Demodex mites induce TRPV1–NGF–TrkA pathway–mediated cutaneous neurogenic inflammation in rosacea

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Demodex mites induce TRPV1–NGF–TrkA pathway–mediated cutaneous neurogenic inflammation in rosacea

Directed by Professor Ju Hee Lee

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ABSTRACT

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Background: Rosacea is a common chronic inflammatory skin condition that is often refractory to treatment, with frequent relapses. Alterations in the skin immunological response and Demodex mite infestation are the primary etiologic factors targeted for treatment. Transient receptor potential cation channel subfamily V member 1 (TRPV1) is a nociceptive cation channel that plays a role in cutaneous neurogenic pain and can be activated by various rosacea triggers.

Objectives: We investigated the effects of TRPV1 modulation in rosacea, focusing on Demodex mite colonization and cutaneous neurogenic inflammation.

Methods: We examined mRNA expression levels according to Demodex population counts. An in vitro study using capsazepine as a TRPV1 antagonist was performed to assess

the influence of TRPV1 in keratinocytes. A rosacea-like mouse model was generated by the injection of the 37-amino acid C-terminal cathelicidin peptide (LL37), and changes in the skin, dorsal root ganglion (DRG), and ears were examined.

Results: Increased Demodex mite population counts were associated with increased expression levels of TRPV1, tropomyosin receptor kinase A (TrkA), and nerve growth factor (NGF), and these levels could be reduced by capsazepine treatment in keratinocytes. In an in vivo study, the downstream effects of TRPV1 activation were investigated in the skin, DRG, blood, and ears of the rosacea-like mouse model.

Conclusions: The findings of this study are instrumental for understanding the underlying causes of rosacea and could potentially lead to the development of new treatments targeting the NGF–TrkA–TRPV1 pathway. The identification of this pathway as a therapeutic target could represent a major breakthrough for rosacea research, potentially resulting in more effective and targeted rosacea treatments. This study contributes to an improved understanding of rosacea pathophysiology, which may lead to the development of more effective treatments in the future.

Key words : rosacea, trpv1, cutaneous neurogenic inflammation, ngf, demodex

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I. INTRODUCTION

Rosacea is a chronic, inflammatory condition that is often accompanied by a stinging or burning sensation and pruritus, which significantly impacts the quality of life.[1,2] Rosacea is often treatment-resistant; can frequently be aggravated by physical stimuli during daily activities, such as heat and sun exposure; and is representative of a family of skin diseases that present with a sensitive skin phenotype and are characterized by barrier disruption.[3,4]

Demodex mites are naturally found in human pilosebaceous follicles and do not display any beneficial or detrimental effects for human skin at low population counts.[5] However, larger *Demodex* mite populations can cause demodicosis, which presents with symptoms such as redness, irritation, itching, and inflammation of the skin.[6] In severe

cases, *Demodex* mites can cause skin diseases, including rosacea. *Demodex* mites interact with Toll-like receptor 2 (TLR2) and kallikrein-related peptidase 5 (KLK5) on skin cells, which increases the production of the 37-amino acid C-terminal cathelicidin antimicrobial peptide (LL37)[7], an important factor in the innate immune response. Increased LL37 production is thought to contribute to rosacea pathogenesis by inducing inflammation, promoting angiogenesis, and activating the innate immune system.[7]

Transient receptor potential cation channel subfamily V member 1 (TRPV1) is a receptor expressed on the surface of skin cells that responds to various external stimuli, including heat, cold, and certain chemicals.[8] TRPV1 is also expressed in other cell types, including neuronal cells, in which TRPV1 is involved in nociception, and keratinocytes.[9-11] In addition to inflammatory conditions, capsaicin in spicy foods can activate TRPV1 and aggravate rosacea, triggering the burning sensation that is a primary rosacea symptom.[4, 12] TRPV1 interacts with neuroinflammatory factors, such as nerve growth factor (NGF) and tropomyosin kinase receptor A (TrkA)[13], suggesting that TRPV1 may be involved in cutaneous neurogenic inflammation associated with rosacea.

To better understand rosacea development, we explored the relationship between rosacea onset and progression and the presence of *Demodex* mites. We also investigated the relationship between TRPV1 and rosacea to determine whether TRPV1 represents a novel therapeutic target for cutaneous neurogenic inflammation associated with rosacea.

II. MATERIALS AND METHODS

1. Thumbnail-squeezing method

The Demodex population was counted using the previously published thumbnail-squeezing method.14 In brief, the designated area of skin was coated in mineral oil and squeezed, and the obtained contents were placed on a slide (n = 38). The protocol was approved by the Institutional Review Board of Yongin Severance Hospital (IRB protocol no. 9-2021-0008). Slides were covered with cover glass, and the Demodex mite population was counted by viewing the slides under a microscope (x100). Samples were grouped according to the Demodex population counts as follows: 0 mites (n = 12), 1–9 mites (n = 16), and >10 mites (n = 10). The slide contents were then dissolved in Trizol reagent (Invitrogen, Waltham, MA, USA), scraped, and transferred to a microcentrifuge tube.

2. Rosacea mouse model

The female BALB/c mice were purchased from ORIENT BIO Animal Center (Seongnam, Korea). All experiments used 15-18 week-old mice and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The experiment protocols were approved by the Animal Care and Used Committee (IACUC) at the Yonsei University College of Medicine (IACUC_2022-0142). According to the protocol referred to in the previous study, LL37 was injected intradermally to induce the rosacea mouse model 15. The brief experiment scheme was shown in Figure 2A. To shortly explain, 24 hr before the LL37 treatment, the mice were shaved and injected with 40 μl of 320μM LL37 on the back skin for two days twice a day. The mice's ears on day

3 were topically rubbed with capsaicin. After a topical rub on mice ears, the ears were imaged and the mice were euthanized within 30 minutes for subsequent analysis. The skin biopsy specimens were for histological analysis, western blot, real-time quantitative PCR (RT-qPCR), immunohistochemistry, and immunofluorescence; DRG biopsy specimens were for western blot and RT-qPCR; Ear biopsy specimens were for histological analysis, RT-qPCR, and immunohistochemistry; serum samples were for ELISA.

3. Use of biopsy specimens

Skin biopsy specimens were obtained for histological, western blotting, reverse-transcriptase–quantitative polymerase chain reaction (RT-qPCR), immunohistochemical, and immunofluorescence analyses. Dorsal root ganglion (DRG) biopsy specimens were obtained for western blotting and RT-qPCR analyses. Ear biopsy specimens were obtained for histological, RT-qPCR, and immunohistochemical analyses. Serum samples were obtained for enzyme-linked immunosorbent assay (ELISA). The histology protocol is described in detail in Supplementary Materials and Methods.

4. Hematoxylin and Eosin (H&E) Staining.

The tissue samples obtained from the mice's skin and ears were fixed with 4% formalin for 24 hr and embedded in paraffin to make a paraffin block. According to the manufacturer's protocol, the samples were cut into sections of 4-μm thick on the slide and stained with H&E Stain Kit (ab245880, Abcam, Cambridge, Ma, USA). To confirm the histological features change, the stained sections were observed and images were obtained via the microscope (BX43, Olympus, Tokyo, Japan).

5. Immunohistochemistry

The tissue samples obtained from the mice's skin and ears were fixed with 4% formalin for 24 hr and embedded in paraffin to make a paraffin block. The samples were cut into sections (4-μm thick) and deparaffinized. For antigen retrieval, the slides were boiled in EnV FLEX TRS High pH 50X (K800421-2, DAKO, Santa Clara, CA, USA) or 10X citrate buffer (C9999, Sigma-Aldrich). The slides were incubated using 3% H2O2 solution for 10 min and blocked with Protein Block Serum-Free reagent (X0909, DAKO) for 2 hr. Afterward, the slides were incubated with anti-TrkA (1:300, 06-574, Merck, Rahway, NJ, USA), anti-TRPV1 (1:500, OSR00246W, Invitrogen), anti-NGF (1:100, MA5-32067, Invitrogen), anti-CD31 (1:100, 77699S, Cell signaling), and anti-c-kit (1:4000, ab256345, Abcam) at 4 ℃ overnight. The slides were covered with a DAKO peroxidase/DAB detection kit (K5007, DAKO) for detection and stained with Mayer's hematoxylin (s3309, DAKO) to observe the nuclei. All IHC images were obtained using the microscope (BX43) and staining intensity was quantified via ImageJ software. The staining intensity was calculated using the randomly chosen 3 images in each sample.

6. Immunofluorescence (IF)

The IF was conducted using the sectioned slides of the mice's skin embedded in a paraffin block. After antigen retrieval, the slides were blocked with PBS buffer containing 5% BSA and 0.3% TritonTM X-100. With the PBS-T (PBS containing TrionTM X-100; DAEJUNG, Busan, Korea), the slides were rinsed three times for 5 min, followed by incubation with anti-TRPV1 (1:1000, NB100-1617, Novus Biologicals, Englewood, CO, USA), anti-CD31 (1:100, 77699S, Cell signaling), and anti-PGP 9.5

(1:200, MA1-83428, Invitrogen) at 4 ℃ overnight. After rinsed with PBST three times for 5 min, the slides were incubated with the secondary antibodies (Goat anti-Rabbit IgG (H+L) Cross-adsorbed Secondary Antibody-Alexa Fluor™ 633, A-21070; Goat anti-Mouse IgG (H+L) Cross-adsorbed Secondary Antibody-Alexa Fluor™ 488, A-11001, Invitrogen) for 2 hr at room temperature to combine primary antibodies. Using the fixation solution (VECTASHIELD® mounting medium with 40,6-diamidino-2-phenylindole (DAPI); Vector Laboratories Inc., Burlingame, CA, USA), the slides were fixed. All fluorescence images were obtained via a laser-scanning microscope (LSM 700, Carl Zeiss, Jena, Germany), and fluorescence intensity was measured using ImageJ software. The staining intensity was calculated using the randomly chosen 3 images in each sample.

7. In vitro study for rosacea-liked model

Immortalized human keratinocytes (HaCaT cells) were grown in Dulbecco's modified Eagle medium (Lonza, Walkersville, MD, USA), supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin–streptomycin (Gibco), in a humidified atmosphere containing 5% carbon dioxide (CO2) at 37℃. HaCaT cells were seeded onto 6-well plates, grown to >80% confluence, and treated with LL37 (10 μg/ml) or capsazepine (CAZ, 1 μM, Abcam) for 24 h.

8. Total RNA isolation and RT-qPCR

From the content obtained by the thumbnail-squeezing method, the HaCaT cell treated by LL37 and/or CAZ, and mouse skin, ear, and DRG, the samples were homogenized through TissueLyser II (Qiagen, Hilden, Germany). The total RNA was

extracted via Trizol reagent according to the manufacturer's protocol. The extracted total RNA was quantified using NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Carlsbad, CA, USA) and performed reverse transcription with RNA to complementary DNA EcoDry Premix Kit (Takara Sake, Berkley, CA, USA). The RT-qPCR was performed using SYBR Green Master MIX (4309155, Promega Co., Madison, WI, USA) and specific primer pairs on the QuantStudio 3 Real-Time Polymerase Chain Reaction System (Applied Biosystems, Waltham, MA, USA). The used primer sequence pairs were shown in supplementary table 1. To normalize and calculate relative mRNA expression levels, GAPDH, and $2^{-\Delta\Delta Ct}$ method were used. All experiments were independently conducted at least three times.

Table 1. RT-qPCR Primer sequences

		Human
TLR2	F	CTTCACTCAGGAGCAGCAAGCA
	R	ACACCAGTGCTGTCCTGTGACA
KLK5	F	CGTCCCACTAAAGATGTCAGACC
	R	TCAAGCACTGGAGGACCTTAGG
LL37	F	TCGGATGCTAACCTCTACCG
	R	GGGTACAAGATTCCGCAAAA
TGF-β	F	GAGGGATCTAGGGTGGAAATGG
	R	AGGACCCTGCTGTGCTGAGT
TNF-α	F	CTCTTCTGCCTGCTGCACTTTG
	R	ATGGGCTACAGGCTTGTCACTC

IL-1β	F	CCACAGACCTTCCAGGAGAATG
	R	GTGCAGTTCAGTGATCGTACAGG
IL-6	F	AGACAGCCACTCACCTCTTCAG
	R	TTCTGCCAGTGCCTCTTTGCTG
IL-8	F	GACCACACTGCGCCAACAC
	R	CTTCTCCACAACCCTCTGCAC
TRPV1	F	ACTGCCATCATCACTGTCATCT
	R	CTTCACAGCCAACAGGTCTACCA
NGF	F	ACCCGCAACATTACTGTGGACC
	R	GACCTCGAAGTCCAGATCCTGA
TrkA	F	CACTAACAGCACATCTGGAGACC
	R	TGAGCACAAGGAGCAGCGTAGA
TRPV3	F	ATCCTACTGCGGAGTGGCAACT
	R	CGCTTCTCCTTGATCTCACGAC
TRPV4	F	TCACTCTCACCGCCTACTACCA
	R	CCCAGTGAAGAGCGTAATGACC
GAPDH	F	TGTTGCCATCAATGACCCCTT
	R	CTCCACGACGTACTCAGCG
		Mouse
Trpv1	F	CATCTTCACCACGGCTGCTTAC
	R	CAGACAGGATCTCTCCAGTGAC
Trka	F	GGTCTTTCTCGCTGAGTGCTAC
	R	GCTGAAAGTCCTGCCGAGCATT

Ngf	F	GTTTTGCCAAGGACGCAGCTTTC
	R	GTTCTGCCTGTACGCCGATCAA
Vegf	F	AAAGGCTTCAGTGTGGTCTGAGAG
	R	GGTTGGAACCGGCATCTTTATC
Trpv3	F	ATCCTGCTGAGGAGTGGCAACT
	R	TTGATCTCGCGGCTGAGGATGT
Trpv4	F	TCACCGCCTACTATCAGCCACT
	R	GAACAGGACTCCTGTGAAGAGC
Vip	F	GATGCCGTTTGAAGGAGCAGGT
	R	GAAGTCTGCTGTAATCGCTGGTG
Cgrp	F	GGACTTGGAGACAAACCACCA
	R	GAGAGCAACCAGAGAGGAACTACA
Gapdh	F	AGGTCGGTGTGAACGGATTTG
	R	TGTAGACCATGTAGTTGAGGTCA

Abbreviations: F, forward; R, reverse; RT-qPCR, reverse-transcriptase–quantitative polymerase chain reaction

9. Western blot

From the mouse skin and ear, the proteins were lysed using RIPA buffer contained with Halt™ Protease and Phosphatase Inhibitor Cocktail 100X (78440, ThermoFisher Scientific). The samples were chopped and homogenized with beads and TissueLyser II. Obtained proteins were quantified via bicinchoninic acid assay and the ELISA microplate reader (VersaMax; Molecular Devices, San Jose, CA, USA). A total of 30 μg/μL of

protein in each group was separated into 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk at room temperature for 1 hr and incubated at 4 ℃ overnight with anti-TrkA (1:1000, 06-574, Merck), anti-TRPV1 (1:500, ACC-030, Alomone, Jerusalem, Israel), anti-NGF (1:250, MA5-32067, Invitrogen), anti-PI3K p85 (1:1000, 4257S, Cell signaling), anti-p-PI3K p85 (1:1000, 4228S, Cell signaling), anti-Akt (1:1000, 4691S, Cell signaling), anti-p-Akt (1:2000, 4060S, Cell signaling), anti-VEGF (1:500, ab46154, Abcam), anti-CGRP (1:200, sc-57053, Santa Curz), anti-VIP (1:500, sc25347, Santa Curz), anti-Substance P (1:300, BS-0065R, Bioss), and anti-GAPDH (1:1000, 2118S, Cell signaling). Afterward, each membrane was washed 3 times for 5 minutes with TBST buffer and incubated with secondary antibodies (1:2000, Anti-mouse IgG, HRP-linked Antibody, 7076S; 1:2000, Anti-rabbit IgG, HRP-linked Antibody, 7074S, Cell signaling) at room temperature for 2 hr. The antigen-antibody complexes band were detected using Western Blotting Luminol Reagent (sc-2048, Santa Curz), visualized on the Amersham ImageQuant 800 (Cytiva, Marlborough, MA, USA), and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). All experiments were independently conducted at least three times.

10. ELISA

The serum samples separated from mice blood were used for ELISA. IL-4 (88-7044-22) and IL-5 (88-7054-22) ELISA kit for the mouse were purchased from Invitrogen and LL37 ELISA kit for the mouse was purchased from BT-Lab (E1739Mo, Birmingham, UK). All ELISA kits were performed according to the manufacturer's

instructions. All experiments were independently conducted at least three times.

11. Statistical analysis

Data are presented as the mean ± standard deviation. All statistical analyses were performed using SPSS statistical software version 25.0 (IBM Corp., Armonk, NY, USA). Unpaired Student's t-test was used for comparisons of two groups. One-way analysis of variance, followed by Tukey's multiple comparisons tests, was used for comparisons of multiple groups. Differences were considered significant at $p < 0.05$.

III. RESULTS

1.The images of Demodex mites and mRNA expression levels according to the Demodex mites population.

The images of patient-derived samples were shown in Figure 1A and were grouped according to *Demodex* population counts as follows: 0 mites, 1–9 mites, and >10 mites. After cDNA synthesis, cDNAs were combined for each group, and RT-qPCR was performed to examine mRNA expression levels (Fig. 1B and C). The mRNA expression levels of genes related to rosacea, such as *TRL2*, *KLK5*, and *LL37*, were significantly increased in groups with higher *Demodex* population counts than in the group with 0 mites (Fig. 1B, ***$p < 0.005$). And that depended on the *Demodex* population. The mRNA expression levels of *TRPV1*, *NGF*, and *TRKA* were markedly increased in groups with higher *Demodex* population counts than in the group with 0 mites (Fig. 1C, ***$p < 0.005$).

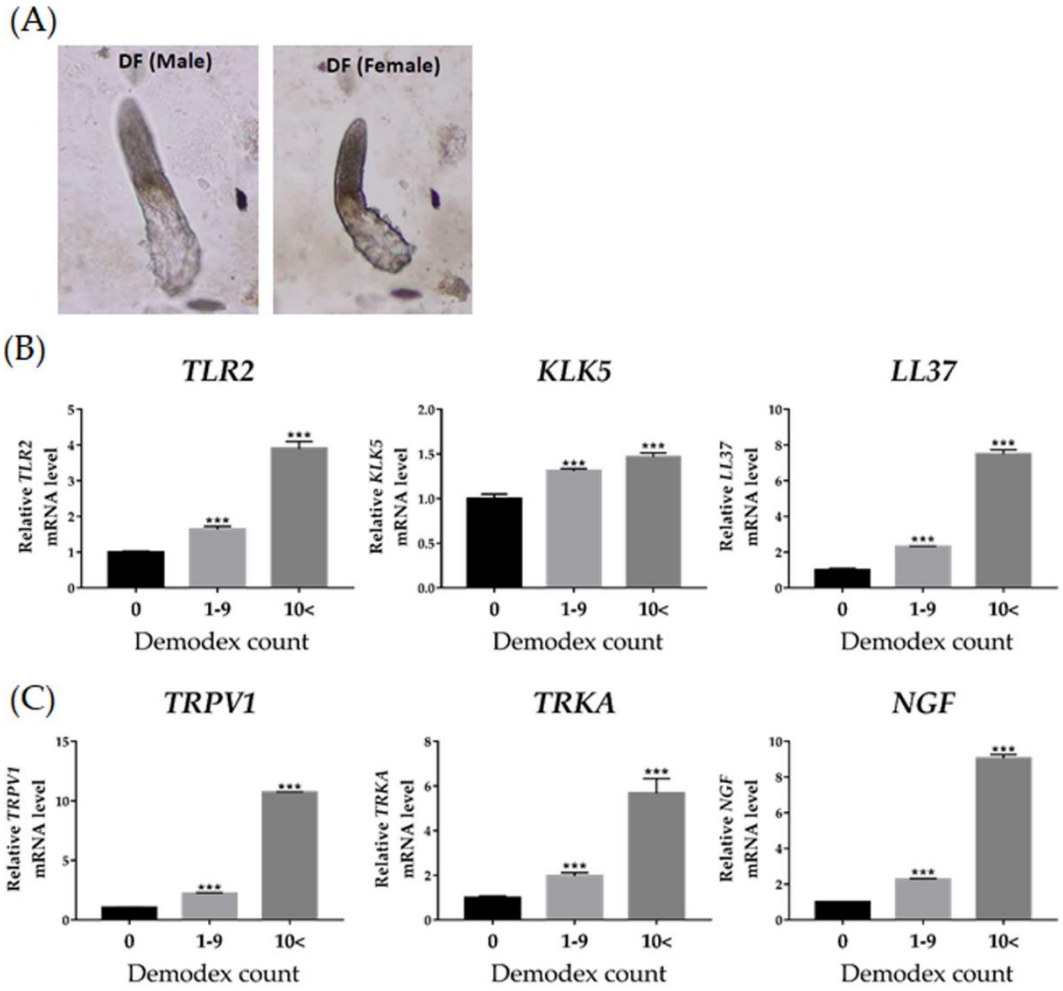

Figure 1. Images of *Demodex* mites obtained from patient samples and comparisons of mRNA expression levels according to *Demodex* population counts. (A) The images of Demodex mites were obtained using the microscope. (B and C) Groups were separated according to *Demodex* population counts. After cDNA synthesis, cDNAs were combined for each group. mRNA expression levels of genes related to rosacea (B) and cutaneous neurogenic inflammation (C) increased with increasing *Demodex* population counts: 0

mites (n = 12), 1–9 mites (n = 16), and >10 mites (n = 10). All experiments were independently repeated at least 3 times. ***$p < 0.005$. TLR2, Toll-like receptor 2; KLK5, kallikrein-5; LL37, 37-amino acid C-terminal cathelicidin peptide; TRPV1, transient receptor potential cation channel subfamily V member 1; TRKA, tropomyosin receptor kinase A; NGF, nerve growth factor.

2. Relative mRNA expression levels of LL37 and/or TRPV1 antagonist treatment groups on the HaCaT cells

To examine the effects of LL37-induced increases in TRPV1 expression in keratinocytes, HaCaT cells were treated with LL37 with and without the TRPV1 antagonist CAZ, and mRNA expression levels were examined via RT-qPCR (Fig. 2). The mRNA expression levels of *TRPV1*, *TRKA*, *NGF*, *IL-1β*, *IL-6*, and *LL37* were increased by LL37 treatment and significantly reduced by CAZ treatment ($p < 0.05$, Fig. 2). These results imply that TRPV1 activity is involved in the observed increases in TRKA and NGF mRNA expression induced by LL37 treatment in HaCaT cells and can be modulated through treatment with a TRPV1 antagonist.

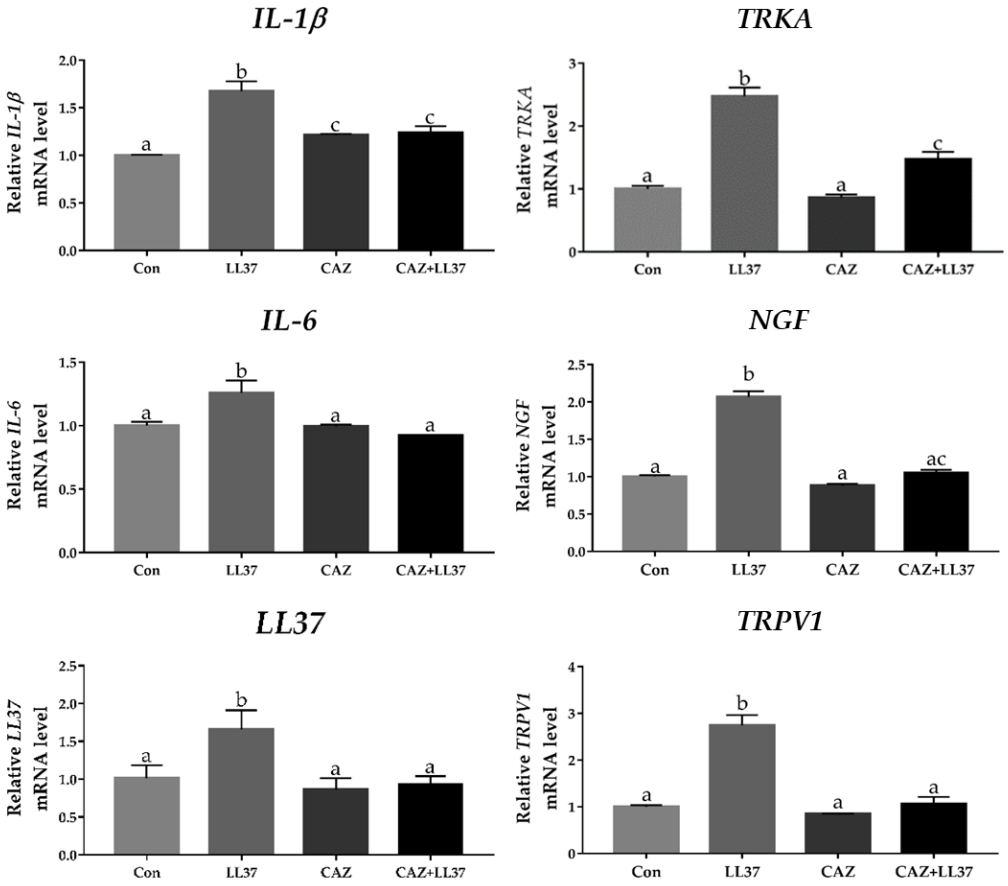

Figure 2. The effects of the TRPV1 antagonist capsazepine on mRNA expression levels in HaCaT cells. Treatment with capsazepine (CAZ) reduced mRNA expression levels induced by LL37 treatment in human keratinocyte HaCaT cells. All experiments were independently repeated at least 3 times. Values followed by the same small letter are not significantly different ($p < 0.05$). Con, control; LL37, 37-amino acid C-terminal cathelicidin peptide injection; CAZ, capsazepine injection; CAZ+LL37, co-treatment with capsazepine and LL37; TRPV, transient receptor potential cation channel subfamily V; TRKA, tropomyosin receptor kinase A; NGF, nerve growth factor; IL, interleukin.

3. The verification of rosacea-like mouse model via phenotype and histological changes.

Phenotypic and histological changes were visually confirmed following LL37 injection in the rosacea-like mouse model, which presented with erythema and increased inflammatory responses (Fig. 3A-C). Examination of c-kit expression on the skin following LL37 injection revealed increased mast cell infiltration compared with control conditions (p = 0.0046, Fig. 3C).

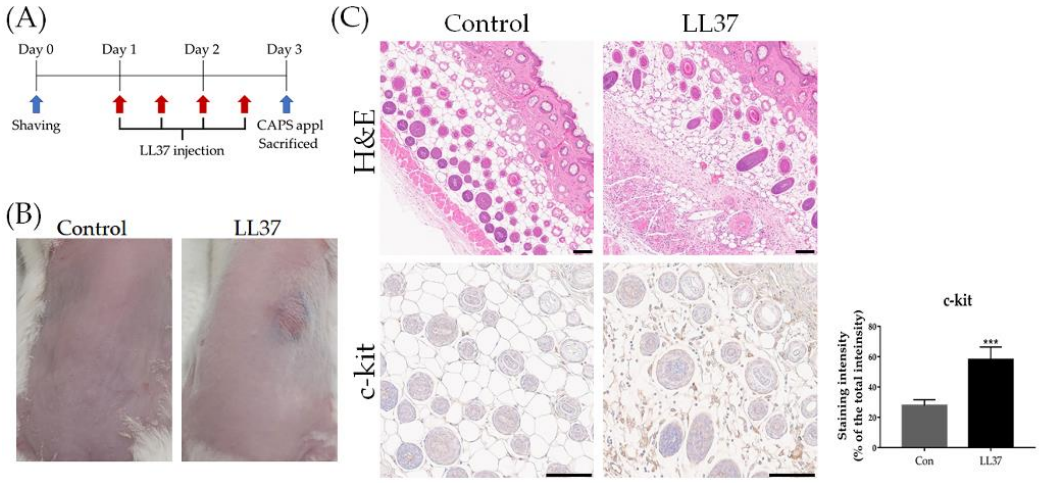

Figure 3. Induction of a rosacea-like mouse model via LL37 injection (A) Schematic showing the in vivo study design. (B) Erythema developed following the injection of LL37 into the skin on the back. (c) Haematoxylin and eosin (H&E) staining following LL37 injection showed histological changes, and c-kit staining showed increased mast cell marker expression (n = 4 mice for each group). Scale bar, 100 μm. *** $p < 0.005$. LL37, 37-amino acid C-terminal cathelicidin peptide; CAPS, Capsaicin

4. Immunohistological changes of TRPV1, TrkA, and NGF protein expression on rosacea-like mouse model skin.

The TRPV1 staining intensity increased significantly in the LL37 injection group compared with the Control group (p = 0.0178, Fig. 4A and B). TrkA expression was detected in 46.98% ± 4.38% of cells in the Control group and in 77.64% ± 4.57% of cells in the LL37 injection group (p = 0.0024). Additionally, NGF expression was detected in 48.73% ± 4.94% of cells in the control group and in 68.52% ± 2.60% of cells in the LL37 injection group (p = 0.0074).

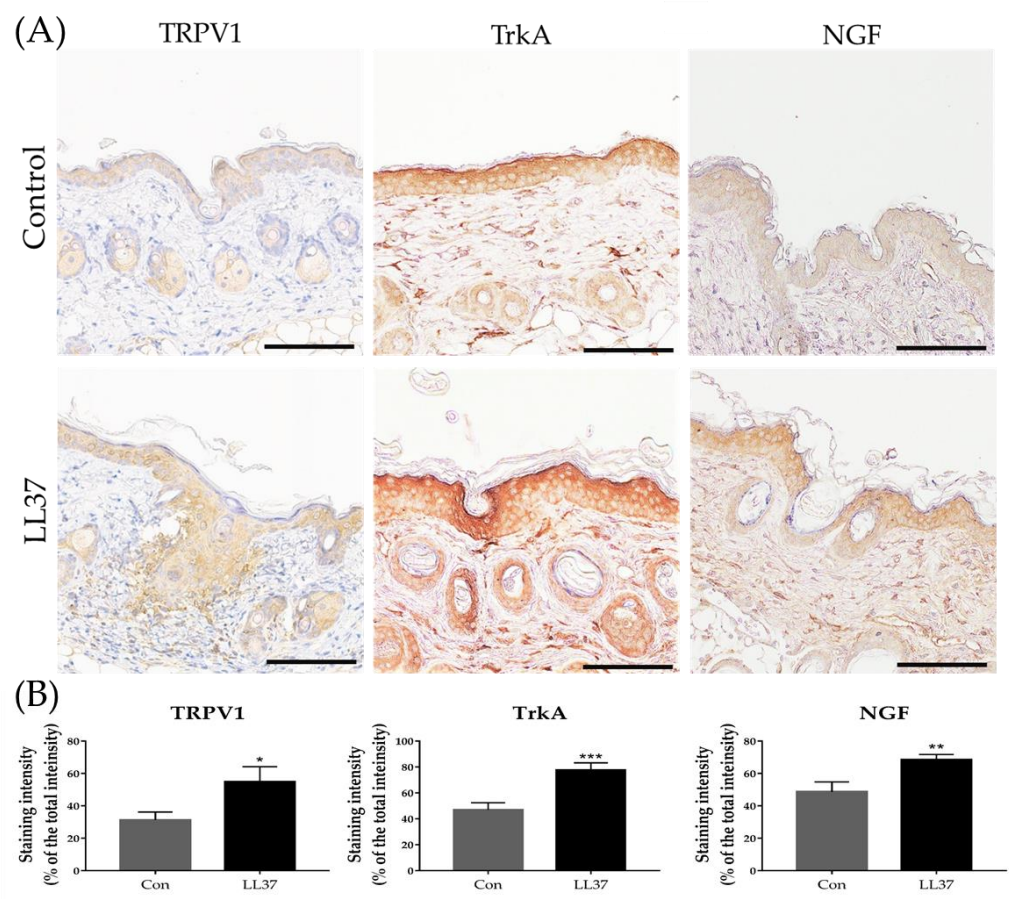

Figure 4. Immunohistochemical staining showing TRPV1, TrkA, and NGF localization in mouse skin. (A) LL37 injection significantly increased TRPV1, TrkA, and NGF staining intensity relative to the Control treatment. (B) The staining intensity of TRPV1, TrkA, and NGF increased significantly in the LL37 injection group compared with the Control group (n = 4 mice for each group). Scale bar, 100 μm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. LL37, 37-amino acid C-terminal cathelicidin peptide; TRPV1, transient receptor potential cation channel subfamily V member 1; TRKA, tropomyosin receptor kinase A; NGF, nerve growth factor.

5. The changes of protein and mRNA expression levels regarding various factors on rosacea-like mouse model skin.

Through western blot and RT-qPCR analyses, we investigated changes in the protein and mRNA expression levels on the skin of the rosacea mouse model (Fig. 5A and B). The LL37 injection group presented with significantly higher protein levels of TrkA ($p = 0.0101$), TRPV1 ($p = 0.0336$), and NGF ($p = 0.0002$) than the control group (Fig. 3a). The protein levels of calcitonin gene–related peptide (CGRP; $p = 0.0002$) and vascular endothelial growth factor (VEGF; $p = 0.0001$) were significantly higher in the LL37 injection group than in the control group (Fig. 5A). The phosphorylation levels of phosphoinositide 3-kinase (PI3K) p85 ($p = 0.0019$) and protein kinase B (Akt; $p = 0.0021$) were increased in the LL37 injection group compared with the levels in the control group (Fig. 5A). These results suggest that LL37 injection altered the protein levels of factors involved in cutaneous neurogenic inflammation. The observed increases in mRNA expression levels for *Trpv1* ($p = 0.0003$), *Trka* ($p = 0.0390$), and *Ngf* ($p = 0.0035$) mirrored the observed increases in protein levels (Fig. 5B). The mRNA expression levels of *Vegf* ($p = 0.0056$), *Trpv3* ($p < 0.0001$), and *Trpv4* ($p = 0.0002$) in the LL37 injection group were significantly higher than those in the Control group (Fig. 5B).

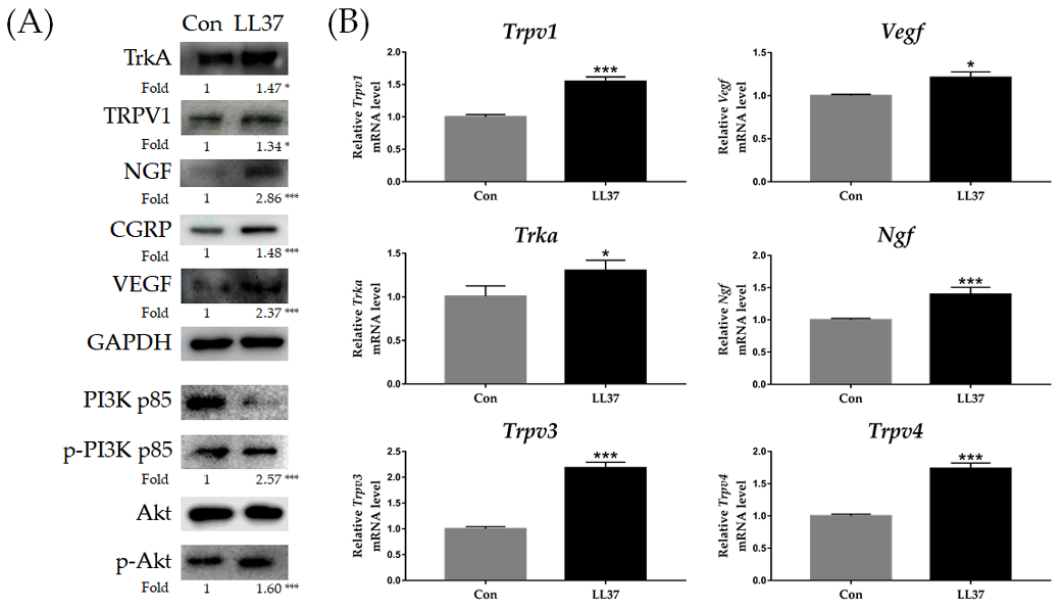

Figure 5. Protein and mRNA expression levels in rosacea-induced mouse skin. (A) In mice, LL37 injection into the skin resulted in increased protein expression levels of TrkA, TRPV1, NGF, CGRP, and VEGF and the phosphorylation of PI3K p85 and Akt. (B) The mRNA expression levels increased significantly in the LL37 injection group compared with the Control group. (A and B) n = 4 mice for each group. * $p < 0.05$, *** $p < 0.005$ Con, control; LL37, 37-amino acid C-terminal cathelicidin peptide injection; TRPV, transient receptor potential cation channel subfamily V; TRKA, tropomyosin receptor kinase A; NGF, nerve growth factor; CGRP, calcitonin gene–related protein; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B;

6. Immunofluorescence changes of TRPV1, CD31 and PGP9.5 protein expression on rosacea-like mouse model skin.

We performed immunofluorescence to examine TRPV1 and protein gene product 9.5 (PGP9.5) protein expression levels in the skin. The fluorescence intensities of both TRPV1 (p = 0.007) and PGP9.5 (p = 0.068) increased in the LL37 injection group compared with the Control group (Fig. 6A-C). To investigate whether LL37 injection affected the relationships between nerves and blood vessels, immunofluorescence was used to examine PGP9.5 and CD31 expression. PGP9.5 expression near $CD31^+$ endothelial cells increased significantly in the LL37 injection group compared with the Control group (Fig. 6B and C, p = 0.0072).

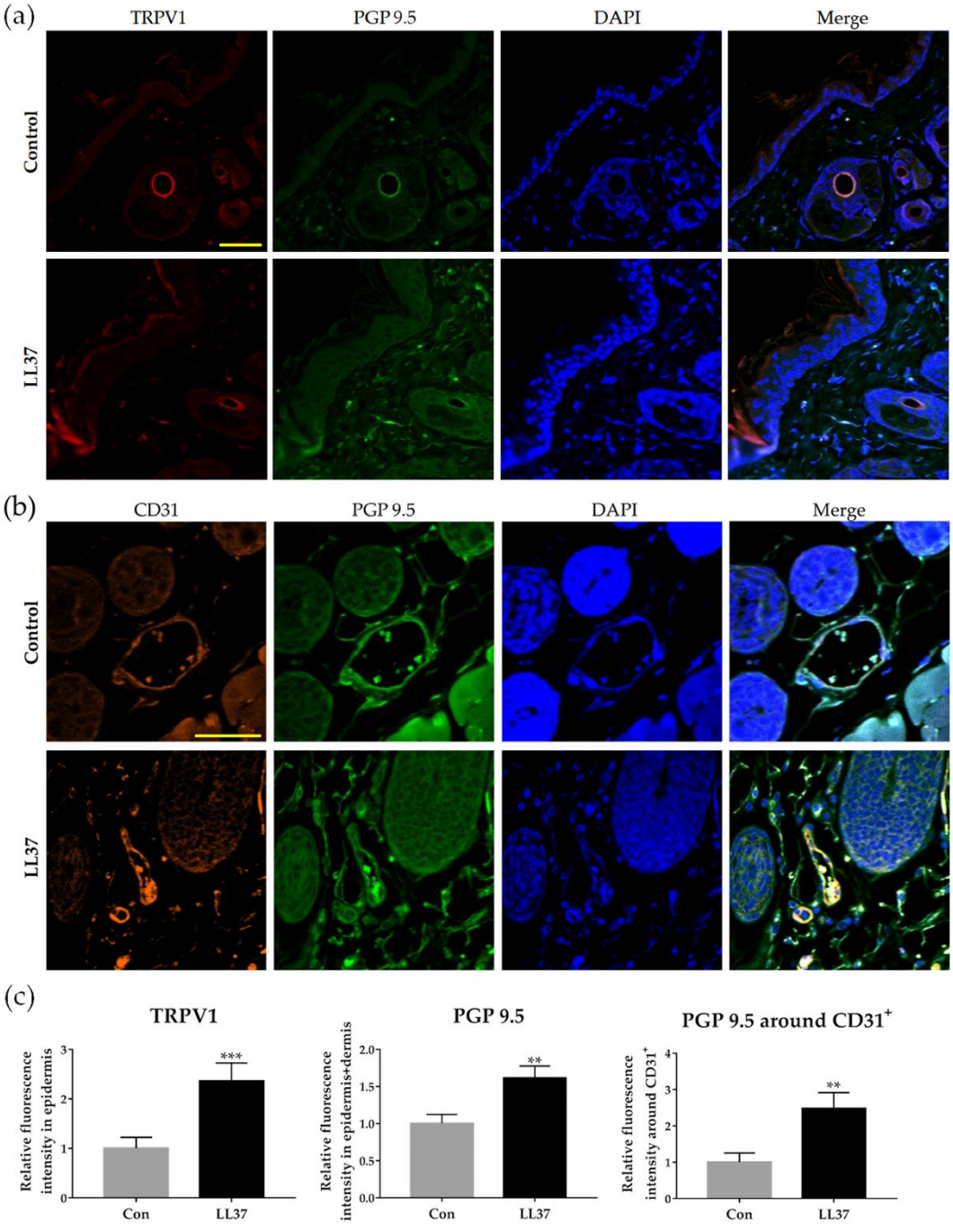

Figure 6. TRPV1, CD31, and PGP9.5 expression levels in mouse skin after LL37 injection. (A and B) Immunofluorescence staining showing TRPV1 (red, A), CD31 (red, B), and PGP9.5 (green) staining in mouse skin with (LL37) and without (control) LL37 injection. Nuclei are identified by 4',6-diamidino-2-phenylindole (DAPI). (C) The TRPV1 protein levels in the epidermis and PGP9.5 protein levels in the epidermis and dermis increased significantly following LL37 injection compared with Control (Con) treatment. PGP9.5 fluorescence intensity around CD31+ cells increased significantly following LL37 injection compared with the Control treatment. n = 4 mice for each group. All experiments were independently repeated at least 3 times. Scale bar, 50 μm. ** $p < 0.01$, *** $p < 0.005$. TRPV1, transient receptor potential cation channel subfamily V member 1; PGP9.5, protein gene product 9.5.

7. The changes of protein and mRNA expression levels regarding cutaneous neurogenic inflammation on rosacea-like mouse model DRG

LL37-induced changes in protein and mRNA expression levels in the mouse DRG were examined (Fig. 7A and B). The protein levels of TrkA (p = 0.004), TRPV1 (p = 0.0065), NGF (p = 0.0045), VIP (p = 0.0272), CGRP (p = 0.0002), Substance P (Sub P; p = 0.0011), and VEGF (p = 0.0065) increased significantly in the LL37 injection group compared with the DRG of Control group (Fig. 7A). mRNA expression levels of *Trka* (p = 0.002), *Trpv1* (p = 0.0121), *Ngf* (p < 0.0001), *Vip* (p = 0.0129), and *Cgrp* (p < 0.0001) in the LL37 injection group increased significantly compared with those in the Control group (Fig. 7B).

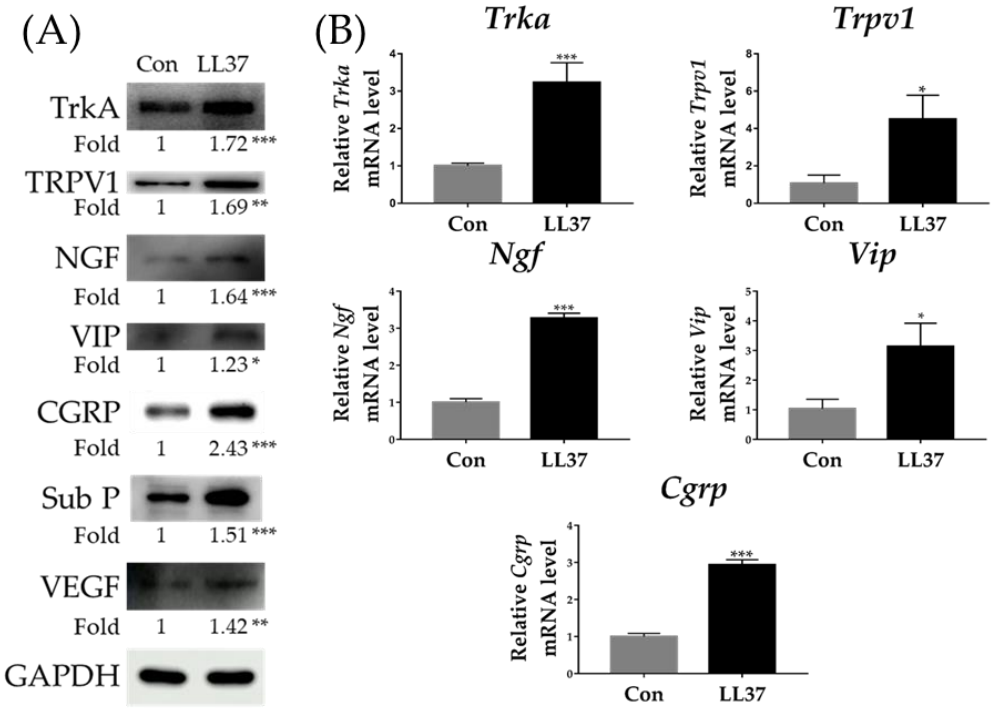

Figure 7. Comparison of protein and mRNA expression levels in the DRG and serum protein levels following LL37 injection in mice. (A) LL37 injection into the mouse skin resulted in increased dorsal root ganglion (DRG) protein expression levels for TrkA, TRPV1, NGF, CGRP, Sub P, and VEGF. (B) mRNA expression levels of Trka, Trpv1, Ngf, Vip, and Cgrp increased significantly for the LL37 injection group compared with the Control group. (n = 4 mice for each group) All experiments were independently repeated at least 3 times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. LL37, 37-amino acid C-terminal cathelicidin peptide; TRPV, transient receptor potential cation channel subfamily V; TRKA, tropomyosin receptor kinase A; NGF, nerve growth factor; CGRP, calcitonin gene–related protein; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Sub P, Substance P; Vip, vasoactive intestinal peptide; B.

8. The changes of the serum levels of IL-4 and IL-5 on rosacea-like mouse model blood

The serum levels of interleukin (IL)-4 ($p = 0.004$) and IL-5 ($p < 0.0001$) were assessed by ELISA (Fig. 8A and B), which showed significant increases in the LL37 injection group compared with the Control group.

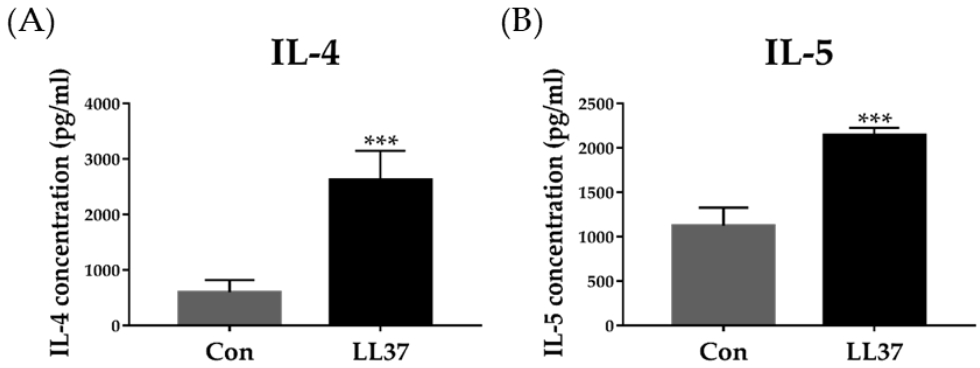

Figure 8. Comparison of serum protein levels in the blood following LL37 injection in mice. (A and B) LL37 injection significantly increased serum IL-4 and IL-5 levels compared with control treatment. n = 4 mice for each group. All experiments were independently repeated at least 3 times. *** $p < 0.005$. LL37, 37-amino acid C-terminal cathelicidin peptide; IL, interleukin

9. The changes of erythema phenotypes and mRNA expression levels on the rosacea-like mouse model ear.

The induced erythema phenotypes observed before and after capsaicin application are shown in Fig. 8A. Additionally, the mRNA expression levels of *Trka* ($p = 0.0264$), *Trpv1* ($p = 0.0003$), *Ngf* ($p = 0.0022$), *Vegf* ($p = 0.0075$), and *Vip* ($p < 0.0001$) were significantly increased in the LL37 injection group compared with the Control group (Fig. 8B).

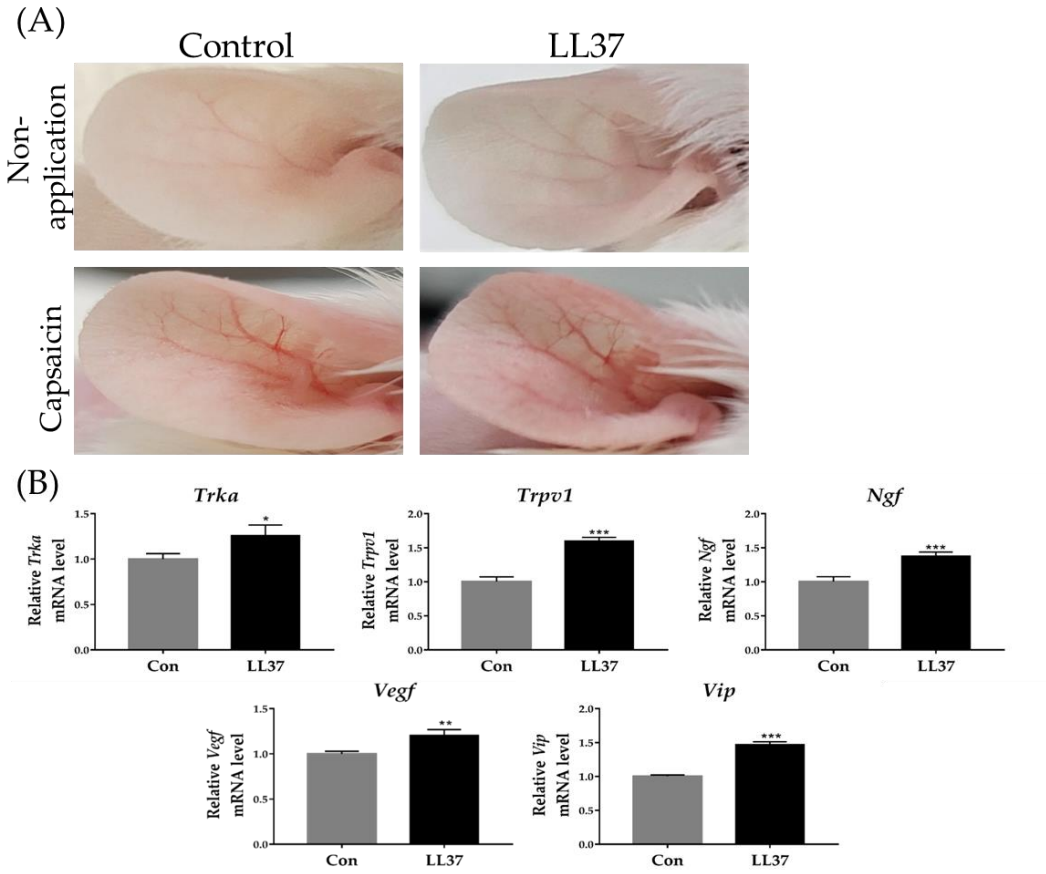

Figure 9. The images of rosacea-like erythema and changes in mRNA expression via LL37 injection. (A) Increased rosacea-like erythema was observed in the ears. (B) The mRNA expression levels were significantly increased in the LL37 injection group compared with the Control group. n = 4 mice per group. Scale bar, 100 μm. * $p < 0.05$, *** $p < 0.005$ (B). Con, control; CAP, capsaicin application; LL37, 37-amino acid C-terminal cathelicidin peptide injection; L+C, LL37 injection + capsaicin application. Trpv1, transient receptor potential cation channel subfamily V member 1; TrkA, tropomyosin receptor kinase A; Ngf, nerve growth factor; Vegf, vascular endothelial growth factor; Vip,

vasoactive intestinal peptide.

10. Immunohistological changes of rosacea-like mouse model ear according to the external stimulation.

Differences between the Control and LL37 injection groups were examined following the application of capsaicin to the ears of mice in each group. the expression of TRPV1 was significantly increased by capsaicin application in both groups (Fig. 8A and B). CD31 immunohistochemistry revealed an increase in the blood vessel areas following capsaicin application to the ears of the mice in the LL37 injection group compared with the ears of mice in the Control group (Fig. 9C and D).

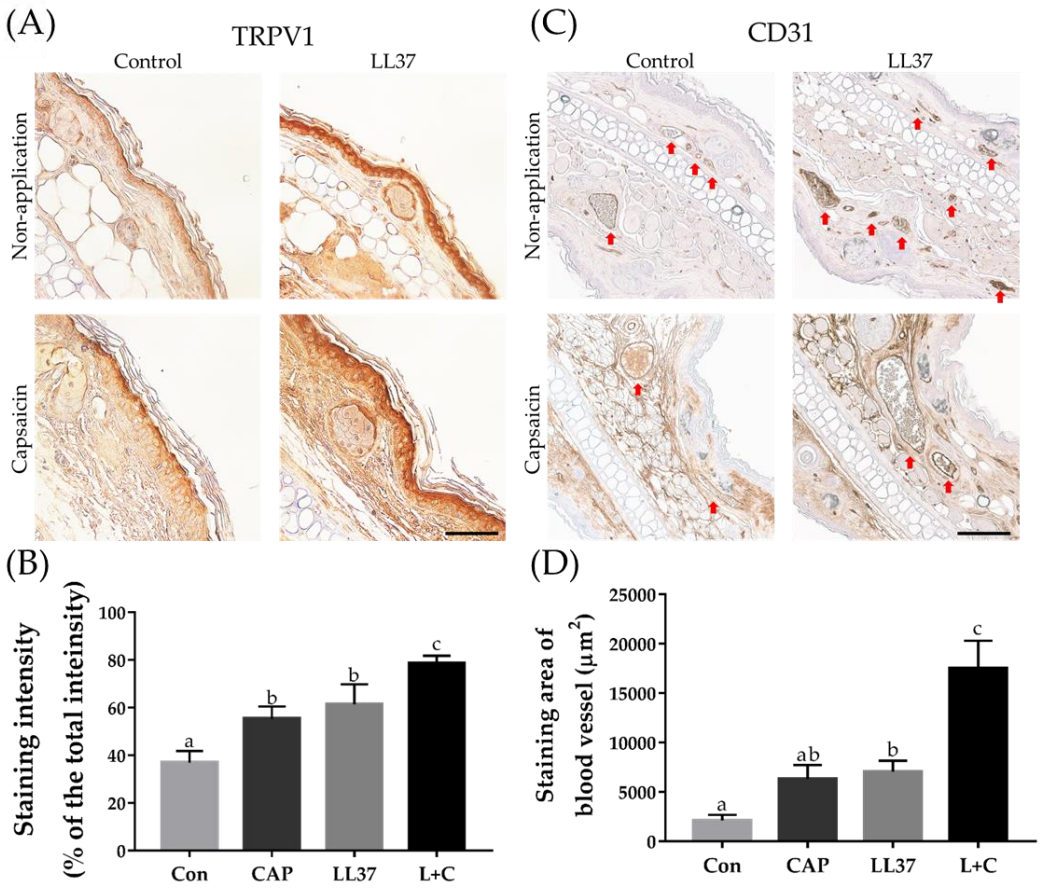

Figure 10. External stimulation of mouse ears after LL37 injection revealed skin sensitization. (A and B) TRPV1 protein expression in mouse ears was significantly increased following LL37 injection, and the LL37 injection group was remarkably more responsive to capsaicin application. (C and D) The blood vessel area in the mouse ear significantly expanded following LL37 injection, and the LL37 injection group showed significantly increased vessel area in response to capsaicin application compared with the Control group. Scale bar, 100 μm. Values followed by the same small letter are not significantly different ($p < 0.05$, B and D). Con, control; CAP, capsaicin application; LL37,

37-amino acid C-terminal cathelicidin peptide injection; L+C, LL37 injection + capsaicin application. Trpv1, transient receptor potential cation channel subfamily V member 1.

IV. DISCUSSION

The increased colonization of *Demodex* mites has been suggested to serve as a potential exacerbating trigger for rosacea.[16] TLR2 activates KLK5, resulting in the cleavage of cathelicidin to activate the well-known LL37 peptide form.[17] In the present study, we demonstrated significantly increased mRNA expression levels of *TLR2*, *KLK5*, and *LL37* in samples separated according to the Demodex population counts. Additionally, *TRPV1*, *TRKA*, and *NGF* were overexpressed in the presence of high *Demodex* population counts. A rosacea mouse model induced by LL37 injection also demonstrated significantly increased expression levels of TRPV1, NGF, and TrkA compared with Control mice.

NGF is released under inflammatory conditions onto peripheral nerve endings in the skin, playing key roles in various types of pain, such as chronic pain, acute pain, and hyperalgesia.[19] NGF levels are increased in damaged or irritated tissues, assisting in the transmission of pain signals by nociceptive neurons through multiple pathways.[18] NGF is produced by skin cells and affects the development of sensory axons.[20] TrkA is a tyrosine kinase receptor that binds to NGF, initiating a signal transduction pathway that leads to TRPV1 activation, resulting in the sensation of pain.[13] TRPV1 has been identified as a key factor in the establishment of pain and is thought to play a role in the development of neuropathic pain.[21] Upon LL37 injection in mice, TrkA, TPRV1, and NGF protein levels increased (Fig. 4A and B), suggesting that LL37 injection may induce NGF production, ultimately activating TrkA and TRPV1.

TrkA activation stimulates the phosphorylation of PI3K p85, leading to the activation of TRPV1 and the phosphorylation of Akt, which is downstream of PI3K p85.[22, 23]

Phosphorylation activates Akt, which is able to regulate VEGF expression and activity [24]. In the rosacea mouse model, the phosphorylation levels of PI3K p85 and Akt were increased (Fig. 5A), as were the protein expression levels of VEGF. Akt activation stimulates VEGF production, resulting in the formation of new blood vessels to increase blood flow to the skin.[25] VEGF can also induce vasodilation, increasing vascular permeability.[26] Increased vasodilation in the skin allows inflammatory factors produced in the skin to spread throughout the body, which can lead to adverse effects. Our study detected increases in serum IL-4 and IL-5 levels following LL37 injection into the skin (Fig. 8). The various relationship between rosacea and cytokines has previously been described.[27-29] As the result of Figures 2 and 8, the treatment of LL37 induced pro-inflammatory cytokines such as IL-1β and IL-6 on keratinocytes and Th2 cytokines such as IL-4 and IL-5 in the rosacea-liked mouse model. T-helper (Th) 2 cytokines are known to emerge in allergic inflammation or infection caused by parasites such as *Demodex*, and their increase regulates Th2 immunity.[30] Rosacea is associated with increased *Demodex* population and chronic inflammation of the skin, and Th2 cytokines, are involved in promoting inflammation and immune responses. Also, the previous study has suggested that rosacea may involve an imbalance in the immune system, with an over-activation of certain immune responses, including Th2-mediated responses.[31,32] The increased Th2 cytokines in the blood of the rosacea-liked mouse model was evidence that the LL37 injection evoked vasodilation more absorbing and spreading various factors induced by LL37, thereby contributing to exacerbation of inflammation and type 2 immunity in rosacea. Further investigation into cytokines regarding Th1 or Th17 other than Th2 cytokine could suggest complex and still poorly unrevealed mechanisms for inflammatory responses and disruption of the immune system in rosacea.

CGRP, a neuropeptide released from sensory nerve endings in the skin, is involved in the regulation of vasodilation and neurogenic inflammation.[33] CGRP also activates mast cells, which release inflammatory mediators, such as cytokines, leading to neurogenic inflammation.[34] Increased CGRP protein expression induced mast cell activation in our study (Fig. 3C) and may have affected angiogenesis or cutaneous neurogenic inflammation, which may also have affected the increase in parallel of protein and mRNA expression levels of TRPV1, VEGF, TrkA, and NGF in the skin (Fig. 5B). To investigate the effects of TRPV1 following LL37 treatment, we treated HaCaT keratinocyte cells with LL37 in the presence and absence of the TRPV1 antagonist CAZ (Fig. 2). The LL37-induced increase in mRNA expression levels could be reduced by co-treatment with CAZ in HaCaT cells, suggesting that LL37-induced cutaneous neurogenic inflammation involves TRPV1 activation and that TRPV1 may represent a key mediator of rosacea.

PGP9.5 in peripheral nerves is involved in the detection and transmission of sensory information in the skin. PGP9.5 is widely used as a marker for the identification and quantification of cutaneous nerve fibres[35,36] because PGP9.5 expressed in the dermis of the skin is involved in nerve fibre formation. Increased PGP9.5 expression has been linked to inflammation and itching in inflammatory skin diseases, such as atopic dermatitis.[37] In our study, we demonstrated that LL37 injection induced increases in TRPV1 and PGP9.5 expression, which were associated with rosacea symptoms (Fig. 6A and C). The fluorescence intensity of PGP9.5 increased in proximity to $CD31^+$ cells (Fig. 6B and C), suggesting that peripheral nerve growth can lead to increased blood vessel formation in the skin, resulting in redness, itching, and other symptoms.

In the skin, sensory nerves derive from the DRG and represent the initial somatic portion

of the afferent sensory pathway. A previous study showed that increased TRPV1 activation in response to NGF in the DRG leads to skin sensitization.[38] We demonstrated that TrkA, TRPV1, and NGF protein levels increase in the DRG of mice treated with LL37 compared with the DRG of Control mice. These results may explain the increased sensitivity of rosacea patients to environmental factors and the associated development of neuropathic pain. Furthermore, the protein expression levels of VIP, CGRP, Sub P, and VEGF in the DRG were significantly higher in the LL37 injection group than in the Control group (Fig. 7A). In the DRG, VIP, CGRP, Sub P, and NGF are involved in pain sensitivity and inflammation. The increased protein expression levels of VIP, CGRP, and Sub P suggest that LL37 injection triggered an inflammatory response and increased pain sensitivity. These results, similar to the results shown in Figs. 2, 5, and 6, provide evidence to support the involvement of these factors in the development of skin sensitization in rosacea. VEGF protein expression was also significantly increased in the LL37 injection group compared with the Control group. VEGF expression is induced by NGF in DRG sensory neurons, and exogenous intravascular VEGF injections cause pain and increase peripheral nerve density.[39,40] The overexpression of VEGF in the DRG also supports angiogenesis and sensitization in rosacea.

To investigate the potential sensitization effect of rosacea, capsaicin was applied to the ears of mice (Figs 9 and 10). After the application of capsaicin, increased TRPV1 staining intensity was observed in samples from the ears of mice in the LL37 injection group compared with those in the Control group. Moreover, blood vessel area also increased in the LL37 injection group compared with the Control group, which was consistent with the TRPV1 staining results. The high TRPV1 expression levels elicited by capsaicin exposure

imply that increased LL37 levels in response to increased *Demodex* mite colonization may increase susceptibility to rosacea aggravation triggers. These findings could have implications for the treatment of rosacea-related symptoms, as targeting TRPV1 could potentially reduce the severity of erythematotelangiectatic rosacea.

V. CONCLUSION

In conclusion, we propose a comprehensive mechanism underlying the skin sensitization and cutaneous neurogenic inflammation symptoms in rosacea based on studies performed in a rosacea-like mouse model generated by the injection of LL37 into the skin. In detailed and visualized, a graphical summary of our present study findings is shown in Figure 11. This research offers an explanation for the skin sensitization and cutaneous neurogenic inflammation symptoms associated with rosacea, which are caused by increased LL37 in the presence of larger *Demodex* population counts in the skin of patients with rosacea. Although previous studies have indicated that TRPV1 is involved in the skin sensitivity associated with rosacea, the detailed downstream pathways and related factors have remained unknown. We found that higher *Demodex* population counts resulted in TRPV1, TrkA, and NGF overexpression. Our *in vitro* study showed that a TRPV1 antagonist was able to reduce LL37-induced increases in TRPV1, TrkA, and NGF gene expression, which may be able to attenuate the vicious cycle associated with NGF–TrkA–TRPV1 pathway activation in rosacea. We also confirmed that angiogenesis in rosacea is caused by the activation of the PI3K–Akt pathway elicited by TrkA. Skin sensitivity to external stimuli increased in our rosacea mouse model, which suggests that the initial diagnosis and detection of rosacea onset is important for treatment. LL37 can also affect the DRG, causing cutaneous neurogenic inflammation, which may be responsible for the neurogenic symptoms of rosacea. Taken together, the findings of this study provide valuable insight into the pathogenesis of rosacea and may be beneficial for the development of novel rosacea therapies that target the NGF–TrkA–TRPV1 pathway.

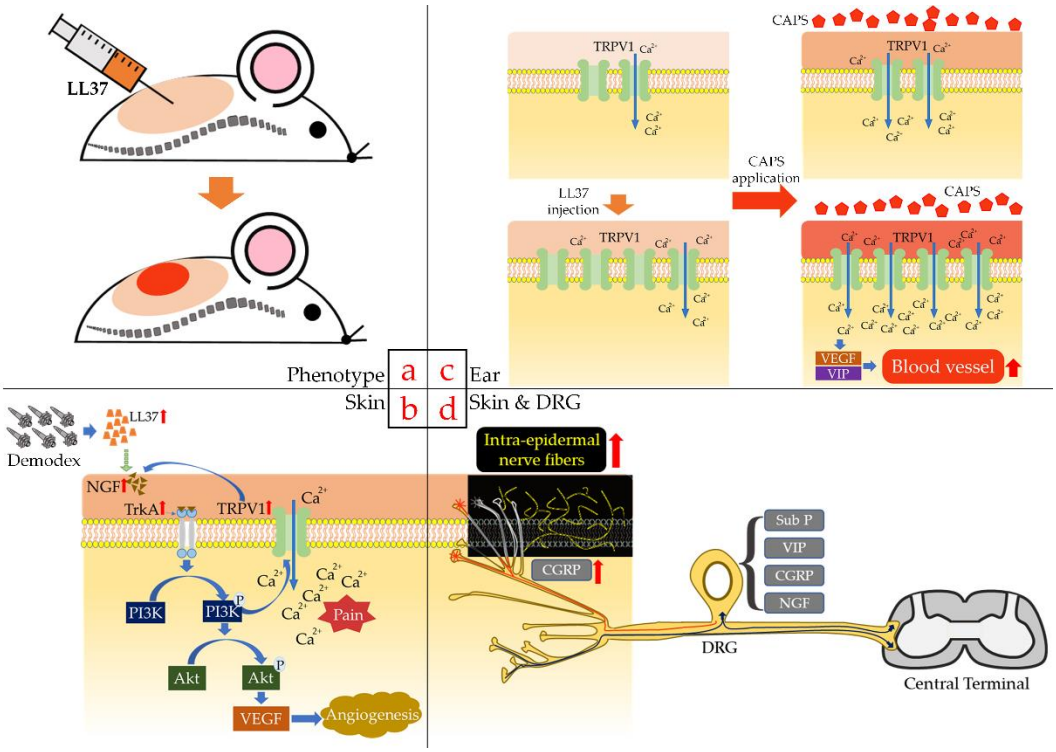

Figure 11. The proposed mechanism underlying skin sensitization and erythematotelangiectatic rosacea symptoms induced by LL37 injection in the rosacea-like mouse model. We propose a comprehensive mechanism underlying the skin sensitization and cutaneous neurogenic inflammation symptoms in rosacea based on studies performed in a rosacea-like mouse model generated by the injection of LL37 into the skin. (A) The intradermal injection of LL37 into the skin on the back causes an erythema phenotype that resembles rosacea symptoms. During erythema, mast cells migrate to the dermis, indicating the induction of an inflammatory response. The overexpression of genes related to rosacea, including TRPV1, TrkA, and NGF, was detected with increased Demodex population counts. (B) The LL37 injection also resulted in increased TRPV1, TrkA, and NGF expression levels in the mouse skin, indicating that the cutaneous

neurogenic symptoms associated with rosacea, such as itching, burning, and stinging, are associated with the induction of the NGF–TrkA–TRPV1 pathway. In addition, the PI3K–Akt pathway is regulated by TrkA activation, leading to the overexpression of VEGF and causing erythema-associated angiogenesis. (C) Changes in the mouse ear following LL37 injection suggest that rosacea alters skin sensitivity to external stimuli (such as capsaicin), which may lead to a vicious cycle that makes rosacea symptoms difficult to improve. (D) Injection of LL37 also altered gene and protein expression of Sub P, VIP, CGRP, and NGF in the DRG, which links peripheral nerves in the skin to a central terminal that eventually reaches the brain, suggesting that these changes may be involved in pain sensitivity and inflammation. Increased CGRP expression in the skin can result in the growth of intradermal peripheral nerve fibres, leading to skin sensitization. LL37, 37-amino acid C-terminal cathelicidin peptide; Trpv1, transient receptor potential cation channel subfamily V member 1; TrkA, tropomyosin receptor kinase A; Ngf, nerve growth factor; PI3K, glyceraldehyde 3-phosphate dehydrogenase; Akt, phosphoinositide 3-kinase; VEGF, vascular endothelial growth factor; Sub P, Substance P; Vip, vasoactive intestinal peptide CGRP, calcitonin gene-related protein

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ABSTRACT(IN KOREAN)

데모덱스 진드기는 TRPV1-NGF-TrkA 경로에 의해 조정되는 피부 신경원성 염증을 유발한다.

<지도교수 이주희>

연세대학교 대학원 의학과

이 상 규

주사는 치료가 매우 어렵고 재발이 잦은 흔한 만성 염증성 피부 질환입니다. 또한 햇빛, 온도, 매운 음식, 바람 등의 다양한 자극에 의해서 악화되며 그 증상으로는 홍반, 가려움증, 따끔거림, 화끈거림 등이 있습니다. 데모덱스 진드기는 피부에 사는 모낭충으로 불리며 주사 환자의 피부에서 일반인 보다 더 많이 발견됩니다. 따라서 피부 염증학적 반응의 변화와 데모덱스 진드기의 감염은 주사의 치료 대상이 되는 주요 생리학적 요인입니다. Transient receptor potential vanilloid 1 (TRPV1)은 피부 신경성 염증에 관여하는 통각성 양이온 채널로 주사를 악화시키는 다양한 자극에 의해 활성화 될 수 있습니다. 본 연구는 데모덱스 진드기 수의 증가와 피부 신경성 염증을 중심으로 주사 질환에서의 TRPV1 조절의 효과를 조사하였습니다.

데모덱스 진드기의 수에 따른 검체를 수집한 후에 그룹을 나는 후 mRNA 발현 수준을 평가하였고, 각질세포에서 TRPV1의 영향을 평가하기 위해 Capsazepine을 TRPV1 억제제로 사용하였습니다. 주사를 유발하는 인자 중

가장 대표적인 37-amino acid C-terminal cathelicidin peptide (LL37)를 마우스에 주입하여 주사 유도 마우스 모델을 만들어 피부, dorsal root ganglion (DRG), 귀, 혈액을 채취하여 변화를 조사하였습니다.

데모덱스 진드기의 수가 높은 그룹의 검체에서 신경 성장 인자 (NGF)와 그의 수용체인 TrkA 와 TPRV1 의 mRNA 발현량이 증가한 것을 확인할 수 있었고, 각질세포에서도 LL37처리를 통한 주사 유도 모델에서 세가지 인자 모두 증가한 것을 확인할 수 있었습니다. 또한 Capsazepine을 처리하였을 때. 세가지 인자의 mRNA 발현량이 감소하였습니다. 이것은 TRPV1의 조절이 NGF와 TrkA의 발현량 조절에 관여한다고 제시할 수 있습니다. 동물실험에서도 이와 비슷한 결과가 피부, DRG, 귀에서 발견되었습니다.

본 연구의 결과는 주사 질환이 신경성 증상이 나타나고 자주 악화되며 재발이 되는 것에 대한 근본적인 원인을 이해하는 데 중요한 역할을 합니다. 주사 유도 마우스 모델에서 DRG에서의 변화를 비교한 연구는 이 연구가 유일하며, 이 결과를 바탕으로 피부 질환과 신경성 증상의 연결고리를 찾을 수 있고, 그 치료에 한걸음 다가갈 수 있습니다. 또한 추후 TRPV1-NGF-TrkA 경로를 표적으로 하는 새로운 치료법의 개발로 이어질 수 있으며, 이 경로를 치료 대상으로 하면 주사 질환의 신경성 증상을 약화 시키거나 재발을 방지할 수 있는 치료가 될 것이며, 더 효과적이고 특이적인 주사 치료법을 개발할 수 있습니다.

핵심되는 말 : 주사, TRPV1, 피부 신경성 염증, NGF, 데모덱스 진드기

PUBLICATION LIST

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