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RESEARCH ARTICLE

S1P/S1PRs-TRPV4 axis is a novel therapeutic target for persistent pain and itch in chronic dermatitis

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Abstract

Background and Purpose: While pain and itch are both commonly associated with chronic dermatitis (CD), the molecular mechanisms underlying these debilitating symptoms is not well understood. This study aims to identify novel, endogenous compounds that mediate CD-associated pain and itch.

Experimental Approach: Lesional skin of CD model mice was examined using unbiased metabolomic analysis to identify candidate pain or itch inducing compounds in CD. Sphingosine-1-phosphate (S1P) concentration in CD model skin was analysed using UPLC/MS/MS. Behaviour, calcium imaging and immunofluorescence staining were used to determine the pain and itch effects and mechanisms of the identified CD-related compounds.

Key Results: In the lesional skin of CD model mice, 136 compounds were significantly changed. These compounds are predominately associated with the sphingolipids metabolism pathway. S1P is significantly increased in the lesional skin . The TRPV4 channel was critical for S1P induced itch and pain. Sphingosine kinase 2 (SPHK2), the key enzyme controlling S1P synthesis, was significantly increased in lesional skin. ABC294640, a SPHK2 inhibitor, significantly decreased S1P concentration in lesional CD model skin, as well as in model associated epidermal hyperplasia and chronic pain and itch. In CD patients, SPHK2 expression and S1P concentration were significantly elevated compared to healthy control skin.

Conclusion and Implications: Our results indicate that, in CD, increased S1P induces chronic pain and itch partly through TRPV4. Inhibition of S1P synthesis or the S1P/S1P receptor-TRPV4 pathway are promising treatment strategies for CD-associated pain and itch.

KEYWORDS

chronic dermatitis, itch, pain, S1P, TRPV4

Abbreviations: ABC, ABC294640; CD, chronic dermatitis; CHS, contact hypersensitivity; GSK219, GSK2193874; MrgprA3^{+}, Mas-related G protein-coupled receptors A3 positive; HC, healthy control; OPLS-DA, orthogonal partial least squares discriminant analysis; OXA, oxazone; S1P, Sphingosine-1-phosphate; S1PRs, S1P receptors; TG, trigeminal ganglion; VIP, variable projection importance.

Xinyu Zhang, Yuan Zhou, Changming Wang contributed equally.

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1 | INTRODUCTION

Pain and itch are unpleasant and sometimes debilitating sensations that involve sensory, cognitive and affective components. While pain and itch elicit distinctive behavioural responses and are often antagonistic, both pain and itch are commonly associated chronic dermatitis (CD) conditions, including atopic dermatitis, allergic and non-allergic contact dermatitis. Tremendous progress has been made in the identification and characterisation of pain and itch sensing nociceptive and pruritoceptive primary sensory neuron populations (respectively) of the dorsal root ganglia (DRG) and trigeminal ganglia (TG).

The delineation between pain and itch mechanisms, especially under pathological conditions, is not always clear. One study reported that differential activation of Mas-related G protein-coupled receptor A3 positive (MrgprA3^{+}) neurons elicited pain or itch responses in a context dependent manner in mice. While activation of these neurons through Gq coupled metabotropic receptors, like MrgprA3 or DREADDs (designer receptors exclusively activated by designer drugs), evokes itch, activation through ionotropic receptors like **P2X3** or channel rhodopsin, predominantly evoked nociceptive responses (Sharif et al., 2020). However, some ion channels, such as transient receptor potential vanilloid 1 (**TRPV1**) and transient receptor potential ankyrin 1 (**TRPA1**), that are involved in pain transduction also play pivotal roles in itch transduction. (Bíró et al., 2007; Yu et al., 2016). Indeed, selective activation of pruriceptive neurons using capsaicin, in the absence of activation of nociceptive neurons, elicits itch. (Han et al., 2013; Yu et al., 2016). As a result, there is mounting evidence suggesting that most noxious chemicals are not specific for itch or pain, highlighting the complex and contextualised nature of itch and pain detection mechanisms (Ross, 2011).

Moderate and severe cases of CD is almost always associated with intense, chronic pruritus. Excessive scratching feeds into a vicious itch–scratch cycle by further damaging the skin barrier (Hu et al., 2021), which, in turn, leads to significantly increased pruritogen (e.g. thymic stromal lymphopoietin) and inflammatory factors (e.g. interleukin 13 and interleukin 31) in the skin (Adhikary et al., 2021; Mitamura et al., 2018; Wilson et al., 2013).

Sphingolipids are essential for the maintenance of skin barrier function but have also been implicated in itch. In atopic dermatitis, glucosylsphingosine was reported to induce pruritus via the activation of serotonin 2A (**5-HT$_{2A}$**) receptors and transient receptor potential vanilloid 4 (**TRPV4**) in sensory neurons. Moreover, Sphingosine-1-phosphate (**S1P**) was reported to induced both pain and itch in mice (Li et al., 2020). Our present study aimed to identify pain- and itch-related molecules in CD. We used an unbiased metabolomic approach to identify enriched compounds in lesional CD skin and analysed differentially activated pathways of these metabolites in a mouse CD model. Our findings directly implicated sphingolipids and S1P. Moreover, our results showed that inhibition of the S1P/**S$_1$P receptor**–TRPV4 pathway alleviated CD-associated chronic pain and chronic itch in mouse model, highlighting the potential of this treatment strategy for CD-associated pain and itch in patients. Consequently, our results advance the understanding of sensory encoding and crosstalk between pain and itch under CD, and provide critical information for CD management.

What is already known?

- Metabolism of sphingolipids is crucial to the maintenance of skin barrier integrity.
- S1P induces pain and itch through TRPV1 and TRPA1 channels in mice.

What does this study add?

- S1P was increased in lesional skin of mice and patients with chronic dermatitis (CD).
- S1P-induced pain and itch are partly dependent on the expression of TRPV4 channels.

What is the clinical significance?

- Inhibition of S1P synthesis or S1P/S1P receptor-TRPV4 pathway might alleviate CD-associated pain and itch.

2 | METHODS

2.1 | Skin donors

Human skin punch biopsies were obtained from healthy individuals and patients with CD. All CD patients exhibited active CD and did not receive any topical (for 2 weeks) or systemic (for 4 weeks) therapy

before biopsy. All procedures were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University (IRB number 2023 [068]). Informed consent was obtained from all donors. Summarised clinical data as well as detailed demographics of the healthy control (HC) and CD donors are listed in Table S1.

2.2 | Animals

Animals were housed and tested in a climate-controlled environment with 20–24°C, 45–65% humidity, and a 12-h day/night cycle. Animals used in behavioural experiments were all 8- to 10-week-old males. C57BL/6J mice, *Trpv4*$^{-/-}$ mice and their WT littermates, and *Trpa1*$^{-/-}$; *Trpv1*$^{-/-}$ mice and their WT littermates were used for experiments. *Trpv4*$^{-/-}$ and *Trpa1*$^{-/-}$; *Trpv1*$^{-/-}$ mice were generously gifted by Dr. Zongxiang Tang, PhD, of Nanjing University of Chinese Medicine. Animal experiments were approved and conducted in accordance with the relevant guidelines and regulations of the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine (ACU190601). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

2.3 | Generation of the mouse chronic dermatitis (CD) model

To induce CD, mice were shaved and treated with oxazolone (OXA) as previously described (Hu et al., 2023; Li et al., 2022). Briefly, mice were sensitised by topical application of 150 µl OXA (2%) in acetone/olive oil (4:1) onto their shaved abdominal skin. Starting from 5 days after sensitisation, mice were challenged with 50 µl of topically applied OXA (0.5%) on their shaved cheek skin once every 48 h. Spontaneous scratching and wiping behaviour was filmed and quantified 24 h after each challenge.

2.4 | Evaluation of skin damage

Skin lesion severity was scored based on a previously published study (Hu et al., 2021). Four symptoms (dryness, dandruff, bleeding and swelling) were scored on the following scale: 0 = *none*, 1 = *light*, 2 = *medium* and 3 = *severe*. Skin lesion severity score was the sum of the four sub-scores, with a maximum of 12.

2.5 | Behavioural assay

All animal experiments were performed and analysed by researchers blinded to animal genotypes and treatment groups. Animals were acclimated to the testing environment for 15 min before the initiation of behavioural tests. Spontaneous scratching and wiping behaviour in the OXA-treated CD model animals were observed for 30 min. Intradermal injection of chemicals was performed as previously described (Li et al., 2022). In brief, S1P and other compounds were intradermal injected into the cheek after acclimation, and scratching and wiping behaviour were observed for 30 min.

2.6 | Trigeminal ganglion (TG) neuron culture and Ca^{2+} imaging

Mice were anesthetized with isoflurane, then killed by cervical dislocation. TG were dissected and collected in cold DH10 medium (90% Dulbecco's Modified Eagle Medium (DMEM/F-12, 10% Fetal Bovine Serum (FBS), 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, Gibco, New South Wales, Australia) and digested using an enzyme solution (1 mg ml^{-1} Collagenase Type I and 5 mg ml^{-1} Dispase in HPBS without Ca^{2+} and Mg^{2+}, Gibco) at 37°C for 25–30 min. Digested TGs were digested by passing through a fire-polished Pasteur pipette, pelleted centrifugation, and re-suspended in warm (37°C) DH10 medium supplemented with nerve growth factor (NGF, 20 ng ml^{-1}) and glial-cell derived neurotrophic factor (**GDNF**) (25 ng ml^{-1}). Cells were plated onto glass coverslips coated with poly-D-lysine (0.5 mg ml^{-1}) and laminin (10 µg ml^{-1}), and cultured in an incubator (95% O_2 and 5% CO_2) at 37°C for 24 h before use. For Ca^{2+}, imaging neurons were loaded with Fura 2-acetomethoxy ester (Molecular Probes) for 30 min at room temperature. After washing and recovery for 5 min, cells were imaged at 340 and 380 nm excitation to detect free intracellular calcium. Cells were considered responsive if the increase in 340/380 fluorescence ratio after stimulation was at least 50% of baseline fluorescence ratio ($\Delta F/F_0 \geq 0.5$, F_0 is defined as the average baseline fluorescence ratio, and ΔF is defined as the maximum fluorescence ratio minus F_0). All experiments were performed by experimenters blinded to animal genotypes and treatment groups. Each experiment was performed with cells from at least three mice.

2.7 | Untargeted metabolomics analysis

Acetonitrile in water (6:1) was used to treat skin tissues. Skin lysates were desalted using Ziptip C18 columns before ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) analysis using the UHPLC Acquity™ system (Waters Corp., Milford, MA, USA) coupled to a Synapt™ Q-TOF mass spectrometer with electrospray ionisation (ESI) in the positive and negative modes. Sample separation was performed using ACQUITY UPLC BEH-C18 chromatographic columns (2.1 × 100 mm, 1.7 µm) at 30°C. Flow rate of the mobile phase was set to 0.4 ml min^{-1}, and the injection volume for each sample was 2 µl. Phase A contained water with 0.1% formic acid and phase B was 100% acetonitrile. Gradient elution programme used was 0–2.5 min, 5–35% B; 2.5–4 min, 35–45% B; 4–10 min, 45–65% B; 10–11 min, 65–85% B; 11–16 min, 85–90% B; 16–18 min, 90–95% B; 18–19 min, 95% B; 19–19.5 min, 95–5% B; 19.5–20 min, 5% B. To

maximise metabolite identification, mass data were scanned from *m/z* 100 to 1000 using both negative and positive ESI modes. Optimised MS detection included the following parameters: ion source temperature of 140°C, nitrogen desolvation gas temperature of 400°C and 800 l h^{-1} flow rate, 50 l h^{-1} cone gas flow rate, 0.15 ml min^{-1} collision gas flow rate, capillary voltage of 3 kV for the positive mode and 2.5 kV for the negative mode, cone voltage of 30 V, ion guide at 1 V, nebuliser pressure at 40 psi, etc. Two functions were performed in MSE mode, one at 6 eV and a second at 20–60 eV ramp to induce fragmentation. Mass calibration was performed using leucine–enkephalin (ESI+: *m/z* 556.2771, ESI−: *m/z* 554.2615) as the locked mass solution to ensure accuracy and reproducibility.

Raw liquid chromatography/mass spectrometry (LC/MS) data were processed using MassLynx™ v4.1 workstation software (Waters Corp.) for metabolic fingerprint profile analysis, including peak detection, noise removal, filtering and alignment to generate a data matrix that composed of retention time (tR), *m/z* value and normalised ion intensity for each peak area.

2.8 | S1P assay

UHPLC analysis was performed using a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Germering, Germany). Acquity UPLC BEH-C18 column (2.1 mm × 100 mm, 1.7 µm, Waters, Milford, USA) was used for all analyses. A linear gradient elution was performed with 0.1% formic acid/water (*v/v*, solvent A) and acetonitrile (solvent B) at a flow rate of 0.4 ml min^{-1}. Injection volume was 2 µl. Separation was carried out over 10 min under the following conditions: 0–2.5 min, 95%–65%A; 2.5–4 min, 65%–55%A; 4–7 min, 55%–35%A; 7–8 min, 35%–5%A; 8–8.5 min, 5%A; 8.5–9.5 min, 5%–95%A; and 9.5–10 min, 95%. Column temperature was maintained at 35°C. An LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, USA) equipped with an electrospray ionisation (ESI) source was used to acquire mass spectra in profile. Optimised operating parameters in the positive ion mode were as follows: spray voltage of 3.8 kV, nitrogen sheath and auxiliary gas with purity > 99.99%, helium collision gas with purity > 99.99%, sheath gas flow rate of 25 arbitrary units, auxiliary gas flow rate of 15 arbitrary units, ion source temperature of 350°C, and capillary temperature of 320°C. MS data were collected from *m/z* 100 to 1000 Da in centroid mode. Data acquisition and processing were performed using XcaliburTM v2.2 (Thermo Scientific, USA).

S1P concentration in the skins of atopic dermatitis patients and HCs were measured by S1P enzyme-linked immunosorbent assay (ELISA) kit (Mlbio, Shanghai, China) according to the manufacturer's instructions.

2.9 | Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA was used for cDNA synthesis, and reverse transcription was performed using a commercially available kit (Yeasen, Shanghai China). SYBR Green Master Mix (Yeasen) was used for qPCR. Primer sequences are listed in Table S2. Relative quantification for each mRNA was calculated by the $2^{-\Delta\Delta CT}$ method, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. All procedures were repeated three times.

2.10 | Western blot

Mice were anesthetized with isoflurane, then killed by cervical dislocation. Total protein from freshly dissected skin was isolated and purified using RIPA Lysis Buffer (Beyotime, Shanghai, China). Protein concentration was determined by BCA assay. Polyclonal **SPHK2** antibody (Cat# 17096-1-AP, RRID: AB_10598479, Proteintech, Wuhan, China) was used at 1 per 1000 dilution, and a monoclonal GAPDH antibody (Cat# ab8245, RRID: AB_2107448, Abcam, Shanghai, China) was used at 1 per 1000 dilution. Equal quantities of protein (60 µg per lane) were resolved on 12% SDS-polyacrylamide gels. Western blotting was performed as detailed previously (Hu et al., 2023). Membranes were incubated sequentially with primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. Results were quantified using Image J software (National Institutes of Health) and normalised to internal controls. All the procedures were repeated three times.

2.11 | Fluorescence imaging of skin section

Mice were anaesthetised with 1% sodium pentobarbital (50 mg kg^{-1}, i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4, 4°C) followed by 4% paraformaldehyde in phosphate buffer saline (PBS, pH 7.4, 4°C). TG and skin were dissected and cryoprotected in 30% sucrose at 4°C for 24 h. Skin was then embedded in an optimum cutting temperature compound (OCT, Leica, Wetzlar, Germany) and rapidly frozen at −20°C (CM1950, Leica). Cryoembedded skin was then cut into 10-µm thick slices using a sliding microtome (CM1950, Leica). Slices were incubated in blocking solution (10% fetal bovine serum in PBS containing 0.1% Triton X-100) for 30 min at room temperature, followed by primary antibody at 4°C overnight. Afterwards, tissue sections were washed with 0.1% PBST and incubated in secondary antibody (Beyotime, Shanghai, China) at room temperature for 2 h in the dark. Polyclonal SPHK2 antibody (17096-1-AP, Proteintech) was used at 1 per 500 dilution. After staining, sections were washed with 0.1% PBS and mounted in glycerol. All imaging was performed with an Olympus fluorescence microscope (BX51, Olympus Japan). Three mice from each group were analysed.

2.12 | Data analysis

Data and statistical analysis complied with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022). Simple randomisation was used to select animals and to assign testing groups. Statistical analysis

was conducted only for assays that had at least five independent experiments for each group. **Each independent experiment is defined as the use of a different mouse, not technical replicates.** All data is presented as the mean ± SEM. Unpaired Student's *t* test (two-tailed) or Mann–Whitney *U* test with Welch's correction was used for comparison between two groups, based on the distribution of data. One-way analysis of variance (ANOVA) was used for the comparisons among multiple groups. **Post-hoc tests were run only if F achieved P<0.05 and there was no significant variance inhomogeneity.** All statistical analysis was performed using GraphPad Prism 8 software. $P < 0.05$ was considered statistically significant, with 95% CI.

2.13 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/23 (Alexander, Christopoulos et al., 2023; Alexander, Fabbro et al., 2023; Alexander, Mathie et al., 2023).

3 | RESULTS

3.1 | Oxazone induced chronic dermatitis (CD) model produced intense pain- and itch-related behavioural responses in mice

Oxazone (OXA) is a small molecule hapten that is commonly used to generate contact hypersensitivity (CHS) in mouse models. In both the OXA induced allergic and non-allergic CD models, animals exhibit significant pain- and itch-related behaviours. A schematic describing a mouse cheek model of OXA-induced CD used for this current study is shown in Figure 1a. After four OXA challenges, CD model mice exhibited intense scratching (179 ± 11.7 OXA vs. 7 ± 2.5 Control) and wiping (200 ± 24.7 OXA vs. 11 ± 2.3 Control) directed at the treated cheek skin (Figure 1b,c). In addition, the CD model resulted in severe skin damage (Figure 1d,e) and epidermal hyperplasia (Figure 1f,g).

3.2 | Unbiased metabolomic analysis revealed metabolic changes in the lesional skin of chronic dermatitis (CD) model mice

Since significant itch- and pain-related behaviours were associated with the OXA-induced CD model, we performed unbiased metabolomic analysis to identify endogenous itch and pain inducing compounds that are produced under CD conditions. Lysate from the lesional skin of CD model mice was generated and analysed using an UHPLC-LTQ-Orbitrap MS system. Significant differences were found between control skin and CD skin. Metabolites were considered differentially abundant if the variable projection importance (VIP) value in orthogonal partial least squares discriminant analysis (OPLS-DA) was > 1 and *t*-test *P*-value < 0.05. Differentially abundant metabolites were then identified by database search, using the Human Metabolome Database (HMDB: https://hmdb.ca/) was well as other databases, and their related metabolic pathways were analysed using Metabo analyst 5.0 (https://www.metaboanalyst.ca/) (Yu et al., 2024). In total, we identified 136 compounds that were significantly different between control skin lysate and CD model skin lysate after four OXA challenges. Among these compounds, 132 were increased and only four

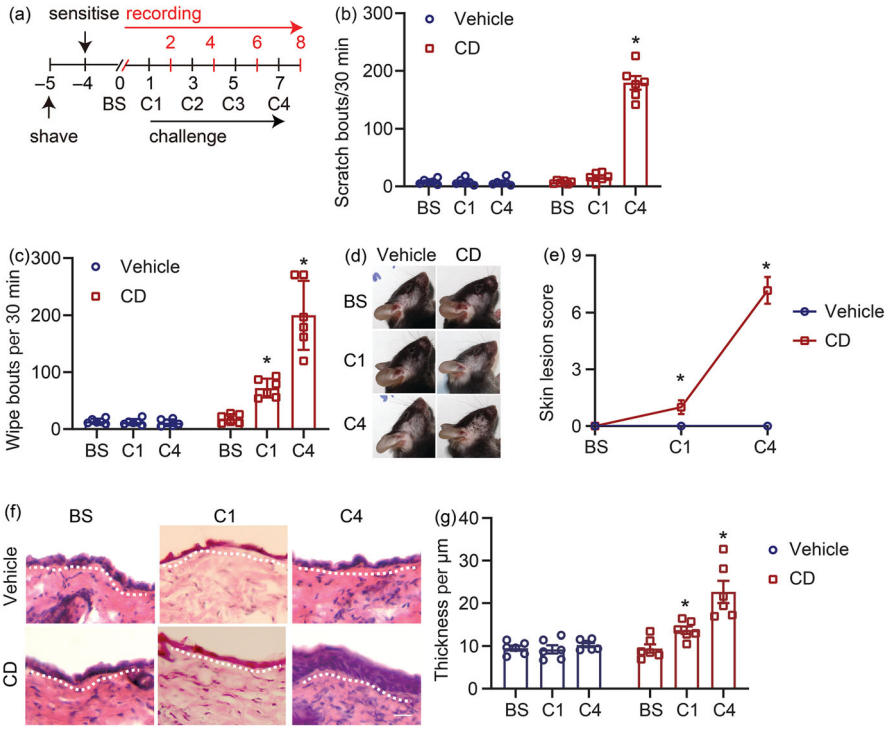

FIGURE 1 The OXA-induced chronic dermatitis (CD) model produced intense pain and itch related behavioural responses in mice. (a) Schematic describing the OXA-induced mouse CD model. (b) Scratching behaviour in the mouse cheek CD model ($n = 6$). (c) Wiping behaviour in the mouse cheek CD model ($n = 6$). (d, e) The CD model resulted in significant skin damage ($n = 6$). (f, g) H&E staining of lesional skin of CD model mice, showing epidermal hyperplasia and inflammatory cell infiltration ($n = 6$). BS: baseline; C1: after the first challenge; C4: after the fourth challenge. *$P < 0.05$. The scale bar represents 50 µm. All data are presented as mean ± SEM.

were decreased (Figure 2a,b and Table S3). Metabolic pathways showing the most significant changes were related to sphingolipid and glycerophospholipid (Figure 2c and Table S4). Compound enrichment analysis was consistent with the pathway analysis findings, and CD was associated with significantly increased abundance of sphingolipids in the skin (Figure 2d and Table S5). Similar differences were observed in lesional CD model skin after two OXA challenges (Figure S1a,b).

3.3 | S1P concentration is significantly increased in lesional chronic dermatitis (CD) model skin

Since the most prominent differences identified by metabolomic analysis were in sphingolipids, we specifically examined S1P availability in the skin using the Waters ACQMITY UPLC system. S1P is a bioactive sphingolipid that was previously reported to induce both pain and itch (Hill et al., 2018). S1P compound (860492P, Sigma-Aldrich,St. Louis, USA) was used as a marker to determine retention time ($t_R = 6.95$ min) and precursor ion ($m/z = 81.899$) in our LC–MS system (Figure S2a). S1P MS spectrum is shown in Figure S2b. Moreover, a standard curve of S1P concentration was generated (Figure S2c,d). While S1P was found in both control skin and lesional skin of CD model mice (Figure 3a,b), S1P concentration was significantly increased in CD model skin (Figure 3c). Significantly, increased S1P was also found after only two OXA challenges (Figure S3).

3.4 | S1P-induced pain and itch is partly TRPV4 dependent

To confirm that S1P is a pain or itch mediator in the OXA-induced CD model, S1P was injected intradermally into cheeks of naïve mice. Remarkably, S1P induced both pain-related wiping and itch-related scratching behaviour in a dose-dependent manner (Figure 4a,b). To mimic cowhage spiculae, a microneedle was used to apply S1P that can be inserted into superficial skin layers, and compared to normal needle injection the scratching number was increased from 13 ± 1.8 to 18 ± 2.9, although there are no significant difference, and there are also no significant differences in pain behaviour (Figure S4). Calcium imaging of cultured TG neurons further confirmed that S1P directly activated small-diameter neurons, with an EC_{50} of 360.2 nM (Figure 4c). S1P-induced calcium influx and neuronal response were eliminated when the experiment was repeated using calcium-free extracellular buffer (Figure 4d,e). To confirm that the S1P responsive neurons innervate OXA-treated cheek skin, the dye dil was injected into OXA-treated skin to label TG neurons. Subsequent immunostaining of TG cell bodies showed that dil labelled-TG neurons express S1PR3 (Figure S5).

A previous study reported that S1P induce itch and pain sensations through TRPA1 and TRPV1, respectively (Hill et al., 2018). However, using TG neurons from *Trpv1*$^{-/-}$/*Trpa1*$^{-/-}$ mice, ~ 9% of neurons remain responsive to S1P (Figure S6a,b). In addition to TRPV1 and TRPA1, TRPV4 is another important ion channel involved

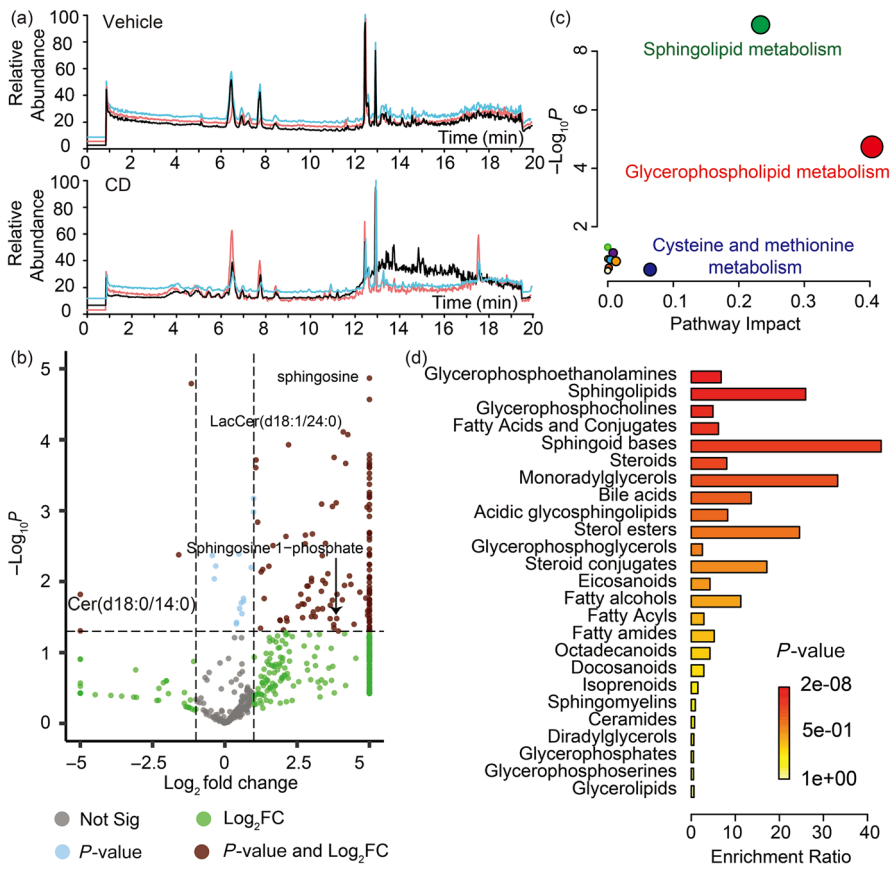

FIGURE 2 Unbiased metabolomic analysis revealed metabolic changes in the lesional skin of chronic dermatitis (CD) model mice. (a) Total ion chromatograms of skin lysates generated from control and CD model mice. (b) Volcano plot showing differentially abundant metabolites in lesional CD model skin, according to the $-\log_{10}$ (*P*-value). (c) Pathway analysis of differentially abundant compounds. (d) Enrichment analysis of differentially abundant compounds.

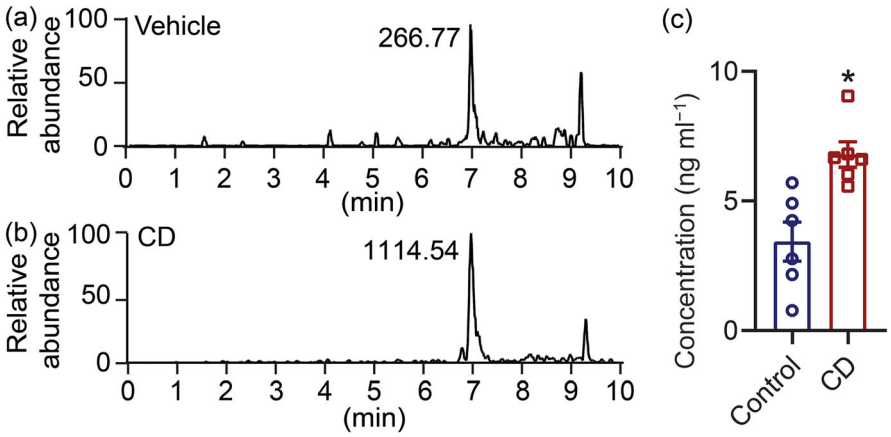

FIGURE 3 **The** S1P concentration is significantly increased in chronic dermatitis (CD) model skin. (a, b) Extract ion chromatograms of S1P from control and CD model skin. (c) Quantification of S1P concentrations in control and CD model skin ($n = 6$). *$P < 0.05$. All data are presented as mean ± SEM.

in itch and pain signalling (Kim et al., 2016; Yan et al., 2021). Remarkably, S1P responsiveness was significantly decreased in cultured TG neurons from *Trpv4*$^{-/-}$ mice (26 ± 4.6 WT vs. 15 ± 2.4 Trpv4$^{-/-}$) (Figure 4f,g). S1P-induced response amplitude was also significantly decreased in neurons from *Trpv4*$^{-/-}$ mice (Figure 4h). Furthermore, the proportion of S1P responsive TG neurons was significantly reduced after pretreatment with the TRPV4 antagonist GSK2193874 (GSK219) (Figure S7). Importantly, S1P-induced wiping and scratching behaviours were both significantly decreased in *Trpv4*$^{-/-}$ mice (Figure 4i).

3.5 | SPHK2 expression was significantly increased in the chronic dermatitis (CD) model

Since S1P availability in the skin is dependent on the expression levels of several key enzymes that control its synthesis and decomposition, we examined the expression levels of ***sphingosine kinase*** *1 (Sphk1)*, *sphingosine kinase 2 (Sphk2)*, *spinster homologue 2* (*Spns2*), *Abcc1*, *sphingosine phosphate lyase 1 (Sgpl1)* and *sphingosine-1-phosphate phosphatase 2* (*Sgpp2)* in the skin of control and CD-model mice by quantitative real-time polymerase chain reaction (qPCR). Among the tested genes, *Sphk2* showed the most significant increase in CD model skin (Figures 5a and S8). Correlating well with this result, western blotting (Figure 5b,c) and immunofluorescence staining (Figure 5d,e) confirmed that SPHK2 expression was significantly increased in CD model skin.

3.6 | Pain and itch associated with the OXA-induced chronic dermatitis (CD) model is dependent on S1P synthesis

Thus far, our data have shown that both S1P and SPHK2, a key enzyme controlling S1P synthesis, are significantly increased in the lesional skin of CD model mice. To confirm the contribution of S1P to CD associated pain and itch, CD model mice were treated with vehicle control or ABC294640 (ABC), a SPHK2 inhibitor, before each OXA challenge. Compared to vehicle-treated CD model skin, S1P abundance in ABC-treated CD skin was reduced by approximately 50% (Figure 6a,b). In addition, ABC treatment significantly attenuated CD-associated skin barrier damage and the clinical severity of skin lesions (Figure 6c,d). Furthermore, ABC treatment also blocked epidermal thickening of CD model skin (Figure 6e,f). Importantly, ABC treatment significantly decreased CD-associated wiping (166 ± 15.9 vehicle vs. 107 ± 17.7 ABC) and scratching (159 ± 11.9 vehicle vs. 83 ± 13.7 ABC) (Figure 6g).

3.7 | SPHK2 expression and S1P abundance is increased in human lesional chronic dermatitis (CD) skin

To confirm the clinical relevance of CD-associated S1P upregulation and chronic itch and pain, we examined if SPHK2 expression is also increased in human CD skin. Immunofluorescence staining of skin biopsies from CD patients revealed enhancement of SPHK2 expression in lesional CD skin, compared to skin from HC donors (Figure 7a,b). Consistent with this observation, western blots also showed increased SPHK2 expression in lesional CD skin (Figure 7c,d). Finally, S1P concentration in lesional CD and HC skin was measured by ELISA. Consistent with our findings using CD model mice, S1P concentration was increased in lesional CD skin (Figure 7e). As n=4 for these experiments, statistical analysis was not carried out, and results should be regarded as preliminary.

4 | DISCUSSION

Pain and itch are two fundamental somatosensory modalities that are initiated and mediated by primary sensory neurons of the DRG and TG. The OXA-induced CD model is commonly used to induce concurrent pain and itch in mice (Li et al., 2022). In this study, we utilised this model to identify endogenous pain and itch mediators associated with CD. Our results specifically implicated sphingolipid metabolites and demonstrated that S1P is significantly increased in lesional CD skin of

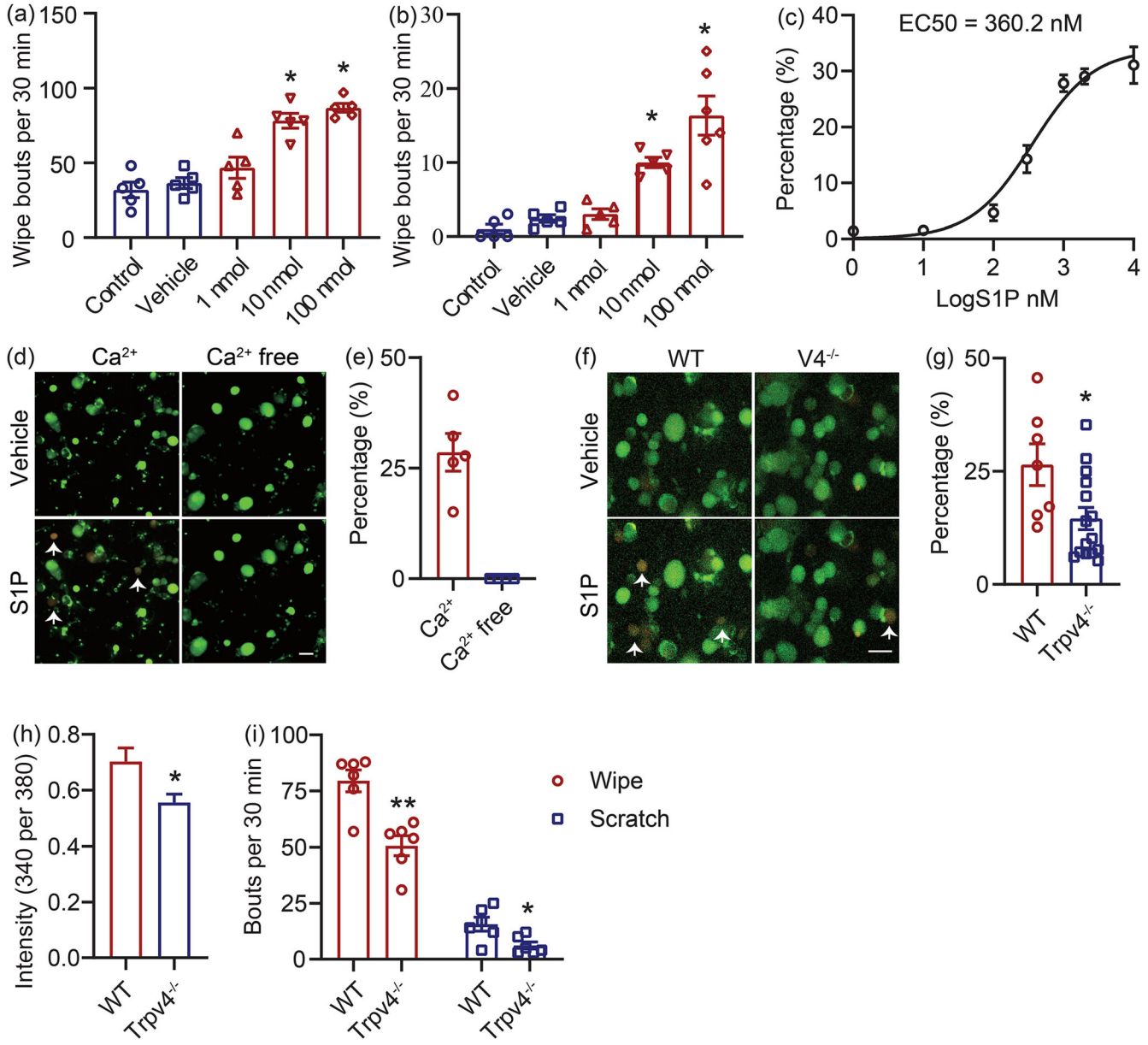

FIGURE 4 S1P-induced pain and itch is partly TRPV4 dependent. (a) S1P cheek injection induced wiping in mice ($n = 5$). (The control group received no injection, and the vehicle group was injected with vehicle at the cheek). (b) S1P cheek injection induced scratching ($n = 5$; 100 nmol, $n = 6$). (c) S1P dose dependently induced calcium influx in cultured trigeminal ganglion (TG) neurons. Calculated EC_{50} was 360.2 nM. (d, e) S1P-induced calcium influx in cultured TG neurons was dependent on extracellular calcium (arrows indicate S1P responsive neurons). (f) Representative stills of S1P responses in cultured TG neurons from WT and *Trpv4*$^{-/-}$ mice (arrows indicate S1P responsive neurons). (g) The percentage S1P responsive TG neurons from WT and *Trpv4*$^{-/-}$ mice. (h) S1P-induced calcium response amplitudes in TG neurons from WT and *Trpv4*$^{-/-}$ mice. (i) S1P (10 nmol)-induced wiping and scratching in WT and *Trpv4*$^{-/-}$ mice ($n = 6$). *$P < 0.05$. The scale bar represents 50 μm. All data are presented as mean ± SEM.

both mice and humans. Inhibition of SPHK2, the key enzyme controlling S1P synthesis, effectively blocked S1P elevation and alleviated CD-associated pain and itch in mice.

Sphingolipids are necessary for the maintenance of the epidermal barrier. Sphingolipids, namely ceramides (CER) and S1P, are signalling molecules that regulate cell growth, apoptosis and immune responses (Borodzicz et al., 2016). One endogenous sphingolipid, glucosylsphingosine (GS), was reported to be highly elevated in the epidermis of patients with atopic dermatitis, due to abnormal ceramide metabolism, and evoked itch in mouse through activating 5-HT_{2A} receptor and TRPV4 (Sanjel et al., 2022). In addition, CER was reported to be reduced and S1P was significantly increased in the total serum of psoriatic patients (Mysliwiec et al., 2017). S1P was further reported to induce pain and itch, through TRPV1 and TRPA1, respectively, in mice (Hill et al., 2018). Long-term abnormalities in S1P signalling was also implicated as a biomarker for previous disseminated viral infections,

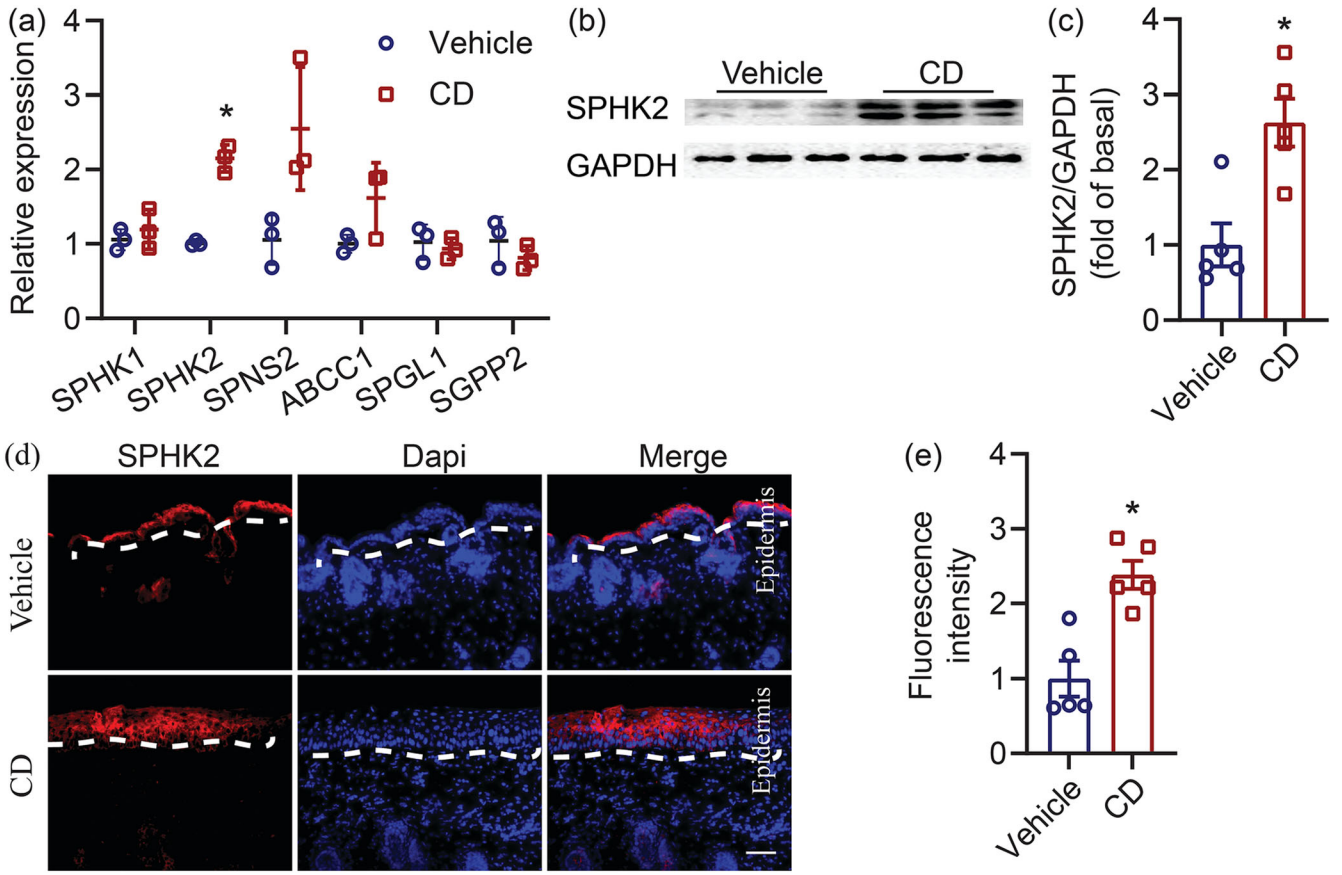

FIGURE 5 SPHK2 expression was significantly increased in chronic dermatitis (CD) model skin. (a) The expressions of key enzymes that control S1P synthesis and decomposition were examined by q-PCR. *Sphk2* and *Spns2* was significantly increased in lesional CD model skin. As n=3 for Spns2 experiments, statistical analysis was not carried out, and results should be regarded as preliminary. (b) Representative western blot bands of SPHK2. (c) Quantification of western blot band intensity showed that SPHK2 expression was increased by approximately threefold in lesional CD model skin, compared with control skin ($n = 5$). (d) Immunostaining of SPHK2 in control and CD model skin. (e) Quantification of $SPHK2^{+}$ fluorescence signal intensity in control and CD model skin ($n = 5$). The scale bar represents 50 μm. $^{*}P < 0.05$. All data are presented as mean ± SEM.

and targeting S1P signalling may provide therapeutic benefits for recurring infections (Berdyshev et al., 2022).

In our current study, we used unbiased metabolomic analysis to examine the lesional skin of CD model mice. The most prominent changes were concentrated in 10 metabolic pathways. Among these, sphingolipid metabolism and glycerophospholipid metabolism showed the most significant differences between control and CD model skin. S1P was identified by our unbiased metabolomic analysis, and S1P concentration in the CD model skin was further measured using a Waters ACQMITY UPLC system. Our results show that, after two and four OXA challenges, S1P abundance was significantly increased in lesional CD model skin and S1P, an endogenous S1PR3 ligand, induced both pain and itch when injected into mice.

Among the enzymes that control S1P synthesis, SPHK2 was persistently overexpressed in the skin of CD model mice. To test if elevated SPHK2-S1P pathway drives increased pain and itch in CD, we treated CD model mice with ABC294640, a potent and selective SPHK2 inhibitor. ABC treatment decreased both SIP skin concentration and pain and itch behaviour in the CD model mice by ~50%. Examination of human skin confirmed that, compared to health control skin, SPHK2 and S1P were also significantly elevated in lesional CD skin. This finding strongly suggests that elevated S1P contributes to CD-associated chronic pain and itch in patients.

It has been reported that S1P induce pain and itch through TRPV1 and TRPA1, respectively (Hill et al., 2018), but may also involve other ion channels (Kittaka et al., 2020). We tested S1P response in cultured TG neurons from $Trpa1^{-/-}/Trpv1^{-/-}$ double knockout mice. Although S1P responsiveness was significantly reduced when compared with TG neurons from wild-type mice, a significant proportion of $Trpa1^{-/-}/Trpv1^{-/-}$ neurons, ~9%, still responded to S1P. In addition to TRPV1 and TRPA1, TRPV4 is another crucial mediator of pain and itch signals. TRPV4 expressed by keratinocytes functions as a pruriceptor during histaminergic and non-histaminergic itch (Chen et al., 2016, 2021; Zhang et al., 2022). Moreover, TRPV4 is required for chronic itch associated with psoriasis, dry skin and atopic dermatitis (Luo et al., 2018; Yan et al., 2021). Indeed, the proportion of S1P responsive TG neurons from $Trpv4^{-/-}$ mice was significantly decreased compared to those from WT mice, and

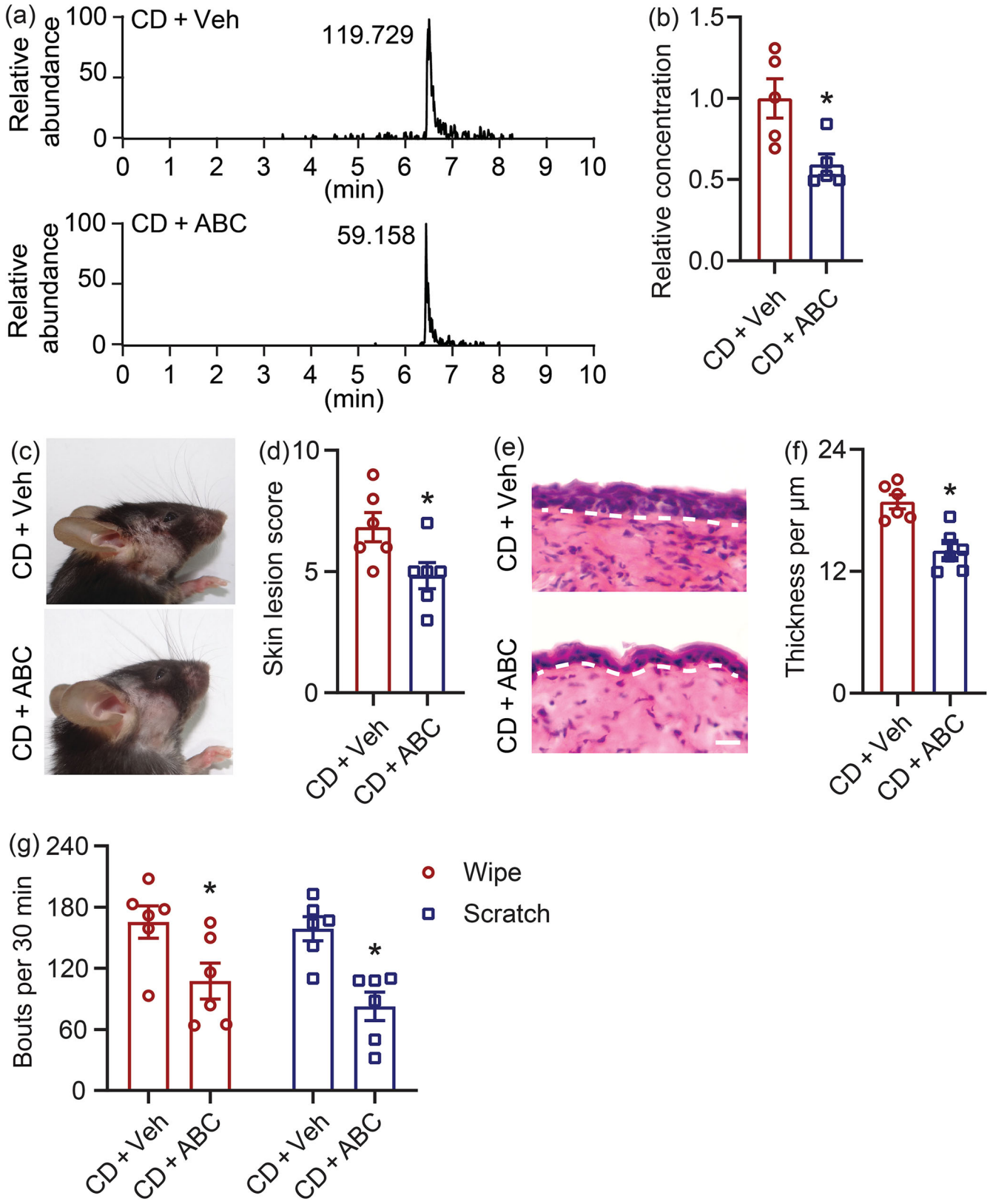

FIGURE 6 Pain and itch associated with the OXA-induced chronic dermatitis (CD) model is dependent on S1P synthesis. (a) Extract ion chromatograms of S1P in vehicle and ABC treated CD model skin. (b) Normalised S1P concentration in vehicle and ABC-treated CD model skin ($n = 5$). (c, d) Quantified skin lesion score of vehicle and ABC-treated CD model mice ($n = 6$). (e) H&E staining of vehicle and ABC-treated CD model skin. (f) The epidermal thickness of vehicle and ABC-treated CD model skin ($n = 6$). (g) Scratching and wiping behaviour of vehicle and ABC-treated CD model mice ($n = 6$). Scale bars represents 50 μm. *$P < 0.05$. All data are presented as mean ± SEM.

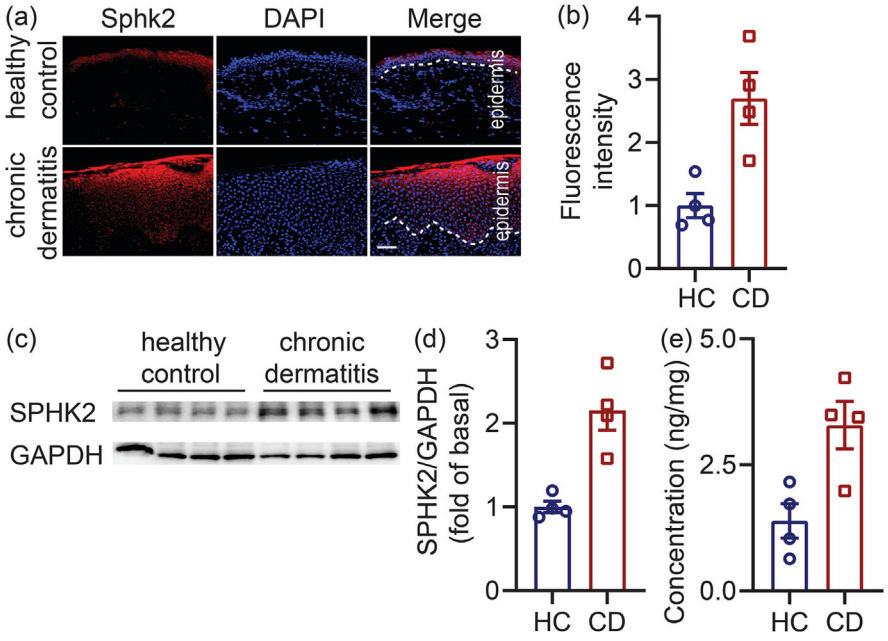

FIGURE 7 SPHK2 expression and S1P abundance are increased in human lesional chronic dermatitis (CD) skin.
(a) Immunostaining of SPHK2 in the skin of HC and CD patients. (b) Normalised SPHK2^{+} signal intensity in the skin of HC and CD patients ($n = 4$). (c) Representative western blot bands of SPHK2. (d) Normalised SPHK2 expression in the skin of HC and CD patients ($n = 4$). (e) S1P concentration in the skin of HC and CD patients ($n = 4$). The scale bar represents 50 μm. All data are presented as mean ± SEM. As n=4 for these experiments, statistical analysis was not carried out, and results should be regarded as preliminary.

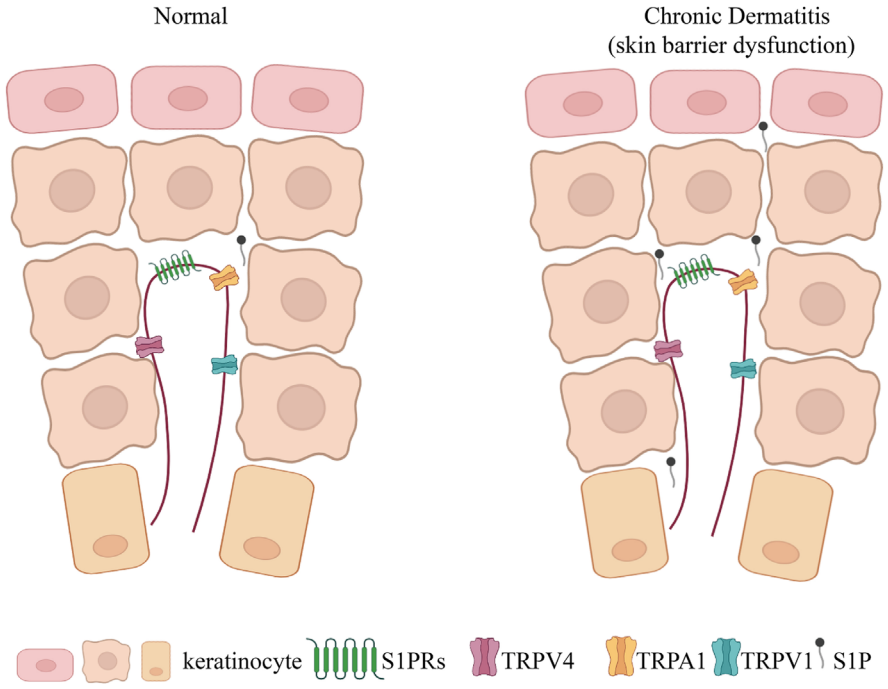

FIGURE 8 The S1P/S1PRs–TRPV4 axis is a novel therapeutic target for persistent pain and itch in chronic dermatitis (CD).

GSK219 pretreatment also significantly decreased the percentage of the responsive neurons, which indicated that about 40% of the TG neuron response to S1P was dependent on the expression of TRPV4. S1P-induced pain and itch was also significantly reduced in the *Trpv4*$^{-/-}$ mice. Our data demonstrate that TRPV4 is another important mediator of S1P-induced pain and itch and implicates this TRP channel in S1P-induced pain and itch signalling in CD.

There are five known S1P receptors (S1PRs) with distributed expression across multiple cell types. Inhibition of S1PR2 not only ameliorates the severity of DNCB-induced atopic dermatitis symptoms but also the Th2 cell-attracting capacity of dendritic cells in this model—suggesting that S1PR2 is a potential therapeutic target for atopic dermatitis (Kang et al., 2020; Park & Im, 2020). S1PR1 facilitates the migration, recruitment and activation of immune cells. S1PR1 and S1PR3 are both reported to be expressed by somatosensory ganglia (Hill et al., 2018), and S1P-induced nociception is largely mediated by S1PR3 receptors in nociceptors (Camprubi-Robles et al., 2013). Our data demonstrate that S1P abundance is significantly elevated in lesional CD skin of both mice and humans. We speculated that S1P directly evokes pain and itch through neuronal S1PR3, and S1P-induced pain and itch signals are propagated by neuronal TRPV4 channels. Although our results also showed that S1PR3 are co-localise

with dil labelled neurons that innervating oxazolone-treated skin, according to our current result, it is difficult to rule out the influence of other S1PR3. At the same time, due to the broad expression of TRPV4 across peripheral neurons, epidermal keratinocytes, fibroblasts, inflammatory cells including macrophages, mast cells and T-cells, and so on, future studies using TG-specific *S1PR3* and *Trpv4* conditional knockout mice are required to confirm our hypothesis.

Taken together, our results demonstrate that the S1P/S1PRs–TRPV4 signalling pathway is required for CD-associated chronic pain and chronic itch. Pharmacological blockade of this pathway may provide substantial pain and itch relief for patients with CD (Figure 8).

AUTHOR CONTRIBUTIONS

Yuan Zhou, Xinyu Zhang, Mingxin Qi, Xue Li, Jiahui Ren, Yin Wang and Weimeng Feng performed the experiments; Yuxiang Ma, Changming Wang and Xinyu Zhang analysed the results. Yuxiang Ma, Changming Wang, Yan Yang, Chan Zhu, Pei Liu, Fang Wang and Zongxiang Tang provided technical assistance. Guang Yu and Xinyu Zhang wrote the manuscript. Guang Yu designed the study. Guang Yu and Zongxiang Tang supervised the entire project. All authors approved the paper as submitted.

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CONFLICT OF INTEREST STATEMENT

The authors have declared that no conflict of interest exists.

DATA AVAILABILITY STATEMENT

All data are available in the main text or in the Supporting Information.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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