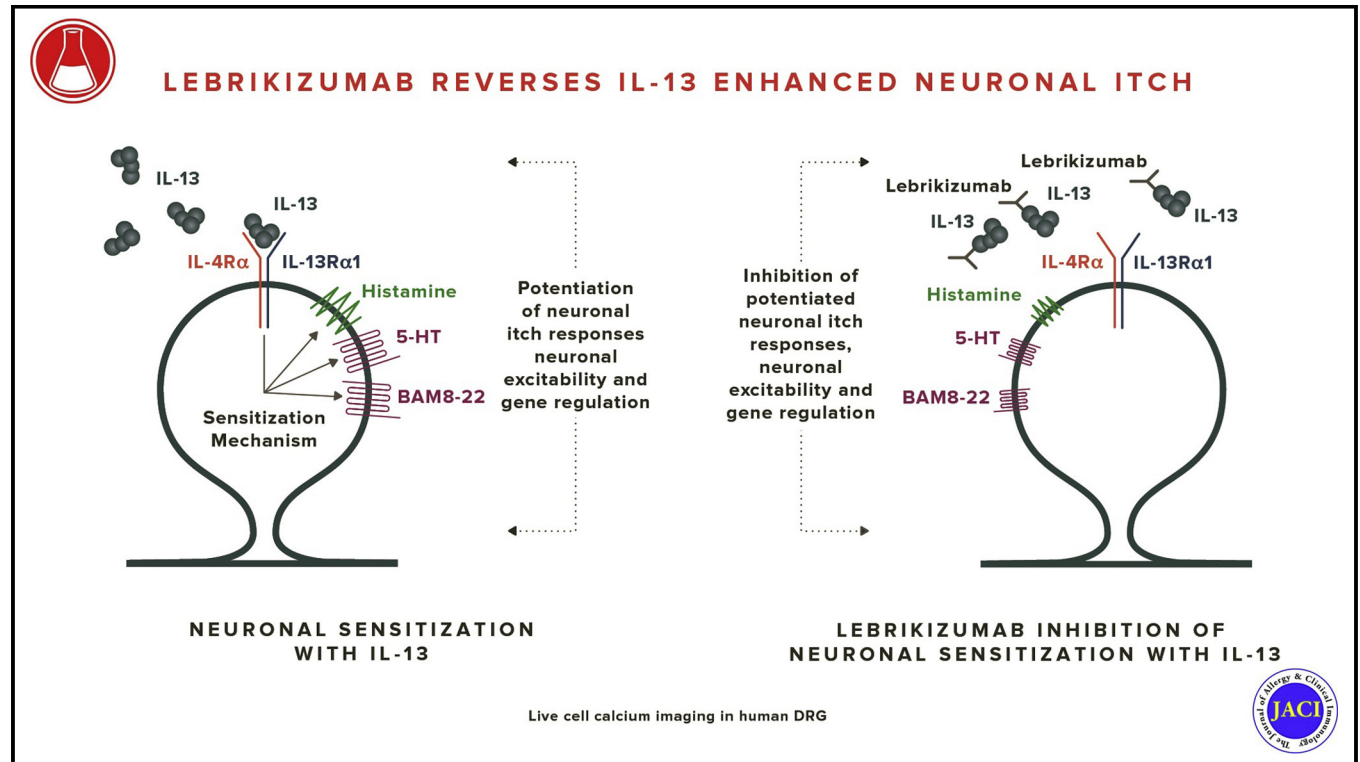


Mechanistic insights into the antipruritic effects of lebrikizumab, an anti-IL-13 mAb

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GRAPHICAL ABSTRACT



Background: Atopic dermatitis is a chronic inflammatory skin disease with persistent and severe itch among its hallmark features. Significant increases in type 2 cytokines (ie, IL-4, IL-13, IL-31) have been documented in acute atopic dermatitis lesions and lead to multifaceted downstream effects, including inflammation, epidermal barrier dysfunction, and itch. **Objective:** The primary objective of preclinical studies reported here was to test direct effects of IL-13 and an anti-IL-13 mAb, lebrikizumab, in a human dorsal root ganglion model in itch amplification, neuronal excitability, and transcriptional downstream targets.

Methods: Neuroactive effects were assessed via live cell calcium imaging, electric field stimulation, and RNA sequencing of

human dorsal root ganglia stimulated with IL-13 alone or in combination with lebrikizumab.

Results: These preclinical findings suggest that IL-13 plays a direct enhancer role in multiple itch and neuroactive pathways as well as transcriptional downstream effects, and provide key insights into the mechanistic basis for lebrikizumab's anti-itch effects.

Conclusion: IL-13 is a potent enhancer of neuronal responses to different itch stimuli, consistent with the neuroimmune axis contributing to chronic itch-associated inflammatory skin disease, and blockade of this cytokine pathway may provide a therapeutic approach. (J Allergy Clin Immunol 2022;■■■■:■■■-■■■.)

Key words: IL-13, lebrikizumab, atopic dermatitis, itch

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Abbreviations used

AD:	Atopic dermatitis
cDNA:	Complementary DNA
CGRPα:	Calcitonin gene-related polypeptide alpha
DEG:	Differentially expressed gene
DRG:	Dorsal root ganglion
EFS:	Electric field stimulation
hDRG:	Human dorsal root ganglion
mRNA:	Messenger RNA
RNA-Seq:	RNA sequencing

Atopic dermatitis (AD) is a chronic, systemic, inflammatory skin disease characterized by persistent itch and dry, scaly, eczematous lesions.¹ Itch is a cardinal feature of AD and is associated with a significant reduction in quality of life.² Like pain, itch is a sensory modality that is transmitted by neuronal innervation of the skin, and itch signaling is mediated by both exogenous substances that affect the skin barrier and endogenous substances released from a diverse array of cell types.³

Different type 2 cytokines, including the interleukins IL-4, IL-13, and IL-31, drive disease pathology; however, the physiological mechanisms underlying the roles played by these cytokines in the debilitating itch reported by patients with AD have yet to be fully elucidated.⁴ Both IL-4 and IL-13 levels are elevated in the skin and serum of AD patients,⁵ while IL-31 was the first cytokine shown to be directly active in neuronal itch.^{6,7} IL-31 is a member of the IL-6 cytokine family and signals through the heterodimeric receptor complex comprising IL-31 receptor alpha and oncostatin receptor beta subunits. Whether IL-31 plays a direct role in human sensory neuron function is debated because the itch triggered by IL-31 is often delayed in translational or mouse neuronal studies,⁸ despite the clinical improvement by IL-31 targeting antibody nemolizumab in the clinic.⁹ In fact, receptor subunits for IL-13 signaling, namely IL-13Rα and IL-4Rα, are expressed in human sensory neurons,¹⁰ suggesting that their afferents are within the skin to sensor itch signals.

Despite the complexity in the underlying pathophysiology of AD, IL-4 and IL-13 are key mediators of proinflammatory processes in AD.¹¹ IL-4- and IL-13-mediated signaling occurs via a heterodimeric receptor complex comprising the IL-13Rα1 and IL-4Rα subunits, which are as a heterodimeric complex expressed primarily on nonhematopoietic cells. After IL-4 and IL-13 cytokine binding and receptor dimerization, downstream signaling occurs through JAK1-mediated phosphorylation of the transcription factor STAT6. IL-4 also signals through the type 1 IL-4 receptor that consists of IL-4Rα and the common gamma chain, which is expressed mainly on hematopoietic cells (eg, T cells and B cells). IL-4Rα appears to be a fundamental component of the chronic itch pathway, as conditional knockout mice that lack the IL-4Rα gene in nociceptive neurons do not develop either chronic itch or histopathologic features in the MC903-induced model of AD.¹⁰

Further, IL-13 overexpression in mice mimics features of AD phenotypes, and IL-13-overexpressing mice exhibit intense scratching behavior.⁷ It is unclear whether the itch in IL-13-overexpressing mice is a result of inflammation or is the result of non-neuronal proinflammatory functions driven by IL-13 overexpression. While application of IL-13 and IL-4 activates

murine dorsal root ganglion (DRG) neurons and increases their responsiveness to histamine, these cytokines did not elicit scratching behavior.¹⁰ In contrast, IL-31 both activates such neurons and evokes scratching behavior.^{6,10,12}

Lebrikizumab is a mAb that specifically binds IL-13 with high affinity and inhibits downstream signaling. In a randomly assigned, double-blind, placebo-controlled, phase 2b clinical trial (NCT03443024), adults with moderate to severe AD showed significantly greater improvement in measures of AD severity (Eczema Area Severity Index, Investigator's Global Assessment, and pruritus numeric rating scale) after lebrikizumab treatment versus placebo at week 16.¹³ Patients receiving lebrikizumab reported itch relief as early as day 2. These findings support a role for IL-13 in the neuronal transmission of itch and its blockade by lebrikizumab.

To better understand the mechanisms underlying this clinical finding, we utilized an *in vitro* human neuronal model to explore the possibility that IL-13 may directly influence the somatosensory system and that lebrikizumab may impact this effect. We evaluated the neuroactive function of IL-13 on isolated human sensory neurons and on pruritogen- and voltage-driven neuronal responses and determined the capacity of lebrikizumab to attenuate effects elicited by IL-13 in neuronal itch responses. In addition, we conducted RNA sequencing (RNA-Seq) analysis of untreated human DRG (hDRG) cells and cells treated with IL-13 alone or in combination with lebrikizumab to identify other potential downstream targets that may be affected by lebrikizumab treatment.

METHODS

All experiments were conducted on isolated hDRG neurons from postmortem organ donors. Ethical consent was provided by the organ donor or next of kin. Three main categories of studies were performed, as follows. Neuronal responses to IL-13 and pruritogens were assessed by monitoring changes in cytoplasmic calcium. Neuronal excitability was assessed via electric field stimulation (EFS). Finally, downstream targets of IL-13 were identified via RNA-Seq. We summarize the methodology for each.

Primary cell isolation and culture

The hDRGs were stripped of connective tissue and enzymatically digested at 37°C for 2 hours using the methods described by Davidson et al.¹⁴ Dissociated cells were seeded in 96-well plastic-bottom plates (Corning, Corning, NY) that had been precoated with poly-D-lysine. Cells were maintained in culture at 37°C with 5% CO₂ in Dulbecco modified Eagle medium/F-12 supplemented with 10% horse serum (Thermo Fisher Scientific, Waltham, Mass), 25 ng/mL human nerve growth factor (hNGF, Cell Signaling Technology, Danvers, Mass), 25 ng/mL GDNF (PeproTech, London, United Kingdom), 1× Gem21 NeuroPlex (Gemini Bio, West Sacramento, Calif), and penicillin/streptomycin (Thermo Fisher Scientific). Half of the culture media was replaced with fresh media every 3 days.

Calcium imaging

All calcium imaging, pruritogen challenges, and EFS were conducted on an IX71 epifluorescence microscope (Olympus, Tokyo, Japan). Images were acquired with a PCO Edge sCMOS camera using MetaMorph (Molecular Devices, Sunnyvale, Calif) at a rate of 0.2 Hz for the pruritogen challenge and 50 Hz for the EFS recordings. The cytoplasmic calcium levels were monitored by loading cells with 3 μM Fluo 8-AM (AAT Bioquest, Sunnyvale, Calif) at room temperature and rinsing after 30 minutes. The fluorescence was recorded using 480 nm excitation and 520 nm emission filters. Imaging experiments were conducted in physiological buffer containing (in mM) 145 NaCl, 3

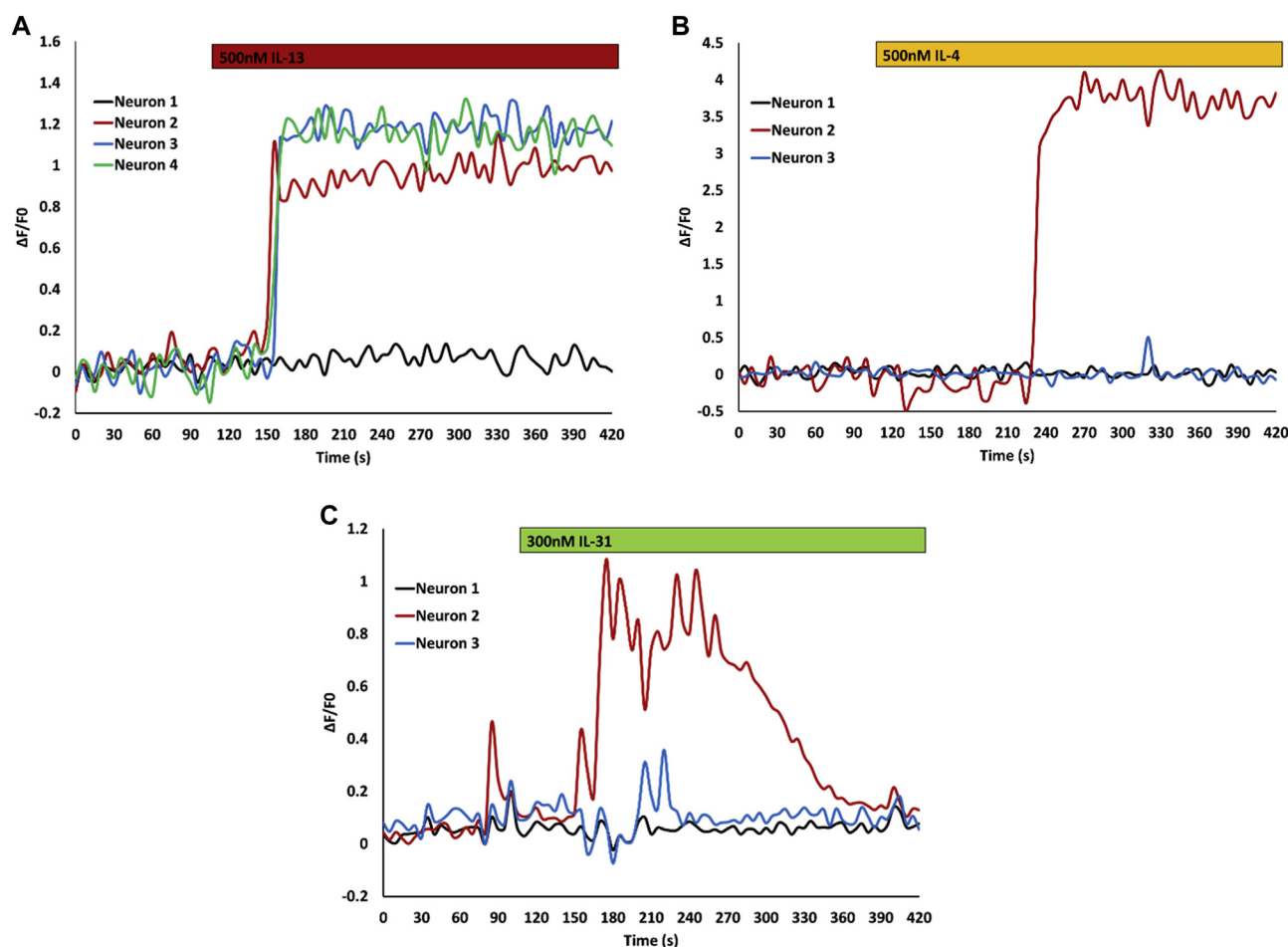


FIG 1. Calcium signaling responses of hDRG neurons by direct activation with cytokines in separate wells with multiple donors for each cytokine. (A) Representative trace of calcium response to IL-13 showing 1 unresponsive (black) and 3 responsive (red, blue, and green) neurons. (B) Representative trace of calcium response to IL-4 showing 2 unresponsive (black and blue) and 1 responsive (red) neuron. (C) Representative trace of calcium response to IL-31 showing 2 unresponsive (black and blue) and 1 responsive (red) neurons.

KCl, 1 MgCl₂, 10 dextrose, 10 HEPES, and 2 CaCl₂ (Sigma-Aldrich, St Louis, Mo) adjusted to pH 7.30.

Neuronal potentiation by cytokines and pruritogens

In these experiments, cytoplasmic calcium levels were monitored after the addition of different pruritogens. IL-13 (500 nM; R&D Systems, Minneapolis, Minn) was applied acutely for 15 minutes or for a prolonged period of 24 hours alone or in combination with lebrikizumab. In the direct cytokine calcium signaling monitoring, IL-4 (R&D Systems) and IL-13 were applied at 500 nM, whereas IL-31 was used at 300 nM.¹⁰ Each cytokine was individually tested in different wells as well as in multiple donors (Fig 1).

Direct pruritogen application. After a 2-minute baseline in different sets of cells, one of BAM8-22 2 μ M (Tocris Bioscience, Bristol, United Kingdom), serotonin 100 μ M (Sigma), or histamine 10 μ M (Sigma) was applied for 30 seconds while the fluorescence signal was monitored. The cells were then rinsed for 5 minutes with buffer under continuous fluorescent monitoring (Fig 1).

Acute desensitization studies. An initial recording was made to identify pruritogen-responsive hDRG neurons as described above. After 25 minutes, a second pruritogen challenge was performed on the neurons. The second application peak was then compared to the first peak to determine the magnitude of the desensitization effect. If the 2 challenges of pruritogens

demonstrated a desensitization effect in vehicle controls, then the cultures would be used to test IL-13 and lebrikizumab effects (Fig 2).

Acute cytokine potentiation and lebrikizumab inhibition protocol. Responsive neurons were identified using the procedure described above, and an initial recording was taken in different sets of cells for each IL-13 (500 nM) sensitization assay, and the combined IL-13 and lebrikizumab (500 μ g/mL or 1 mg/mL) inhibition assay. Before neuronal exposure, IL-13 or the mixture of IL-13 and lebrikizumab was first incubated for 20 minutes at 37°C, then applied to the neurons 5 minutes before the second challenge. The second application peak was then compared to the desensitized second vehicle peak for each study (Fig 2).

Acute normalization and relative effects. In the acute studies, the desensitized second vehicle peak value in each study was normalized, and the relative potentiation effect of IL-13 was calculated as the difference between the desensitized vehicle and IL-13. For the inhibitory effect of lebrikizumab, the IL-13 value was normalized, and the relative inhibition was calculated as the difference between IL-13 and lebrikizumab treatment. If the relative inhibition effect was more than 100% of the potentiation effect, then 100% inhibition was reported for the average across the different donors (Fig 2).

Prolonged cytokine potentiation and lebrikizumab inhibition protocol. The various treatments (vehicle, IL-13 [500 nM] alone, or the mixture of IL-13 [500 nM] and lebrikizumab [500 μ g/mL or 1

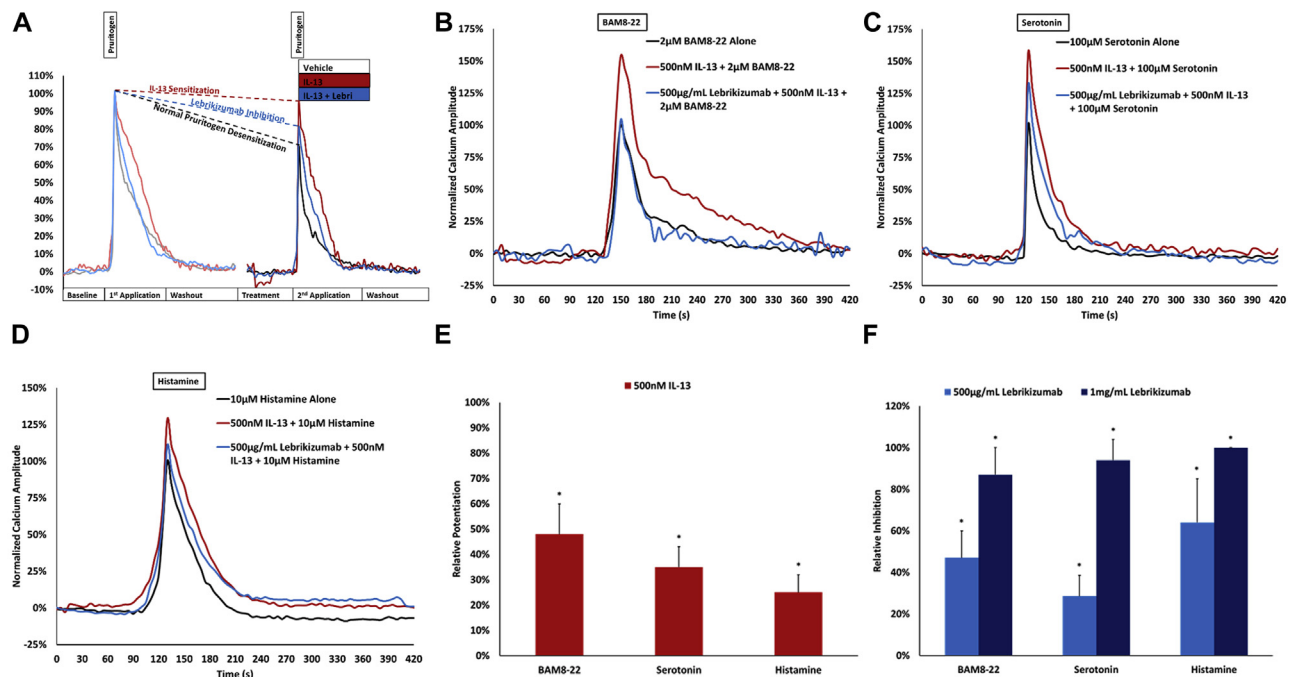


FIG 2. Representative time course of calcium responses to pruritogens with an acute treatment with IL-13 or IL-13 + lebrikizumab. (A) Schematic of dual pruritogen activation protocol. The second application of pruritogen, used as the readout, is typically desensitized, while the IL-13 resensitizes the response. Lebrikizumab inhibits the effect of IL-13 sensitization. (B) Representative traces with the IL-13 sensitization and lebrikizumab inhibition effect over the desensitized BAM8-22 response. (C) Representative trace with the IL-13 sensitization and lebrikizumab inhibition effect over the desensitized serotonin response. (D) Representative trace with the IL-13 sensitization and lebrikizumab inhibition effect over the desensitized histamine response. (E) Average IL-13 sensitization effect over multiple donors for BAM8-22, serotonin, and histamine. *IL-13 potentiation compared to vehicle ($P < .001$). (F) Average lebrikizumab inhibition effect over multiple donors for BAM8-22, serotonin, and histamine. *IL-13 potentiation compared to IL-13 + lebrikizumab ($P < .01$).

mg/mL) were first incubated for 20 minutes at 37°C, then applied to the neurons for 24 hours at 37°C (Fig 3). Subsequently, the neurons were loaded with dye and the direct pruritogen application protocol performed. The peak response of this first application was used to evaluate the effects (vehicle peak, IL-13 peak, and lebrikizumab peak).

Prolonged normalization and relative effects. In the prolonged study, the vehicle peak was normalized, and the relative potentiation effect of IL-13 was calculated as the difference between the vehicle and IL-13. For the inhibitory effect of lebrikizumab, the IL-13 value was normalized, and the relative inhibition was calculated as the difference between IL-13 and lebrikizumab treatment. If the relative inhibition effect was more than 100% of the potentiation effect, then 100% inhibition is reported for the average across the different donors (Fig 3).

Neuronal excitability

EFS was performed by a stimulator (AMPI, Jerusalem, Israel) using a pair of carbon electrodes to deliver current to the loaded Fluo 8-AM neurons. Five trains of 10 biphasic pulses were delivered to the well at different activation voltages from 500 to 2500 mV, with a 1-minute rest between trains. The number of positive steps from the 10 biphasic stimulations was quantified and reported as an average from each individual neuron. For the acute treatment, an EFS response was first established at the 5 different voltages, then evaluated again after 20 minutes of treatment with one of vehicle, 500 nM IL-13, or 1 mg/mL lebrikizumab and 500 nM IL-13. The response was normalized against the first measured response before the treatment. For the prolonged treatment, the neurons were exposed to the same conditions as the acute study, but for 24 hours. Because the EFS response could not be established before the treatment, it was normalized against the vehicle response population (Fig 4).

RNA sequencing

Messenger RNA sequencing. Total RNA was isolated from primary cultured neurons lifted and preserved in RNeasy Lysis Buffer (Qiagen; Thermo Fisher Scientific) using the phenol-based TRIzol method (Invitrogen), and integrity of the purified RNA was confirmed using a Bioanalyzer (Agilent Technologies, Santa Clara, Calif). A messenger RNA (mRNA) sequencing library was prepared from 1 μg total RNA using a TruSeq stranded mRNA sample preparation kit (Illumina, San Diego, Calif). Poly-A-containing mRNA molecules were purified using poly-T oligo-attached magnetic beads, after which the mRNA was fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were copied into first-strand complementary DNA (cDNA) using reverse transcriptase and random primers. Strand specificity was achieved by replacing dTTP with dUTP in the Second Strand Marking Mix, followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. A single 'A' base was added to the cDNA fragments, and the adapter was ligated. The products were then purified and enriched with PCR to create the final cDNA library. Finally, quality and band size of libraries were assessed using an Agilent 2100 Bioanalyzer. Libraries were quantified by real-time quantitative PCR using the CFX96 Real Time System (Bio-Rad, Hercules, Calif). After normalization, sequencing of the prepared library was conducted on the NextSeq system (Illumina) with 75 bp paired-end reads. RNA isolation, sequencing, and primary gene analyses were performed at LAS Inc, Gimpo City, South Korea (Fig 5).

Preprocessing and genome mapping. After trimming the low-quality bases and sequencing adapters in the raw reads with Skewer v0.2.2,¹⁵ the cleaned high-quality reads were mapped to the reference genome by STAR v2.5 software.¹⁶ Because the sequencing libraries were prepared using Illumina's strand-specific library preparation kit, the first-strand library type was applied in the mapping process (Fig 5).

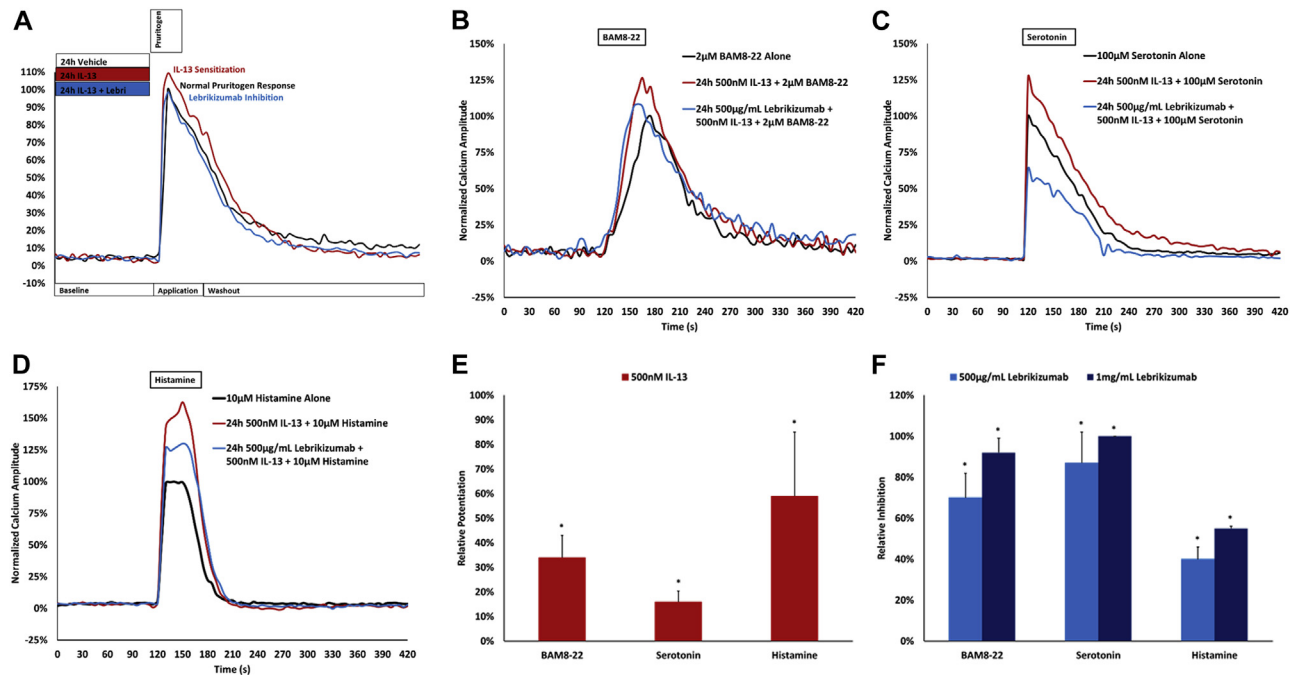


FIG 3. Representative time course of calcium responses to pruritogens after prolonged treatment with IL-13 or IL-13 + lebrikizumab. (A) Schematic of prolonged activation protocol. IL-13 resensitizes the response of sensory neurons compared to vehicle control. Lebrikizumab inhibits the effect of IL-13 sensitization. (B) Representative traces with the IL-13 sensitization and lebrikizumab inhibition effect over the desensitized BAM8-22 response. (C) Representative trace with the IL-13 sensitization and lebrikizumab inhibition effect over the desensitized serotonin response. (D) Representative trace with the IL-13 sensitization and lebrikizumab inhibition effect over the desensitized histamine response. (E) Average IL-13 sensitization effect over multiple donors for BAM8-22, serotonin, and histamine. *IL-13 potentiation compared to vehicle ($P < .05$). (F) Average lebrikizumab inhibition effect over multiple donors for BAM8-22, serotonin, and histamine. *IL-13 potentiation compared to IL-13 + lebrikizumab inhibition ($P < .001$).

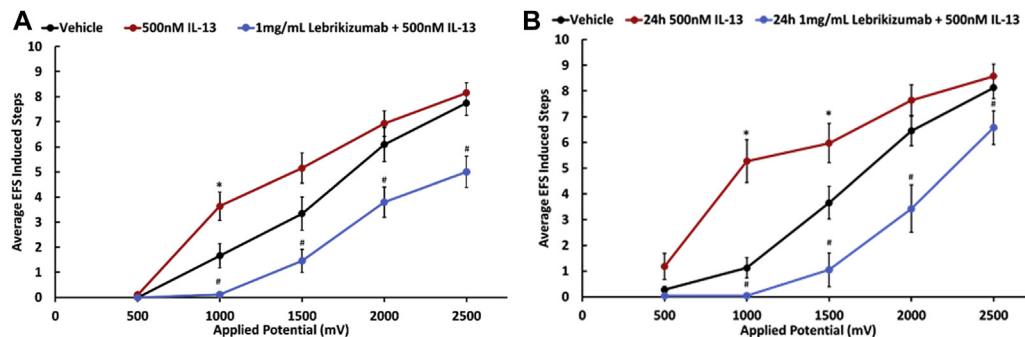


FIG 4. Determination of average changes in sensory neuron excitability with EFS after acute (A) and prolonged (B) treatments with IL-13 and IL-13 + lebrikizumab. *IL-13 potentiation compared to vehicle ($P < .05$). #IL-13 potentiation compared to IL-13 + lebrikizumab inhibition ($P < .05$).

Quantifying gene expression and analysis of differentially expressed genes. The mapped reads on the reference genome were quantified into the gene expression values using Cufflinks v2.2.1¹⁷ with the strand-specific library option ‘first-strand,’ and default options. The gene annotation of the reference genome hg38 (UCSC genome, GTF format; <https://genome.ucsc.edu>) was used as gene models, and the expression values were calculated as fragments per kilobase of transcript per million fragments mapped. The differentially expressed genes (DEGs) between the 2 selected biological conditions were analyzed by Cuffdiff software in the Cufflinks package¹⁸ using the strand-specific library option, library option ‘first-strand,’ and default options. To compare the expression profiles

among the samples, the normalized expression values of a few hundred of the DEGs were clustered by in-house-authored R scripts. Gene expression scatterplots and volcano plots of the expression fold changes and P values between the 2 selected samples were also drawn by in-house-authored R scripts (Fig 5).

Functional category analysis. To better understand the functional role of genes exhibiting differential expression between the compared biological conditions, g:Profiler v0.6.7¹⁹ was used to test gene set overlap between the DEGs and functionally categorized genes, including those involved in gene ontology, KEGG pathways (<https://www.genome.jp/kegg/>), and other functional gene sets (Fig 5).

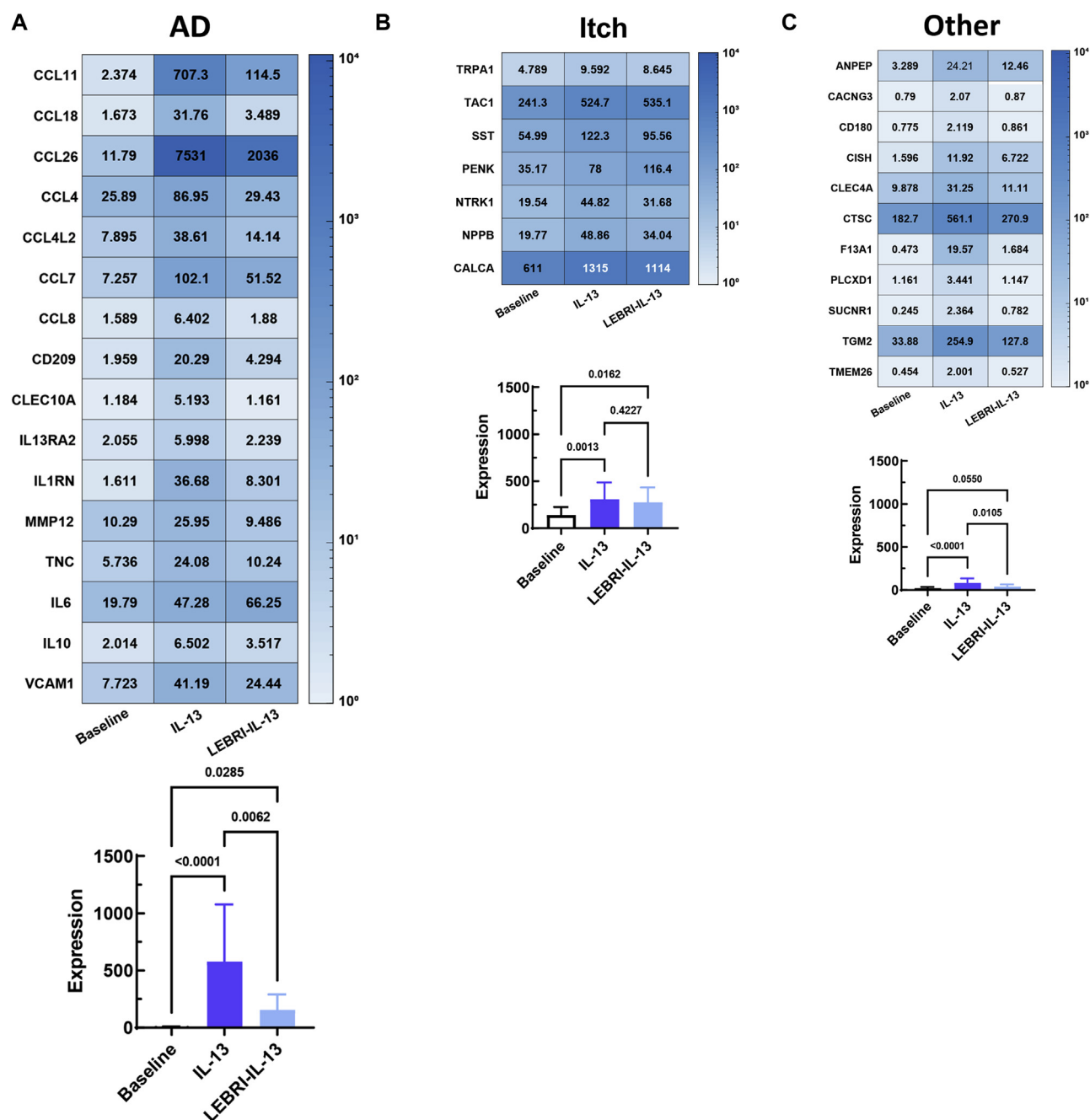


FIG 5. RNA-Seq analysis of untreated, IL-13, and IL-13 + lebrikizumab stimulated hDRG neurons in culture. Heat maps and mean gene expression changes that were upregulated by IL-13 stimulation and reversed by lebrikizumab are shown for AD (A), itch (B), and other conditions (C) (n = 4 donors in each group). Gene expression heat maps were generated by MATLAB (MathWorks, Natick, Mass) using the function 'heatmap.' Color scaling is logarithmic. Color limits are 0 to 9.3. Group differences were determined by nonparametric 1-way ANOVAs with Friedman tests (non-Gaussian distribution of residuals). Repeated measures were genes (baseline vs IL-13 vs IL-13 lebrikizumab-IL-13). Dunn tests were used for multiple comparison in GraphPad Prism 9.0.0 software (GraphPad Software, La Jolla, Calif). No outliers were excluded. Significance values are shown.

RESULTS

Role of IL-13 and lebrikizumab in neuronal potentiation and pruritogen response

Some human sensory neurons are directly activated by IL-13. To test the effects of type 2 cytokine IL-13 on human

sensory neurons, we utilized hDRG from multiple donors after their deaths. Neuronal responses were initially evaluated by measurement of the direct calcium response via fluorescent-based calcium imaging of the DRG neurons in culture. Approximately 1% to 2% of cultured hDRG neurons demonstrated a direct

calcium signal response after application of IL-13 (500 nM) (Fig 1, A, and Table I). Similarly, application of IL-4 and IL-31 also produced a direct calcium signal in <2% of the DRG neurons (Fig 1, B and C). The numbers of neurons responsive to IL-13, IL-4, and IL-31, which were tested using separate experimental setups, are summarized in Table I. Our calcium imaging results suggest that overall, each of the cytokines—namely IL-13, IL-4, and IL-31—directly activates calcium signaling, but only in a tiny subset of sensory human neurons. This percentage of neurons that is in fact much smaller than described in mouse calcium imaging experiments using these cytokines.^{6,10} Of note, IL-31 induced only in single cells calcium signaling at concentrations of both 300 and 500 nM. However, only 500 nM of IL-13 yielded reproducible calcium measurements for different tested donors throughout the studies.

Acute stimulation with IL-13 amplifies neuronal responses to pruritogens, which are effectively blocked by lebrikizumab. To evaluate whether IL-13 plays a neuromodulatory role in human sensory neurons, we acutely stimulated hDRGs with IL-13 for 5 minutes before application of 3 pruritogens known to induce direct neuronal activation: BAM8-22, serotonin, and histamine (Fig 2). Neuronal responses were evaluated first with pruritogen alone, then after the second application of pruritogen in the presence of either vehicle or IL-13. In addition, our test conditions included incubation of IL-13 with lebrikizumab to test its ability to reverse IL-13–elicited neuroactivity. The acute stimulation protocol design is illustrated in Fig 2, A.

All 3 pruritogens induced first responses after initial application and attenuated second responses (desensitization) after reapplication in vehicle controls. Application of IL-13 before the second pruritogen stimulation resulted in sensitized responses to all 3 pruritogens with the following increases: BAM8-22 (mean $\pm$ SEM; $48\% \pm 12\%$, $n = 8$) (Fig 2, B), serotonin ($35\% \pm 8\%$, $n = 8$) (Fig 2, C), and histamine ($25\% \pm 7\%$, $n = 7$) (Fig 2, D). These neuronal responses to IL-13 stimulation and pruritogens are summarized in Fig 2, E, and Table II.

We hypothesized that the sensitizing effects of IL-13 in the human neuron model could be blocked by lebrikizumab. To investigate this possibility, we performed the prestimulation experiments described above with IL-13 in the presence of lebrikizumab. Lebrikizumab (500 μ g/mL) effectively inhibited the IL-13–elicited neuronal sensitization responses to each of the 3 pruritogens: BAM8-22 ($47\% \pm 13\%$, $n = 5$) (Fig 2, B), serotonin ($29\% \pm 10\%$, $n = 4$) (Fig 2, C), and histamine ($64\% \pm 21\%$, $n = 5$) (Fig 2, D). When applied at higher doses (1 mg/mL) lebrikizumab potently blocked neuronal sensitization by over 85% for each of the pruritogens: BAM8-22 ($94\% \pm 10\%$, $n = 3$), serotonin ($87\% \pm 13\%$, $n = 3$), and histamine ($100\% \pm 0$, $n = 2$). The acute inhibition data are summarized in Fig 2, F, and Table III. Thus, lebrikizumab was effective at inhibiting IL-13–potentiated responses to histaminergic and non-histaminergic pruritogens in human sensory neurons in a dose-dependent manner.

Prolonged stimulation with IL-13 amplifies neuronal responses to pruritogens, which are effectively blocked by lebrikizumab. To assess whether prolonged incubation with IL-13 or the combination of IL-13 and lebrikizumab alters the sensitized responses to pruritogens in human sensory neurons, we incubated the neurons with vehicle, IL-13, or the combination of IL-13 and lebrikizumab for 24 hours before challenging them with BAM8-22, serotonin, or histamine. The

TABLE I. Sensory neurons directly activated by cytokines

Cytokine	Donor no.	No. of neurons	No. of cytokine-responsive neurons	%
IL-4	1	179	3	1.7
IL-13	2	630	7	1.1
IL-31	2	358	2	0.6

TABLE II. Acute responses of pruritogens to IL-13

Puritogen	500 nM IL-13	SEM	No.	P value
BAM8-22	48%	12%	8	<.001
Serotonin	35%	8%	8	<.001
Histamine	25%	7%	7	<.001

TABLE III. Acute responses of pruritogens to lebrikizumab

Purritogen	500 µg/mL		P	1 mg/mL		P		
	lebrikizumab	SEM		lebrikizumab	SEM			
BAM8-22	47%	13%	5	<.001	87%	13%	3	<.001
Serotonin	29%	10%	4	<.01	94%	10%	3	<.001
Histamine	64%	21%	5	<.005	100%	0	2	<.001

TABLE IV. Prolonged sensitization with IL-13

Puritogen	500 nM IL-13	SEM	No.	P value
BAM8-22	34%	9%	6	<.001
Serotonin	16%	4%	6	<.001
Histamine	59%	26%	5	<.05

prolonged stimulation protocol design is shown in Fig 3, A. Under these conditions, the IL-13 increased neuronal potentiation of the pruritic signals: BAM8-22 ($34\% \pm 9\%$, $n = 6$) (Fig 3, B), serotonin ($16\% \pm 4\%$, $n = 6$) (Fig 3, C), and histamine ($59\% \pm 26\%$, $n = 5$) (Fig 3, D). Summaries of prolonged responses to IL-13 are shown in Fig 3, E, and Table IV.

Under the same test conditions (Fig 3, A), we tested lebrikizumab's ability to block neuronal sensitization activity in parallel with prolonged exposure to IL-13. Lebrikizumab was effective in blocking the neuronal responses for all 3 pruritogens in a dose-dependent manner. At 500 μ g/mL, lebrikizumab inhibited IL-13 sensitization of BAM8-22 ($70\% \pm 12\%$, $n = 6$) (Fig 3, B), serotonin ($87\% \pm 15\%$, $n = 4$) (Fig 3, C), and histamine ($40\% \pm 6\%$, $n = 4$) (Fig 3, D). At 1 mg/mL, lebrikizumab inhibited IL-13 sensitization of BAM8-22 ($92\% \pm 9\%$, $n = 2$), serotonin ($100\% \pm 0$, $n = 2$), and histamine ($55\% \pm 1\%$, $n = 3$). The inhibitory responses of prolonged exposure of lebrikizumab and IL-13 are summarized in Fig 3, F, and Table V.

Effects of IL-13 and lebrikizumab on neuronal excitability

Pain tolerance and intensity has been linked to neuronal excitability.²⁰ To investigate whether the AD-associated type 2 cytokine IL-13 might influence neuronal excitability, we used an EFS procedure to detect changes in the firing of the sensory

TABLE V. Prolonged inhibition with lebrikizumab

Purritogen	500 µg/mL			<i>P</i>	1 mg/mL			<i>P</i>
	lebrikizumab	SEM	No.		lebrikizumab	SEM	No.	
BAM8-22	70%	12%	6	<.001	92%	7%	2	<.001
Serotonin	87%	15%	4	<.001	100%	0	2	<.001
Histamine	40%	6%	4	<.001	55%	1%	3	<.001

neurons after stimulation with 5 different voltage activation pulses. We observed an increase in neuronal excitability after either acute (20 minutes; Fig 4, A) or prolonged (24 hours; Fig 4, B) treatment with IL-13 compared to vehicle-treated neurons. We then evaluated whether lebrikizumab might act to reverse the enhanced neuronal excitability induced by IL-13 by incubating the neurons in a mixture of IL-13 and lebrikizumab. The addition of lebrikizumab significantly lowered excitability, shifting neuronal firing rates to levels below those of vehicle-treated neurons after both acute (Fig 4, A) and prolonged (Fig 4, B) stimulation. These findings suggest that IL-13 can exert neuronal sensitization through potentially different mechanisms than the pruritogens, which are also effectively blocked by lebrikizumab.

Transcriptional targets of IL-13 signaling identified via RNA-Seq

To gain insight into possible transcriptional effects of prolonged IL-13 on cultured hDRG neurons, we conducted RNA-Seq analysis of RNA samples derived from neurons and the supportive nonneuronal glial cells in culture without treatment and after treatment with IL-13 for 24 hours (each group *n* = 4 donors). Additionally, we tested whether lebrikizumab (1 mg/mL) converts the transcriptional effects elicited by IL-13 on those neurons. Overall, IL-13 stimulation altered expression of 237 genes, inducing a statistically significant upregulation of 143 genes (*P* ≤ .05) and a ≥2-fold increase in expression compared to untreated neurons (data not shown). Among these, we found 24 DEGs, including immune-regulatory and inflammatory chemokines such as *CCL26*, *CCL11*, *CCL18*, *CCL8*, and *CCL4*, which were also significantly downregulated within 24 hours of treatment of lebrikizumab (Fig 5, A). *CCL26*, a chemokine that is induced by type 2 cytokines and that is upregulated in AD,^{21,22} is involved in tissue remodeling during atopic skin inflammation.^{23,24} *CCL26* is produced by keratinocytes and endothelial cells in skin tissue and activates CCR3 on T cells and eosinophils.²⁵ IL-4 and IL-13 upregulate *CCL26* expression in keratinocytes.²⁶ We found that IL-13 potently upregulates the *CCL26* transcripts and that lebrikizumab has countermodulatory effects in cultured hDRG neurons (Fig 5, A). In the nervous system, *CCL26*, also known as eotaxin-3, together with its cognate receptor, contribute to neurodegeneration.²⁷ It is very likely that *CCL26* is involved in the long-term neuronal sensitization elicited by IL-13 stimulation. Among the transcripts, we found that matrix metalloproteinase 12 was upregulated by IL-13 and that lebrikizumab reverted this expression to baseline expression by lebrikizumab (Fig 5, B and C). Matrix metalloproteinase 12 is defined as a marker of general inflammation and is responsible for extracellular matrix degeneration during inflammation, in line with IL-13-driven tissue reorganization functions.²⁸

Of note, IL-13Rα2 was upregulated almost 3-fold by IL-13 stimulation compared to untreated samples, and lebrikizumab converted the expression level back to control value (Fig 5, A).

It has been suggested that IL-13Rα2 acts as a decoy for IL-13,²⁹ and it has been reported that scratching upregulates the IL-13Rα2a receptor, counteracting the effects of IL-13.³⁰ Our results suggest that neuronal IL-13-regulated expression of IL-13Rα2 might serve a similar itch-protective function. A deeper insight into the neurospecific role of IL-13Rα2 will require future studies.

We also observed that *IL1RN*, an interleukin receptor gene that encodes an endogenous antagonist of the IL-1 receptor, is significantly upregulated by IL-13 and significantly downregulated by lebrikizumab (Fig 5, A). Additionally, other DEG transcripts regulated by both IL-13 and lebrikizumab include the genes *SUCNR1*, *CLEC4A*, *TGM2*, *CTSC*, and *TMEM26* (Fig 5, C). The neuronal or nonneuronal function of these transcripts in AD is unknown and will require further studies.

Genes encoding several itch and pain-transmission relevant neuropeptides (*Nppb*, *SST*, *PENK*, *TAC1*, *CALCA*) and receptors (*TRPA1*, *NTRK1*) are expressed in elevated levels after IL-13 stimulation (Fig 5, B) but were not significantly attenuated by lebrikizumab at the tested time points. *Nppb* encodes natriuretic peptide B, which contributes to peripheral itch circuitry, as the loss of *Nppb* in mice reduces itch responses to multiple pruritogens.³¹ *SST* encodes somatostatin, which is present in itch-specific neurons in the mouse DRGs.³² *PENK* encodes proenkephalin-A, which serves as a precursor polypeptide for BAM8-22, a highly pruritogenic peptide that directly activates human sensory neurons.³³ *TAC1* encodes tachykinin-1, which is a precursor for substance P, a well-known pruritogen and mast cell degranulation promoter that acts as a proxy for itch in AD.^{34,35} *CALCA* encodes calcitonin gene-related polypeptide alpha (CGRPα), which is found in nociceptive murine dorsal root ganglion cells that respond to itch and pain. Insight into the functional role of CGRPα was provided by the observation that CGRPα-positive DRG neurons are activated by capsaicin, the painful ingredient in hot pepper, as well as by chloroquine, a nonhistaminergic pruritogen.³⁶

TRPA1, the irritant receptor, is a signal transducer in multiple nonhistaminergic itch pathways.³⁷ Our findings support a role for *TRPA1* in itch transmission in humans similar to that found in mice. IL-13-induced upregulation of *TRPA1* might lead to heightened itch signal transduction in type 2 associated inflammatory diseases. The neuronal receptor transcript *NTRK1*, which encodes the NGF-activated receptor tyrosine kinases A, B, and C, was upregulated by IL-13. Loss of the *NTRK1* gene during development results in congenital insensitivity to pain with anhidrosis, a condition represented clinically by insensitivity to itch in the presence of neurogenic inflammation.³⁸ Such patients lack the NGF-dependent C-nociceptors, as well as the Ad fibers that convey pain and itch signals.³⁸⁻⁴⁰ IL-13 stimulation resulted in the statistically significant upregulation of each of these genes (Fig 5, B). Lebrikizumab partially reversed upregulation of the itch-specific neuropeptide genes *TRPA1* or *NTRK1*. Surprisingly, we found cytokine transcripts such as IL-6 and IL-10 were elevated by IL-13 stimulation, but only IL-6 expression remained high, whereas IL-10 expression was reversed with lebrikizumab treatment (Fig 5, A). The role of these genes in the neuronal environment and the expression sources in the RNA samples that include neuronal as well as nonneuronal glial cells need to be explored further in the context of IL-13-mediated inflammation and neuromodulation. We further conducted RNA-Seq analysis on single hDRG neurons in culture and found that all the DEGs shown in this study were of neuronal origin, highlighting the

fact that IL-13 is directly affecting gene expression in the sensory neurons.

In summary, our transcriptome analysis revealed a multitude of itch-relevant genes that are upregulated within 24 hours by IL-13 stimulation in human sensory neurons (Fig 5). Additionally, we found that lebrikizumab effectively reversed some transcripts to baseline or significantly lower levels within 24 hours. In our mechanistic studies, we found that lebrikizumab reverses neuronal sensitization elicited by IL-13 on itch as well as the increased neuronal firing. Our finding that lebrikizumab reverted IL-13-induced gene transcripts suggests that under prolonged exposure, this antibody underscores that IL-13 regulates gene function in human sensory neurons. These gene transcripts might play a role in subsequent cross-communication between immune and nervous system.

DISCUSSION

Chronic itch is a cardinal feature of AD. In AD, itch likely originates from inflammation in the tissue and the subsequently sensitized nervous system.⁴¹⁻⁴³ The preponderance of type 2 immune cells in the disease signature led to the discovery that type 2 cytokines are key drivers in the pathology of AD. For example, IL-31, which is released primarily by activated T_H2 cells, elicits itch in mice through direct communication with its cognate receptor subunits on sensory neurons.⁴⁴⁻⁴⁸ Results from the current study demonstrate that IL-13 is a potent, neuroactive cytokine that directly modulates human sensory neurons in their neuronal excitability and potentiates the responses in histaminergic and nonhistaminergic itch pathways. The major lack of direct calcium signaling effects on the neurons activated by IL-13 suggests it may play a role in humans different from that in mice, in which IL-13 has been postulated to elicit itch directly.¹² IL-13 has not been reported to trigger itch directly in humans, and it remains to be determined whether IL-13-induced itch is a secondary neuronal enhancer effect of the abundant IL-13 levels found in the skin of AD patients.⁴⁹ Our *ex vivo* mechanistic studies here suggest that IL-13 potentiates pruritogenic responses and sensitizes the sensory neurons.

Results from the phase 2b clinical trial of lebrikizumab, a high-affinity mAb specifically targeting IL-13, suggest that blocking IL-13 systemically alone may be sufficient to improve itch significantly in patients with moderate to severe AD.¹³ The rapid (within days) and robust efficacy observed with lebrikizumab on itch in AD patients in the phase 2b study is consistent with a neuroactive role for IL-13 in itch amplification. Additionally, data from our human neuron model provide the mechanistic insight into the neuroimmune interaction that may contribute to hypersensitivity of AD patients. A similar cellular mechanism might partake for other targeted immune inhibitors providing treatment for AD patients. However, the studies used a concentration of IL-13 that resulted in reproducible data with consideration of donor-to-donor variability, which is likely not physiological. To our knowledge, the plasma as well as the tissue protein levels for IL-13 in the skin have not yet been reported. The *ex vivo* setup for the live calcium detection as well as the other experimental readouts were conducted at concentrations that yielded clear responses. Nevertheless, the complexity within AD patients is solely depicted via cellular and molecular insights focusing on human neuronal responses triggered by IL-13 and reversed by lebrikizumab void of the

multicellular cross-communication occurring within the pathophysiology of the disease.

We found that prolonged IL-13 stimulation of the human sensory neurons did not further amplify nonhistaminergic itch signals for BAM8-22 and serotonin, while histamine-induced responses were potentiated. We interpret these findings as an indication that different mechanisms might underlie itch signaling by distinct pruritogens and that prolonged IL-13 stimulation might emerge in transcriptional priming within the sensory neurons. Because of its mast cell-associated effects, IL-13 could help drive histaminergic itch in allergic reactions such as chronic urticaria, particularly in anti-IgE treatment-resistant forms. It is possible that sustained levels of enhancer molecules (eg, IL-13) as well as the continuous release of pruritogens drive the chronicity of this persistently sensed itch in inflammatory diseases. This chronicity may result from the sensitization or heightened activation of itch-specific neurons, peripherally as well as centrally. Our findings with EFS also suggest a unique but still unknown direct mechanism of IL-13 on neuronal excitation. Moreover, lebrikizumab potentially reverses IL-13-induced neuronal excitability to levels below baseline, suggesting that lebrikizumab may affect more than 1 neuronal pathway. Future investigations of IL-13-associated effects on pain pathways may provide mechanistic insight into possible cross-over pathways of pain and itch dysesthesias as experienced in AD patients.^{50,51}

The data presented here are the first transcriptional RNA-Seq analysis of human sensory neurons stimulated with IL-13 for 24 hours; they also establish the effects of lebrikizumab on IL-13-induced stimulation. This approach advances our understanding of the transcriptional regulation of AD-associated type 2 cytokines on human sensory neurons and of the ability of lebrikizumab to counterregulate IL-13-driven transcriptional changes. We observed that more than 142 DEGs were upregulated >2-fold with IL-13 in human sensory neurons, and that more than 20 of these were downregulated >2-fold within 24 hours of lebrikizumab administration. Our calcium imaging studies demonstrated that prolonged IL-13 stimulation resulted in altered neuronal potentiation to the tested pruritogens acute exposure compared to vehicle, a finding that led us to hypothesize that long-term effects might result from transcriptional changes, which may differ from those underlying the more immediate somatosensory modality: itch. RNA-Seq data analysis demonstrated significant changes in expression of a substantial number of genes, indicating that IL-13 is a potent driver of transcription-based neuronal changes. Among the DEGs, cytokine–cytokine receptor interaction and chemokine signaling pathway–related genes were the top 2 of the enriched KEGG pathways. Lebrikizumab was most potent at downregulating chemokine activity and chemokine receptor binding, followed closely by CCR chemokine receptor binding. For example, *CCL26*, the gene showing the highest degree of upregulation by IL-13, has been reported to be transcriptionally regulated by IL-13 on epithelial cells involved in allergic inflammation.⁵² We have shown that these transcriptional effects are maintained in neuronal cells by IL-13 and the transcripts are readjusted by lebrikizumab. Interestingly, IL-13 upregulated the gene encoding the IL-13R α 2 receptor, which was downregulated to baseline by lebrikizumab. The exact role of IL-13R α 2 is still unclear, although most studies suggest a protective function involving binding IL-13 to prevent signaling via its receptor complex.^{53,54} The functional contribution for IL-13R α 2 requires evaluation in human

disease settings because the concept of scratching as a mechanor-effective regulation to further induce itch is a well-described mechanism and the basis of the itch-scratch cycle.¹¹

We also found that IL-6 transcript was increased, which might promote neuronal excitability, as has been reported for IL-6 in the nervous system.⁵⁵ Some of itch-relevant neuropeptides as well as receptors were significantly upregulated in human neurons with IL-13 stimulation, thus verifying their importance in itch signaling in human tissue (data not shown). Overall, this suggests that IL-13 might increase the transcription of other cytokines but also key itch circuits to exacerbate the neuronal effects in AD. Overall, one might speculate that IL-13 could have a broader role to play in influencing other cellular targets via the transcripts regulated in neurons.

Gene transcripts associated with itch transmission, including *NPPB*, *SST*, *PENK*, *TAC1*, and *TRPA1*, were increased after IL-13 stimulation in human sensory neurons. Upregulation of these genes suggests that IL-13 may modulate long-term neuronal sensitivity and itch responsiveness in addition to potentiating acute responses.^{56,57} Taken together, these results might indicate that IL-13 alters responsiveness to itch through neuronal changes resulting from upregulation of the itch-associated signal transduction receptor *TRPA1*.³⁷ *NTRK1* has been reported to be upregulated by IL-13 in epithelial cells⁵⁸ and is believed to be involved in the development of allergic inflammation. The most prominent function of the neurotrophic receptor encoded by *NTRK1* is in neuronal development in response to NGF.

Taken together, our data indicates that IL-13 is a neuroactive type 2 cytokine that has acute and chronic effects on neuronal excitation, pruritogen sensitization, and transcript regulation in human sensory neurons. To our knowledge, this is the first study exclusively focused on data from hDRG neurons to report that IL-13 is a neuronal enhancer in multiple itch pathways and that it may contribute to the persistence of chronic itch in type 2-associated diseases. The potent ability of lebrikizumab to block the neuronal effects of IL-13 may form part of the mechanistic basis of the beneficial impact of lebrikizumab on chronic itch in AD. Additionally, this is the first study to show that IL-13 is a potent mediator of neuronal gene transcription and that lebrikizumab partially reversed these changes. Inhibition of IL-13 by lebrikizumab might provide multiple therapeutic advantages in the treatment of neuroinflammatory diseases beyond moderate to severe AD.

Graphic illustrations summarizing effects of lebrikizumab were designed by Dani Guralnick (Distill Creative Studio). Prescott Medical Communications Group (Chicago, Ill), with financial support from Dermira Inc, a wholly owned subsidiary of Eli Lilly and Company, and Nancy Tan, an employee of Eli Lilly and Company, provided editorial assistance.

Key messages

- Our findings in human sensory neurons suggest key pathogenic and neuroactive functions of the type 2 cytokine IL-13 that drive the chronicity of itch in AD.
- Lebrikizumab can potentially block the neuronal effects of IL-13 and directly address the neuronal itch sensitization in AD.

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