 7, GraphPad Software, La Jolla, CA, USA).

For Figure 1A,B, the $\log_2\text{FCH}$ of counts were filtered and plotted from DEGs.²⁹ The $\log_2\text{FCH}$ values are presented based with their corresponding Benjamini adjusted p -value of each murine model contrast, for the MADAD DEGs (cut-off absolute $\text{FCH} \geq 2$ and $\text{FDR} \leq 0.05$).

3 | RESULTS

3.1 | IL-20 expression level, but not its receptor IL-20R1, is enhanced in human AD lesion

IL-20 transcripts are known to be upregulated in lesional skin from psoriasis patients^{30–32}; however, whether IL-20 expression is altered in AD remained unclear.

RNA-seq was used to analyze the transcription of IL-20 and its cognate receptor subunits, IL-20R1 and IL-20R2, in skin samples from lesional AD (LAD), non-lesional AD (NLAD) and healthy controls (HC). The differentially expressed genes (DEGs) were expressed as \log_2 fold change (FCH), with equaling or greater than 1 indicating a significant change (False Discovery Rate [FDR] < 0.05). Transcription levels of IL-20 were greatly elevated in LAD versus HC, ($\log_2\text{FCH} = 7.7$), and in LAD versus NLAD ($\log_2\text{FCH} = 4.2$), but not in NLAD versus HC ($\log_2\text{FCH} = 3.5$) (Figure 1A). In contrast, transcription levels of the IL-20 receptor subunits, IL-20R1 and IL-20R2, were decreased in skin from LAD versus HC, with the downregulation of IL-20R1 reaching significance ($\text{FDR} < 0.05$ cut-offs and $\log_2\text{FCH} \geq 1$) (Figure 1A). This change was not observed in either LAD versus NLAD or NLAD versus HC (Figure 1A). Transcription levels of IL-22R, a common

receptor for the IL-20 subfamily, were also analyzed by RNA-seq. However, no changes were identified (data not shown). Thus, human AD lesions display enhanced transcription of IL-20, but not its cognate receptors. Using a published dataset, we also examined the expression of IL-20 in murine models of AD. Here, IL-20 transcriptional levels were increased in both oxazolone (Ova)-challenged and Flaky-tail mouse models²⁹ (Figure 1B), further confirming our findings and strengthening the link between IL-20 and AD lesions.

Skin samples from human LAD and HC were then analyzed by immunohistochemistry. In both LAD and HC ($n = 3$ donors/group), IL-20 protein was predominantly detected in the epidermal keratinocyte layer of skin (Figure 1C). Importantly, IL-20 was highly expressed in LAD skin, with quantification of the fluorescent signal revealing a significant increase in LAD samples versus HC (Figure 1C,D). In contrast, no fluorescence signal was detected when primary antibodies were omitted (Figure S1). In a similar manner to our RNA-seq data, expression of IL-20 receptor subunits was reduced in LAD compared with HC, with quantification of the fluorescent signal demonstrating a significant downregulation of both IL-20R1 and IL-20R2 proteins (Figure 1C,D).

Together, these data reveal a link between AD and IL-20 expression. IL-20 levels are upregulated in AD lesion but not the levels of the IL-20 receptor subunits. Conversely, in psoriasis, both IL-20 and its receptors are increased in the epidermal lesions^{31,33,34} but not in the total RNA extracted from whole skin.^{20,35}

3.2 | IL-20 triggers calcium influx, induces inflammatory cytokine release, and upregulates AD-related gene transcription in primary human keratinocytes

IL-20 is now accepted as a potent pro-inflammatory mediator and a key factor in inflammatory skin disease. Despite this, our understanding of the effect of IL-20 on epidermal keratinocytes remains incomplete.^{10,12,18}

Live-cell calcium imaging was used to measure the effect of IL-20 on cellular calcium in cultured human primary keratinocytes (phKCs). Therefore, phKCs were loaded with a fluorescent calcium dye and, after a baseline recording (200 s), were stimulated with IL-20. Application of IL-20 promoted a rapid calcium spike (Figure 2A) in a fraction of phKCs (41.7% of total cells) (Figure 2B). It is postulated that this subset of phKCs express high levels of IL-20 receptors. Single-cell data were then expressed with respect to two key features, $F\Delta/F0_{\text{max}}$ and Time_{max} , generating an IL-20 calcium signature and demonstrating the

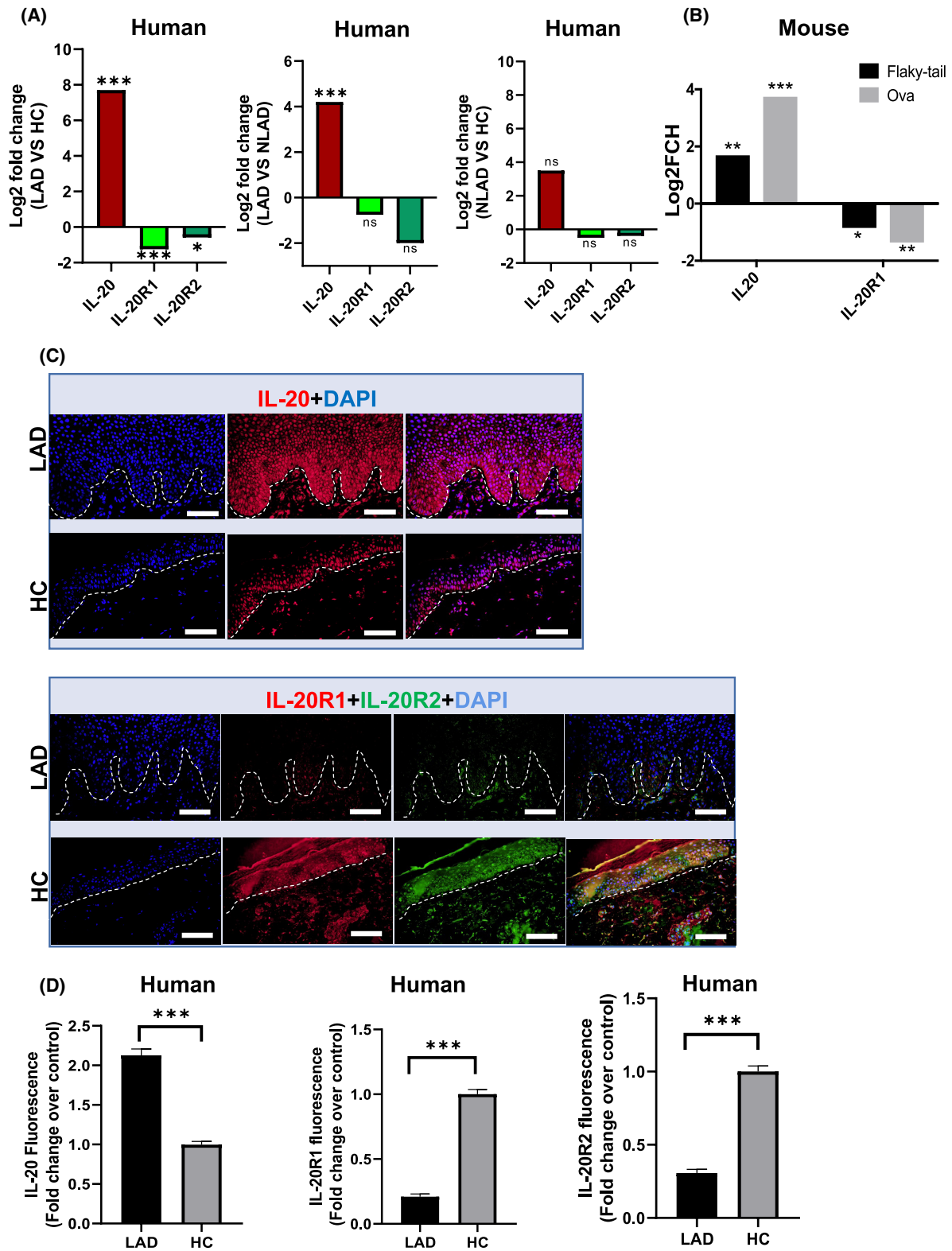


FIGURE 1 In AD skin, transcription, and expression levels of IL-20, but not its receptors, are correlated with disease severity. (A) Transcriptional analysis by RNA-seq of IL-20, IL-20R1, and IL-20R2 in human LAD versus HC, in LAD versus NLAD, and in NLAD versus HC ($n = 5$ donors/group). (B) Transcriptome analysis of IL-20 and its receptors in skin from flaky-tail and ova-induced mice models. Full dataset and methods are available from a previous report.²⁹ (C) Representative immuno-staining of IL-20, IL-20R1 and IL-20R2 in skin samples from human LAD and HC ($n = 3$ donors/group). Scales = 50 μm . (D) Analysis of fluorescence intensity of randomly selected keratinocytes in epidermis in panel C. For (A), DEG threshold: 2-fold up- or down-regulation, ***FDR < 0.001, *0.01 < FDR < 0.05, ^{ns}FDR > 0.05. For (C), data are means \pm SEMs ($n = 3$); *** $p < .001$, a two-tailed Student's t -test followed by Welch's correction was used

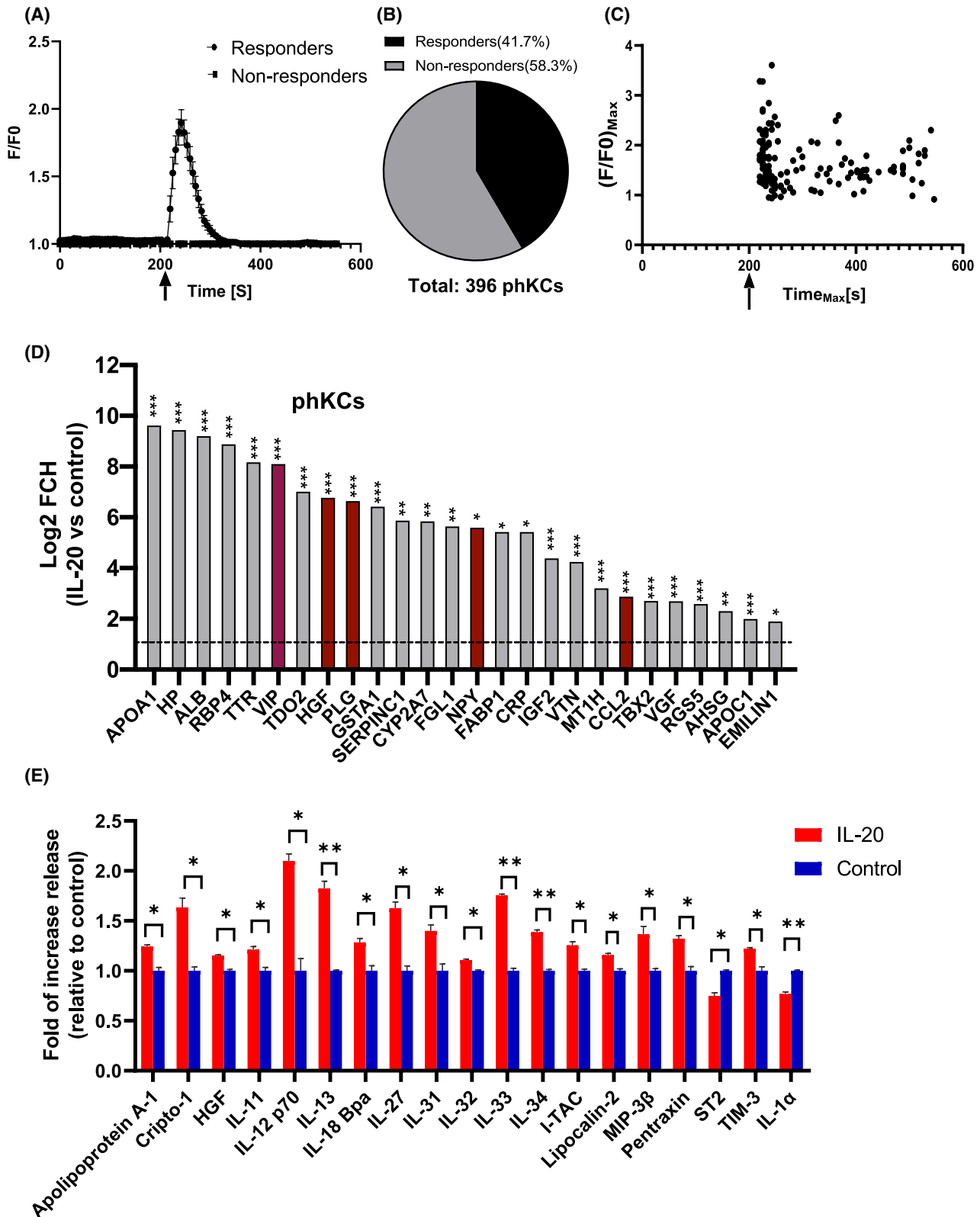


FIGURE 2 IL-20 triggers calcium influx, upregulates transcription levels of AD-related gene transcripts, and induces release of itch mediators in cultured phKCs. (A–C) IL-20-induced calcium mobilization (A), percentage of responders (B), and subpopulation analysis with regard to Time_{max} versus (F/F0)_{max}. (C); *n* = 396 phKCs were recorded. (D, E) IL-20-induced upregulation in gene transcription (D) and cytokine release (E). For data in (D), DEG threshold: 2-fold up- or down-regulation, ***FDR < 0.001, **0.001 < FDR < 0.01, ^{ns}FDR > 0.05. For (E), data are means ± SEMs (*n* = 3); ^{ns}*p* > .05, **p* < .05, ***p* < .01, and ****p* < .001, a two-tailed Student's *t*-test followed by Welch's correction was used

heterogeneity within the IL-20-responsive subpopulation (Figure 2C).

To investigate the impact of IL-20 on transcription in pHKCs, cultured cells were treated with IL-20 (500 ng/ml, 6 h) and analyzed by RNA-seq. Here, IL-20 was associated with enhanced transcription of vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), C-C motif chemokine-2 (CCL2), Plasminogen (PLG), and hepatocyte growth factor (HGF) (Figure 2D). Importantly, these mediators have been linked to skin lesions, with a number being elevated in AD.^{29,36–40} Application of IL-20 promoted upregulation of a variety of other gene transcripts, including Apolipoprotein A-I (APOA1), Haptoglobin (HP), Albumin (ALB), Retinol-binding protein (RBP4), Tryptophan 2,3-dioxygenase (TDO2), VGF peptide, Glutathione S-Transferase Alpha 1 (GSTA1), SERPINC1, and cytochrome P450 (CYP2A7) (Figure 2D), several of which are implicated in long-term inflammation.^{41–43}

To examine whether IL-20 could promote cytokine release in keratinocytes, cultured pHKCs were treated with IL-20 (500 ng/ml, 24 h) and supernatants were analyzed by cytokine array. Application of IL-20 resulted in the release of numerous cytokines, including IL-11, IL-12, IL-18, I-TAC, Lipocalin-2, and ST2 (also known as IL-1R4) (Figure 2E). Among them, IL-11, IL-12, IL-18, I-TAC and ST2 are implicated in AD.^{35,44–47} Interestingly, IL-20 stimulation was also associated with release of IL-13, IL-27, IL-31, IL-33, IL-34 (Figure 2E), each of which represent critical and potent drivers of skin atopy.^{26,48–53}

Thus, IL-20 evokes calcium fluctuations, upregulates AD-related gene transcripts, and stimulates the release of key inflammatory mediators in human keratinocytes. Together, this data suggest that IL-20 may promote atopic epidermal inflammation and ‘drive’ the development of AD skin lesions.

3.3 | IL-20R1 transcripts are increased in sensory ganglia ipsilateral to the AD skin lesion

Though cutaneous IL-20 was enhanced in human AD, the effect of IL-20 on adjacent sensory neurons remained unclear. This potential interaction was investigated using the MC903-treated AD-like murine model. Following topical application of MC903 (to left ear, and ethanol to the right ear), these mice display AD-like skin lesions.²⁵ However, human AD and MC903-induced lesions may be mediated by distinct signaling pathways.⁵¹ At day 12, when the MC903-treated ears displayed clear lesions, ear skin biopsies were taken and both left (ipsilateral) and right (contralateral) trigeminal ganglia (TG) were harvested (from 6 mice/group).

Skin biopsies from control and MC903-treated ears were analyzed using immunohistochemistry. MC903-treated samples displayed a high level of IL-20 immunosignal, with quantification of fluorescence revealing a significant increase in MC903-treated skin compared with vehicle (Ethanol)-treated control skin (Figure 3A,B). Conversely, immunosignals for both IL-20R1 and IL-20R2 were significantly reduced in MC903-treated skin relative to control skin (Figure 3C,D). Importantly, these findings are consistent with our results from other dermatitis models (Flaky tail and Ova-induced) and human AD (cf. Figure 1).

Next, the effect of topical MC903 treatment on the transcription of IL-20 receptors in adjacent sensory neurons was investigated. IL-20R1 and IL-20R2 transcripts in ipsilateral and contralateral TG were analyzed by qPCR. The transcript level for each receptor was expressed as the fold change in the ipsilateral TG relative to the contralateral TG, with sensory neurons from the ipsilateral TG innervating the MC903-treated ear and the contralateral innervating the vehicle-treated (EtOH) ear. We observed a significant upregulation in IL-20R1, but not IL-20R2 (Figure 3E), indicating that topical MC903 treatment stimulates IL-20R1 expression in adjacent sensory neurons. Thus, MC903-treatment enhances IL-20R1 transcription and may alter the sensitivity of sensory neurons to IL-20.

Immunohistochemistry was used to examine the effect of topical MC903 treatment on IL-20R1 protein expression in ipsilateral and contralateral TG. Again, sensory neurons from the ipsilateral TG innervate the MC903-treated ear, and from the contralateral TG innervate the vehicle-treated (EtOH) ear. Importantly, IL-20R1 protein was detected in both ipsilateral and contralateral TG, with neuronal marker PGP9.5 co-staining confirming its neuronal location (Figure 3F). Consistent with qPCR data, ipsilateral TG displayed enhanced IL-20R1 immunosignal, with quantification of fluorescent signal indicating a significant difference (Figure 3F,G). Though images were acquired using identical exposure times and image settings, we note that PGP9.5 fluorescence signals were also increased in ipsilateral versus contralateral TG (Figure 3F). In contrast to IL-20R1, no significant upregulation was detected for IL-20R2 (Figure S2). This finding is consistent with the qPCR result that IL-20R1 but not IL-20R2 is enhanced in TG from MC903-mice.

Taken together, this data provide the first clear evidence of IL-20 receptor regulation in sensory neurons. Moreover, topical MC903 was found to enhance the transcription and expression IL-20R1 in adjacent sensory neurons. Notably, this finding suggests that sensory neurons innervating AD lesions may be hyper-responsive to cutaneous IL-20, a mediator which is overexpressed in AD lesions.

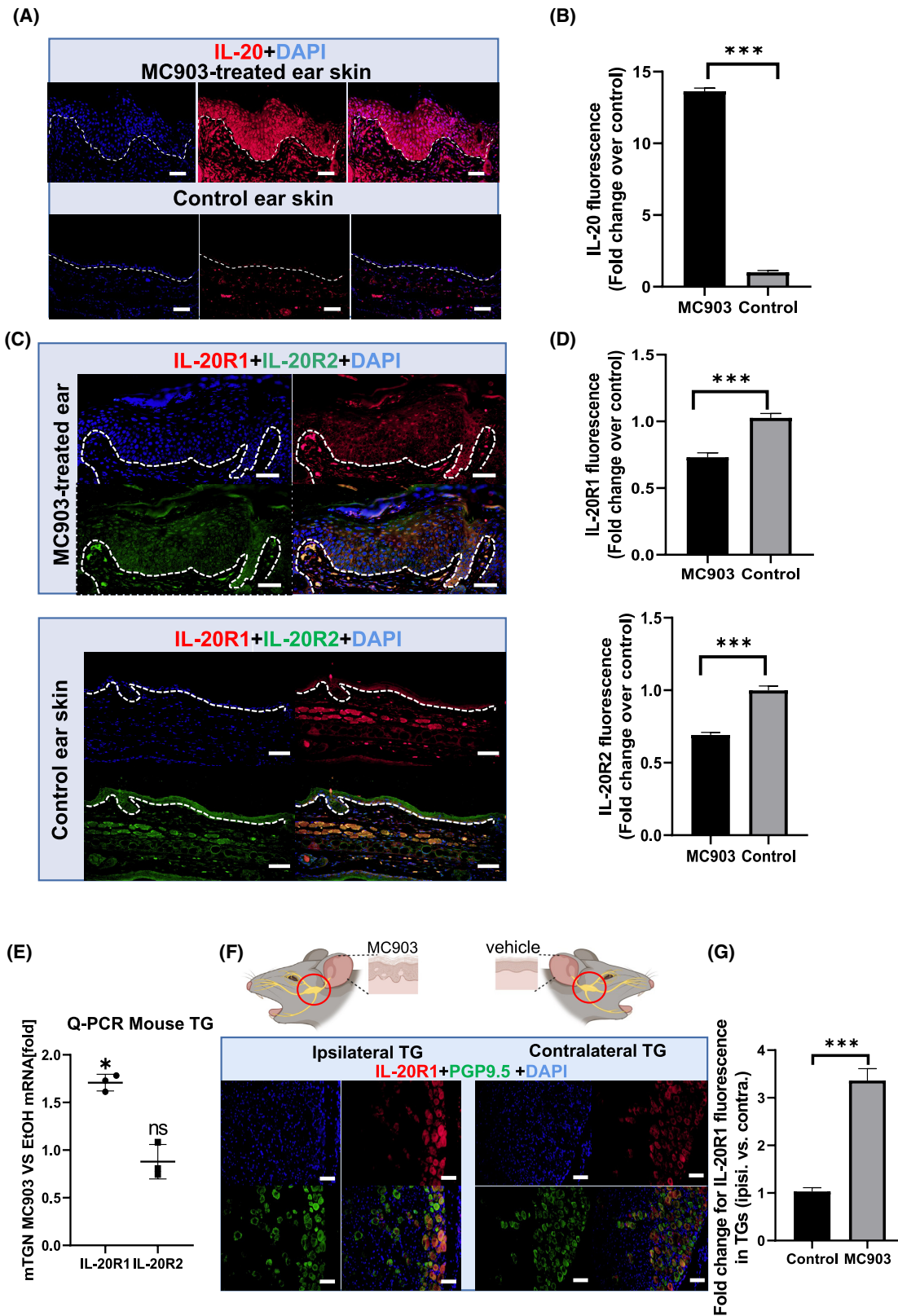


FIGURE 3 IL-20 is upregulated in MC903-treated mouse ear skin when compared to control, whereas IL-20 receptors are downregulated. In contrast, IL-20R1 transcription levels are upregulated in sensory trigeminal ganglia (TG) ipsilateral to MC903-treated ear. Representative immunofluorescence staining of IL-20 (A), and IL-20R1 plus IL-20R2 (C) in MC903- and vehicle-treated mouse ear, and their fluorescence intensity analysis (B, D); $n = 5$ slides/mice ($n = 3$ mice). (E) Q-PCR analysis of IL-20R1 and IL-20R2. (F, G) Immunofluorescence staining of IL-20R1 in ipsi. and contra. TG to MC903-treated mice ear as depicted above (F), and IL-20R1 fluorescence intensity analysis (G); $n = 5$ slides/mice ($n = 3$ mice). For (B, D, E and G), means \pm SEMs ($n = 3$); $^{ns}p > .05$, $^{*}p < .05$, and $^{***}p < .001$, a two-tailed Student's t -test followed by Welch's correction was used. For (A, C and F), scales = 50 μ m

3.4 | IL-20 drives calcium influx, promotes inflammatory cytokine transcription, and induces AD-related cytokine release in murine sensory neurons

Following our discovery of IL-20 receptors in sensory neurons, we sought to investigate the effect of IL-20 on cultured murine trigeminal ganglionic neurons (mTGNs).

We first examined calcium responses in cultured mTGNs following application of IL-20 (500 ng/ml). Interestingly, IL-20 successfully elicited a calcium spike in a small subpopulation (5.42%) of mTGNs (Figure 4A). This finding confirms that IL-20 can directly activate a subset of sensory neurons.

To assess the effect of IL-20 on neurogenic inflammation, cultured mTGNs were treated with IL-20 (500 ng/ml, 24 h) and supernatants were analyzed by cytokine array. Application of IL-20 resulted in the release of numerous mediators, including CXCL1, CXCL5, Proprotein Convertase 9, Angiopoietin-2, CCL12, Chemerin, LIF, MMP-3, VEGF, Coagulation Factor III, Cystatin C (Figure 4B). These findings were supported by Q-PCR, which showed increased transcription of MMP3, CXCL5, CXCL1 and CCL2 following IL-20 (500 ng/ml, 6 h) treatment (Figure 4C). Importantly, previous reports have shown that CCL2 signals both itch and pain-like behavior in mice,⁵⁴ CXCL1 evokes acute itch,⁵⁵ Serpin E1 elicits AD-itch,²⁵ and IL-11 and MMP-3 are implicated in AD pathogenesis.²⁹ Our Q-PCR results also revealed a significant increase in Toll-like receptor 2 (TLR2) expression following IL-20 stimulation of mTGNs (Figure 4D), suggesting that IL-20 may sensitize neurons to innate immune signaling. Thus, IL-20 enhances transcription of inflammatory mediators and promotes neurogenic inflammation.

Thus, IL-20 can promote both neuronal activation and stimulate neurogenic inflammation. Taken together, our murine and human findings describe a complex network of intercellular communication and highlight the role of IL-20 in the pathological cellular alterations and inflammatory cascades associated with AD lesions.

3.5 | Knockdown of IL-13R α 1/2 in keratinocytes results in increased transcription of IL-20, but not IL-20 receptors; Co-injection of IL-20 and IL-13 elicits itch-like behavior in mice

IL-13 is a cytokine which contributes to the hallmark features of AD.⁵⁶ However, the effect of IL-13 on IL-20 signaling remained unknown.

To examine the effect of IL-13 on IL-20 transcript expression, IL-13 receptors were knocked down (KD) in

cultured phKCs and transcripts were measured by RNA-seq. KD was achieved using lentiviral shRNA specifically targeting the IL-13 receptors, IL-13R α 1 and IL-13R α 2, while control cells received the corresponding scrambled control (Figure 5A). Interestingly, transcription levels of IL-20 were increased in IL-13R α 1KD or IL-13R α 2KD phKCs when compared to scrambled control-treated cells, however, IL-20R1 transcription levels were decreased (Figure 5B). As a comparison, we also incubated KD cells with IL-13 and measured transcription of IL-20 and its receptors by RNA-seq. As expected, IL-20 transcripts were increased, while transcription levels of both IL-20R1 and IL-20R2 were decreased (Figure 5B). Thus, IL-13 receptors function to negatively regulate IL-20 expression, with KD of IL-13 receptors effectively alleviating the regulation and resulting in higher IL-20 transcription.

Finally, we examined whether IL-20 and/or IL-13 could promote acute itch-like responses in mice. Using a murine cheek model of itch, scratching responses were assessed following intradermal (i.d.) cheek injection of IL-20 or IL-13 alone, or co-injection of a mixture of IL-20 and IL-13. Mice were recorded for 1 h, starting immediately after i.d. injection, and scratching bouts were quantified. When injected alone, neither IL-20 nor IL-13 elicited a significant scratching response, with both groups being comparable to relevant controls (either PBS or H₂O, respectively) (adjusted *p* value for vehicle vs. IL-13 is greater than 0.9999; for vehicle vs. IL-20 is greater than 0.9999; 2-way ANOVA followed by Bonferroni's post hoc analysis) (Figure 5C), suggesting the chosen concentrations of IL-13 and IL-20 were unable to induce itch-like behaviors in mice. Conversely, co-injection of IL-20 and IL-13 resulted in a significant increase in scratching bouts when compared to same volume injection of all controls and to IL-13- or IL-20-injection alone (adjusted *p*-value for H₂O vs. IL-13 plus IL-20 is 0.0008; for IL-13 vs. IL-13 plus IL-20 is 0.0009; for IL-13 vs. IL-13 plus IL-20 is 0.0464; for IL-20 vs. IL-13 plus IL-20 is 0.0139; 2-way ANOVA followed by Bonferroni's post hoc analysis) (Figure 5C). These findings suggest that IL-20 and IL-13 may act synergistically to promote and intensify itch transduction in mice.

4 | DISCUSSION

IL-20 is produced by a variety of cells, including monocytes, dendritic cells, endothelial cells, keratinocytes, synovial fibroblasts, mesangial cells, with IL-20 functioning as a pro-inflammatory and anti-inflammatory mediator as well as a non-immune signaling compound.^{10,12,13,16} Increased expression of IL-20 has been described in several inflammatory diseases such as rheumatoid arthritis, atherosclerosis, and psoriasis.^{14,18,30,32} The IL-20 serum level

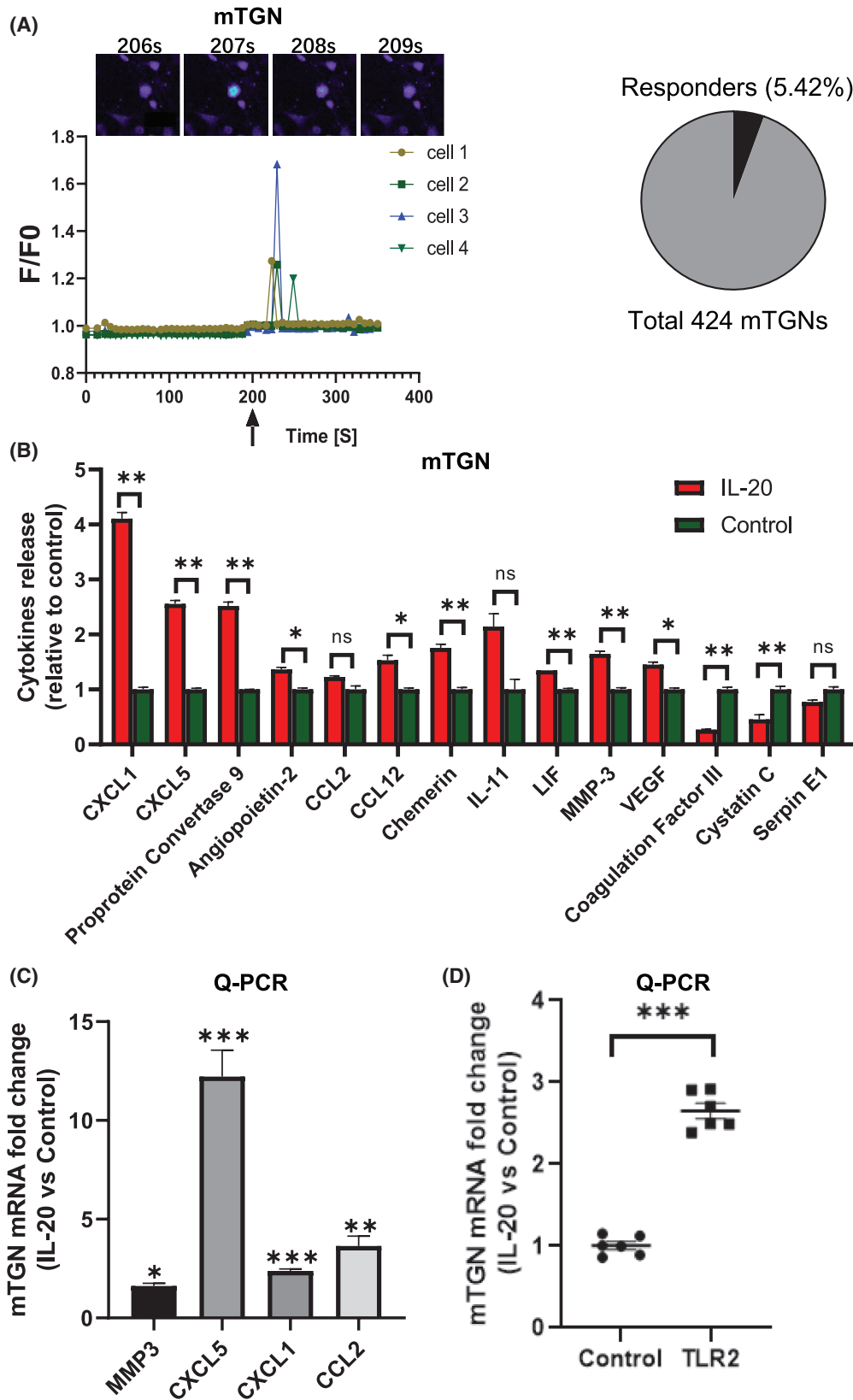


FIGURE 4 IL-20 triggers calcium transients in mTGNs, induces release of AD-related cytokines, and upregulates itch-related gene transcripts. (A) Calcium transients induced by IL-20 in mTGNs and analysis of responders; $n = 424$ mTGNs; representative micrographs for calcium spikes are shown on the top panel. (B) Cytokine release induced by IL-20 versus vehicle. (C, D) Q-PCR analysis of IL-20-induced gene transcripts (C) and TLR2 mRNA (D) relative to vehicle control; $n = 6$. For (B–D), means \pm SEMs ($n = 3$); $^{ns}p > .05$, $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$, a two-tailed Student's t -test followed by Welch's correction was used

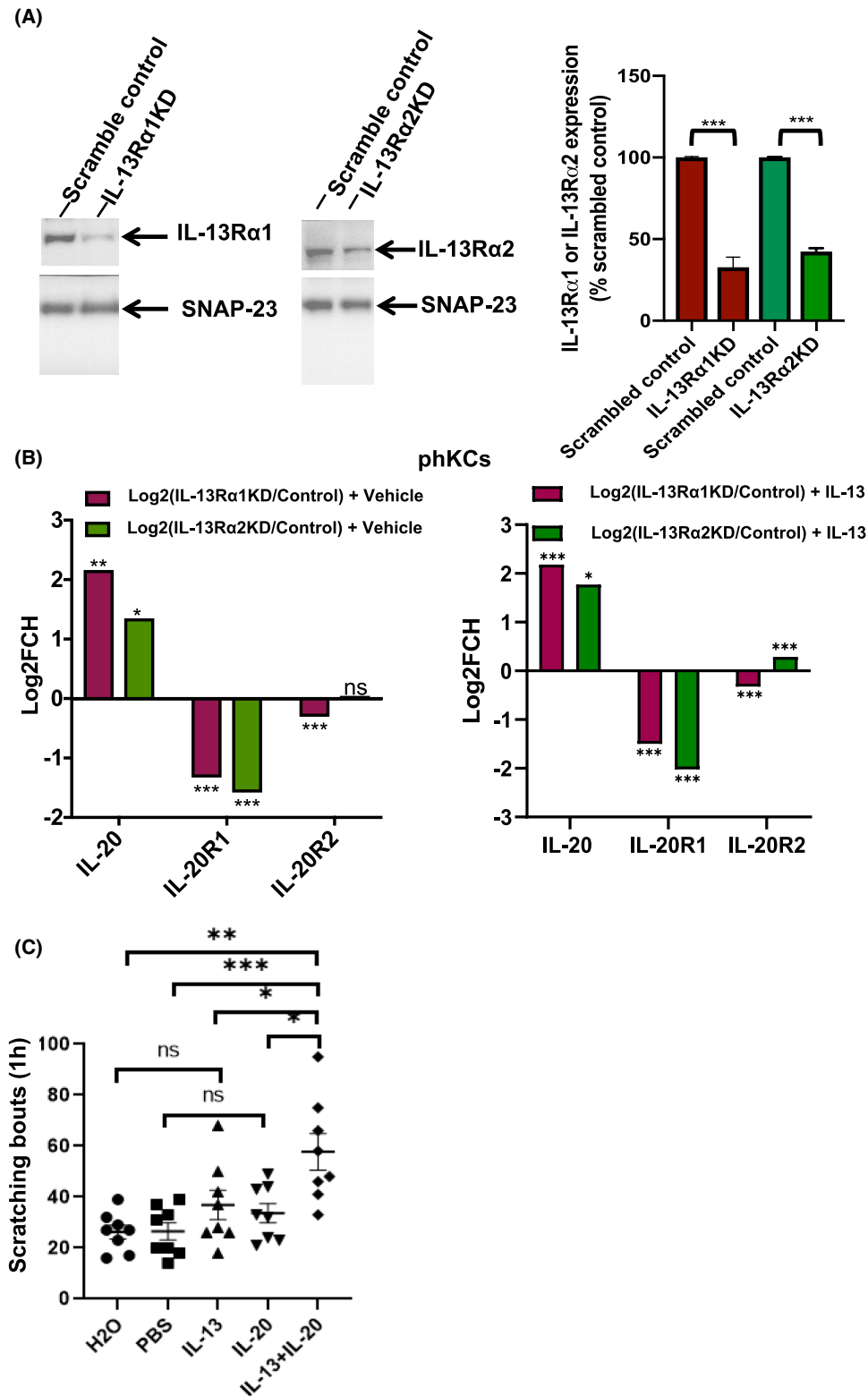


FIGURE 5 In pHKCs, KD of IL-13 receptors results in increased transcription levels of IL-20 and reduced levels of IL-20 receptors. Injection of IL-20 and IL-13 in mouse cheek induces itch-like behavior, though only when co-injected. (A) Lentiviral shRNA-mediated KD of IL-13R α 1 and IL-13R α 2 in pHKCs. SNAP-23 serves as the internal control in Western-blotting. Percentage (%) KD relative to scrambled controls from $n = 3$ independent experiments. (B) Transcriptome analysis of IL-20 and its receptors in pHKCs after KD of IL-13 receptors. KD cells and scrambled control-treated control cells were incubated with vehicle (left panel) or IL-13 (right panel) before RNA-seq. (C) Scratching bouts in mouse cheek model after injection of vehicle, or IL-13, or IL-20, or following co-injection of IL-13 and IL-20 (2-way ANOVA followed by Bonferroni's post hoc analysis; data are presented as mean \pm SEM; $n = 8$ mice/group.). For (A) and (B) $^{ns}p > .05$, $^*p < .05$, $^{**}p < .01$, and $^{***}p < .001$, a two-tailed Student's t -test followed by Welch's correction was used

are elevated in both moderate-to-severe AD and psoriasis patients, when compared to the healthy controls.⁵⁷ In this report, we demonstrate that IL-20 is upregulated in human AD and several murine models of dermatitis, suggesting a positive association between IL-20 expression and dermatitis severity. Conversely, in the same samples, expression of IL-20 receptors, IL-20R1 and IL-20R2, was unchanged or even downregulated. This finding is in contrast to published reports from lesional psoriasis, where both IL-20R1 and IL-20R2 are upregulated at both transcriptional and protein level in the keratinocyte layer,^{31,33,34} though not in whole skin samples, which emphasizes differences between psoriasis and AD with respect to IL-20/IL-20R regulation. In our study, IL-20 was shown to directly activate both sensory neurons and keratinocytes of skin, stimulating the release of itch peptides and inflammatory cytokines, enhancing AD- and itch-related gene transcription, and promoting IL-13-associated itch. Together, this data reveal a novel neuronal-epidermal link and highlight its role in atopic inflammation and itch.

MC903 is a commonly utilized model for AD-like itch. Here, MC903 treatment was found to induce distinct transcriptional alterations treated ear skin and innervating sensory ganglia. While IL-20 is highly upregulated in

MC903-treated skin, it is undetected in the associated sensory neurons (ipsilateral). Conversely, while IL-20 receptors are downregulated in MC903-treated skin, IL-20R1 is significantly increased in the ipsilateral neurons. Importantly, these results indicate that MC903-mediated dermatitis indirectly regulates the sensitivity of sensory neurons to IL-20, suggesting crosstalk between the damaged or diseased skin and innervating sensory neurons. Together, it is reasonable to postulate that increased cutaneous IL-20 could act on the upregulated IL-20R1 in sensory neurons, thus resulting in neuronal hypersensitivity and atopic itch (Figure 6).

Keratinocyte-derived inflammation is a potent driver of lesional AD. Our findings showed that IL-20 can activate keratinocytes, generating cellular calcium transients, releasing AD-related cytokines and upregulating itch-related gene transcripts. Together, these data suggest that IL-20 represents a robust inducer of cellular communication, acting both directly and indirectly to generate potent inflammatory cascade. Specifically, the application of IL-20 enhanced the synthesis of VIP, NPY, and CCL2 and stimulated the release of AD-related cytokines, including IL-13, IL-27, IL-31, IL-33, and IL-34. Though these cytokines are predominantly produced by activated antigen-presenting

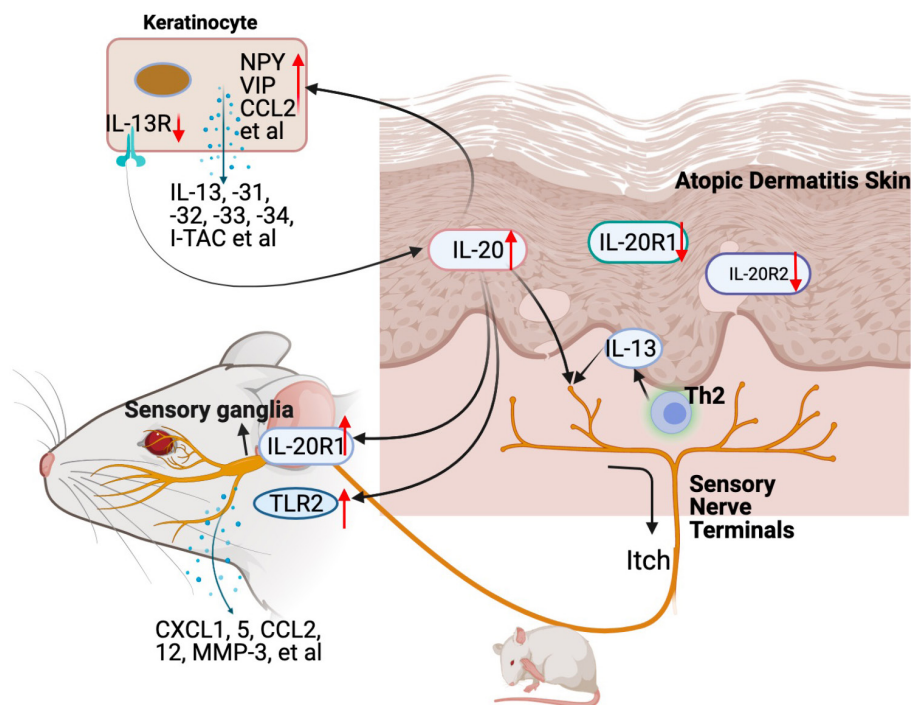


FIGURE 6 Schematic showing pathway of IL-20 in AD. IL-20 is upregulated in skin from AD, particularly in lesional AD. Skin lesion induces upregulation of IL-20 receptors in sensory ganglia. IL-20 triggers calcium influx in both keratinocytes and sensory neurons, promoting their AD-related molecule release, and transcription of itch-related genes. In sensory neurons, IL-20 increases TLR2 transcripts, implicating a link between innate immune response and IL-20. Intradermal co-injection of IL-13 and IL-20 induces itch-like behaviors in mice, but this is not observed in mice injected with IL-13 or IL-20 alone. Our findings provide novel insights into IL-20 function in peripheral (skin-derived) itch and clinically relevant intercellular neuron-epidermal communication and highlight IL-20 is functionally linked with lesional AD in human, thus forming a new basis for the development of a novel antipruritic strategy via interrupting IL-20 signaling pathways

cells (APC) cells, mast cells, basophils, eosinophils, dendritic cells, macrophages, T cells, a number of reports have proposed that they may also be synthesized and released by keratinocytes under certain conditions (i.e. inflammation conditions or chronic eczema).^{48,53,58–63} Release of these compounds from epidermal keratinocytes would likely further activate resident and infiltrating immune cells and potentiate chronic atopic inflammation. In addition, the in situ upregulation of IL-13 (Figure S3) in keratinocyte layer was also detected in MC903-ear. Delineation of these complex and previously unrecognized pathways enhances our knowledge of AD pathogenesis, solidifies IL-20 as an important factor in AD, and reveals novel therapeutic options for future drug development.

In keratinocytes, we also observed that KD of IL-13 receptors resulted in enhanced IL-20 transcription, suggesting that IL-13 receptors may negatively regulate IL-20 transcription in keratinocytes. Because IL-13 is a critical cytokine communicating with keratinocytes to enrich Th2-cells in lesional skin of acute AD patients,^{64–66} IL-20 might compensate the reduced IL-13 signaling in IL-13 receptor KD cells to attract Th2 cells to the epidermis,⁶⁷ thereby contributing to epidermal hyperplasia. The mechanisms of IL-20 regulation in keratinocytes might be different from that in sensory neurons, particularly, IL-20 is not expressed in sensory ganglia. The mechanisms by which the down-regulation of IL-13 receptors enhances IL-20 transcription in keratinocytes requires further investigation, but one can conclude that it is independent from sensory signaling in acute itch sensation, as the latter is seen in a short time window (1 h).

For the first time, our results also showed that IL-20 can directly activate sensory neurons. In addition to calcium transients, IL-20 triggered the release of CXCL1, CXCL5, CCL2, CCL12, MMP3, etc., from sensory neurons, some of which were also found to be upregulated at transcription levels (Figure 6). Importantly, CXCL1 and CCL2 have been defined as pruritogenic cytokines in mice.^{54,55} Additionally, we found that TLR2 transcripts were upregulated by IL-20 in sensory neurons, highlighting a link between the innate immune response and IL-20 signaling. Together, these results highlight a previously unknown neuro-epidermal link and demonstrate the role of IL-20 in neurogenic inflammation and pruritogen release.

Several AD-linked cytokines are now recognized as direct inducers of itch signaling (pruritogens). Here, injection of IL-20 failed to elicit scratching behaviors in a murine model of acute itch. Similarly, and in line with an earlier report and our own recent study,^{27,51} IL-13 was also unable to elicit itch-like behavior. However, co-injection of IL-20 and IL-13 generated a significant scratching response. These findings suggest that while IL-20 may not be pruritogenic alone (especially at our selected dose), but

may act synergistically with other cytokines (IL-13) resulting in heightened and aberrant itch transmission. Thus, within the inflammatory AD lesion, cutaneous IL-20 may represent a key inducer of atopic itch.

5 | CONCLUSIONS

Overall, this work provides the first evidence that IL-20 regulates neuro-immune and neuro-epidermal communication in AD. Thus, blocking peripheral IL-20 activity could alleviate pathological inflammation, interrupt aberrant disease-driven intercellular communication, and ultimately target the underlying signaling pathways enhancing clinical AD and atopic itch.

DISCLOSURES

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Supervision: Jianghui Meng, Jiafu Wang; *Conceptualization:* Jianghui Meng, Jiafu Wang; *Data Curation:* Zhiping Lu, Shanghai Xue, Renkai Zhu, Weiwei Chen, Lianlian Li; *Funding Acquisition:* Zhiping Lu, Jianghui Meng, Jiafu Wang; *Formal Analysis:* Zhiping Lu, Song Xiao, Yanqing Li, Jianghui Meng, Jiafu Wang, Shanghai Xue, Zhiping Lu; *Resources:* Wenke Cheng, Shanghai Xue, Hua Yang, Martin Steinhoff, Ruizhen Wang, Wenhao Zhang, Xinrong Yan, Jiafu Wang, Jianghui Meng; *Writing - Original Draft Preparation:* Zhiping Lu, Shanghai Xue, Martin Steinhoff, Ciara Larkin, Jiafu Wang, Jianghui Meng; *Writing - Review and Editing:* ALL.


ETHICS APPROVAL AND CONSENT TO PARTICIPATE











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, China.

DATA AVAILABILITY STATEMENT

Datasets related to this article can be found at <https://www.ncbi.nlm.nih.gov/gds/>, hosted by GEO database (accession number GSE172006 and GSE195538).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Lu Z, Xiao S, Chen W, et al. IL-20 promotes cutaneous inflammation and peripheral itch sensation in atopic dermatitis. *FASEB J.* 2022;36:e22334. doi:[10.1096/fj.202101800R](https://doi.org/10.1096/fj.202101800R)