

Original Article



Effects of Botulinum Toxin A on Rosacea-Like Inflammation in an LL-37-Induced Rosacea Mouse Model

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ABSTRACT

Background: Rosacea is a chronic inflammatory disorder characterized by flushing, erythema, papules/pustules, and telangiectasia. Several clinical studies have investigated the efficacy of botulinum neurotoxin A (BoNT/A) in the treatment of rosacea, but its mechanism of action remains unclear.

Objective: This study aims to examine the potential role of BoNT/A in a mouse model of rosacea-like skin lesions induced by the 37-amino acid C-terminal cathelicidin peptide (LL-37).

Methods: Mice were randomly divided into 4 groups: Control, LL-37, LL-37 + BoNT/A, and LL-37 + dexamethasone.

Results: BoNT/A treatment alleviated skin damage, reduced skin thickness, and decreased mast cell infiltration. Furthermore, BoNT/A improved redness score severity and redness area while enhancing skin barrier function by suppressing transepidermal water loss and increasing skin hydration. At the molecular level, BoNT/A decreased the mRNA levels of interleukin (IL)-6 and tumor necrosis factor- α , which are known as pro-inflammatory cytokines. It also downregulated the expression of pyrin domain-containing protein 3, caspase-1, and IL-1 beta in the LL-37-injected dorsal skin. Furthermore, BoNT/A prevented LL-37-mediated upregulation of neurovascular-associated factors, including CD31, transient receptor potential vanilloid 1, calcitonin-related polypeptide alpha, vascular endothelial growth factor, chymase 1, and tryptase alpha/beta 1.

Conclusion: These results indicate that BoNT/A effectively alleviates inflammatory and vascular responses in a rosacea mouse model, highlighting its potential as a promising preventive approach for rosacea.

Keywords: Angiogenesis; Botulinum toxin A; Inflammasomes; Neurogenic inflammation; Rosacea

INTRODUCTION

Rosacea is a persistent inflammatory skin condition defined by symptoms such as facial redness, dilated blood vessels, and inflammatory papules and pustules, primarily affecting the central region of the face¹. Rosacea typically develops between 30 and

50 years of age but can occur at any age². Globally, the prevalence of rosacea is estimated to be 5.5% among the adult population³. Beyond its dermatological manifestations, rosacea can lead to decreased self-esteem and depression. Additionally, it negatively affects quality of life, as well as social and psychological well-being⁴. Furthermore, recent studies have revealed a correlation

between rosacea and an increased risk of various systemic disorders, including cardiovascular, gastrointestinal, neurological, autoimmune, and psychiatric diseases^{5,6}.

Since the cause and underlying mechanisms of rosacea remain unclear, treatment primarily focuses on symptom management rather than addressing the pathological processes. Current therapeutic options include topical agents such as azelaic acid, oral treatments (including doxycycline and isotretinoin), and vascular-targeting laser. However, these treatments vary in efficacy and carry risks of side effects, including rebound flares⁷⁻⁹.

Rosacea pathogenesis is related to increased levels of the 37-amino acid C-terminal cathelicidin peptide (LL-37)¹⁰. In rosacea skin lesions, Toll-like receptor (TLR) expression and cathelicidin antimicrobial peptide (CAMP, encoding LL-37) levels increase, with CAMP being cleaved into its active form, LL-37, by Kallikrein 5 (KLK-5), which is also upregulated^{11,12}. LL-37 regulates immune responses, induces chemokine and cytokine release, and promotes angiogenesis. Excessive LL-37 exposure activates NOD-, leucine-rich repeat, and pyrin domain-containing protein 3 (NLRP3) inflammasome^{13,14}. Activating NLRP3 inflammasome results in caspase-1 maturation and the subsequent release of interleukin-1 beta (IL-1 β). The released IL-1 β promotes inflammatory cell infiltration and cutaneous inflammation via IL-1 receptor-mediated signaling¹⁵.

Additionally, rosacea pathophysiology involves complex neurovascular interactions. Neurovascular dysregulation contributes to vasodilation and flushing through mechanisms involving transient receptor potential (TRP) channels and neuropeptides such as calcitonin gene-related peptide (CGRP)^{16,19}. These pathological mechanisms highlight the significance of managing neurovascular and inflammatory pathways in rosacea treatment.

Produced by *Clostridium botulinum*, botulinum neurotoxin A (BoNT/A) prevents acetylcholine release from presynaptic nerve endings, thereby disrupting neurotransmission²⁰. BoNT/A is utilized in various clinical applications. BoNT/A primarily exerts its aesthetic effects through neuromuscular blockade. The key action of BoNT/A is as follows: wrinkle reduction, jawline slimming, nose and lip enhancements, eyebrow lifting, pore tightening, sweat reduction, and contouring of the neck and calves²¹. In addition, BoNT/A is utilized as a preventive treatment for chronic migraine due to its inhibitory effects on CGRP and NLRP3 expression²². BoNT/A has also been reported to alleviate craniofacial pain by suppressing the expression of TRP vanilloid 1 (TRPV1)²³. Interestingly, BoNT/A has demonstrated efficacy in treating rosacea-related facial flushing and erythematous symptoms^{24,25}. However, research on the mechanism of botulinum toxin in the treatment of rosacea remains limited.

Therefore, this study was designed to investigate the preventive effects of BoNT/A in the rosacea mouse model induced by LL-37 and to explore its underlying mechanism of action.

MATERIALS AND METHODS

Chemicals and reagents

LL-37 (purity $\geq 97\%$, HOR-041) was obtained from Prospecc (Rehovot, Israel), dexamethasone (DEX) (purity $\geq 97\%$, D4902) from Sigma-Aldrich (St. Louis, MO, USA), BoNT/A from Daewoong Pharmaceutical Co., Ltd (Seoul, Korea).

Animal experiments

Female Balb/c mice, 5 weeks old, were procured from Saeron Bio Inc. (Uiwang, Korea). The mice were provided ad libitum access to antibiotic-free solid feed and water until the experiment. A 1-week acclimation period was conducted under controlled environmental conditions ($23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ temperature, $55\% \pm 10\%$ humidity, and a 12-hour-light/dark cycle). All procedures were approved by the Institutional Animal Care and Use Committee of Chung-Ang University (IACUC, No. 202401030082). To induce rosacea-like lesions, the LL-37 peptide was used based on previously established protocols¹⁰. The dorsal skin was shaved 24 hours prior to any injections. Mice received an intradermal injection of 0.5 units of BoNT/A. After 12 hours, the mice were administered intradermal injections of 40 μl of 320 μM cathelicidin LL-37 or saline twice daily for 2 days. DEX was topically applied to the LL-37 injection site at a dose of 10 mg/kg (200 μl in saline) twice daily for 2 days. The dose of BoNT/A and DEX was set based on previous studies^{26,27}. Mice ($n=4$ per group) were randomly allocated into one of 4 groups: control (saline); LL-37; LL-37 + BoNT/A (0.5 units); and LL-37 + DEX (10 mg/kg). At the end of the experiment, mice were anesthetized, and dorsal skin samples were biopsied for RNA extraction, hematoxylin and eosin staining (H&E), toluidine blue (TB) staining, and immunohistochemistry (IHC). Mice were then sacrificed by CO_2 euthanasia.

Measurement of redness score and area

The anesthesia procedure was performed as previously shown²⁸. The anesthetic solution, composed of Zoletil (0.008 ml/10 g; 40 mg/kg) and Rompun (0.002 ml/10 g; 5 mg/kg), was diluted tenfold in normal saline. After anesthesia, the dorsal skin of mice was captured at close range using a digital single-lens reflex camera (Nikon, Tokyo, Japan). After the experiment, the redness score and affected area were measured to assess the severity of rosacea-like skin lesions. The redness score (ranging from 1–5) was determined by averaging individual scores, which were graded as 1 (none), 2 (mild), 3 (moderate), 4 (severe), and 5 (very severe). The highest possible score was 5. The ImageJ software (National Institutes of Health Image, Bethesda, MD, USA) was utilized to quantify the total area of redness.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RNA extraction and cDNA synthesis were performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and PrimeScript™ RT Master Mix (Takara Bio, Kusatsu, Japan), respectively. RT-qPCR was conducted using SYBR-Green–based detection (Enzynomics, Daejeon, Korea) on a CFX96 Touch Real-Time PCR system (Bio-Rad, Hercules, CA, USA). The thermocycling protocol consisted of an initial 15-minute activation at 95°C, followed by 45 cycles of the following steps: 95°C for 10 seconds (denaturation), 60°C for 15 seconds (annealing), and 72°C for 30 seconds (elongation). Gene expression levels were calculated relative to the glyceraldehyde-3-phosphate dehydrogenase normalization control using the $2^{-\Delta\Delta Cq}$ quantification method. Analysis was performed using RNA extracted from individual mice ($n=4$ per group), and each sample was measured in technical triplicate. All primers used in this study are listed in **Supplementary Table 1**.

Histological analysis

Biopsied dorsal skin samples were fixed in 10% formalin, paraffin-embedded, and sectioned (5 μ m). Epidermal thickness and mast cell (MC) infiltration were evaluated by H&E and TB staining, respectively. For IHC, sections were stained with antibodies against NLRP3 (1:200; Cell Signaling Technology, Danvers, MA, USA), caspase-1 (1:100; Cell Signaling Technology), IL-1 β (1:100; Affinity Biosciences, Cincinnati, OH, USA), and CD31 (1:4,000; Abcam, Cambridge, UK). Slides were washed with phosphate-buffered saline containing 0.1% Tween® 20. Signal detection was achieved using a high-sensitivity 3,3'-diaminobenzidine chromogenic substrate kit (Vector Laboratories). After a tap water wash, sections were counterstained with hematoxylin, washed, dehydrated, and digitized by slide scanner (Pannoramic MIDI; 3DHISTECH Ltd., Budapest, Hungary). Analysis was performed with Case Viewer software (version 2.7; 3DHISTECH Ltd.).

Assessment of skin barrier

Stratum corneum hydration was quantified using a Corneometer® CM 825, and transepidermal water loss (TEWL) was measured with a Tewameter® TM 300 (both from Courage and Khazaka Electronic GmbH, Köln, Germany). Both values were measured at the end of the experiment.

Statistical analysis

Outcome assessments, including redness scoring, histological analysis, and skin barrier assessment were performed by an investigator blinded to the experimental group allocations. Data are expressed as the mean $\pm$ standard deviation derived from independent biological samples ($n=4$ per group). The normality of data distribution was verified using the Shapiro-Wilk test, and

no significant deviations were observed. A one-way analysis of variance followed by Bonferroni's *post hoc* test was used for statistical comparisons. All statistical calculations were performed using GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA).

RESULTS

BoNT/A attenuated LL-37-mediated rosacea-like skin inflammation in mice

We established a rosacea-like lesion mouse model via LL-37 injection to examine the impact of BoNT/A on rosacea inflammation, with DEX serving as a positive control. **Fig. 1A** presents the overall experimental scheme. Photographs of the dorsal skin confirmed that BoNT/A significantly alleviated skin damage compared with those of the control group (**Fig. 1B**). To analyze its effects on skin redness, we scored redness severity and measured the affected area. Skin redness was scored according to predefined criteria (**Fig. 1C**). Analysis based on this scoring system revealed that BoNT/A effectively reduced LL-37-induced redness, decreasing the mean redness score from 4.0 ± 0.6 in the LL-37 group to 1.5 ± 0.3 (**Fig. 1D**). Similarly, the area of redness was significantly reduced by 97.1% following BoNT/A treatment (**Fig. 1E**). These findings suggest that BoNT/A attenuates rosacea-like skin lesions.

BoNT/A downregulated LL-37 expression

BoNT/A suppressed LL-37-mediated upregulation of CAMP (**Fig. 2A**). CAMP expression increased by approximately 2.3-fold in the LL-37 group compared with the control group, whereas BoNT/A treatment reduced its level to approximately 0.25–0.30-fold relative to the LL-37 group. Additionally, BoNT/A decreased the expression levels of TLR2, MMP-9, and KLK-5 (**Fig. 2B–D**). TLR2 and MMP-9 were markedly elevated following LL-37 stimulation (2- to 4-fold increases) compared with the control, and BoNT/A substantially attenuated these increases, reducing their expression to near-baseline levels. KLK-5 expression was also increased by LL-37 (4.5-fold) compared with the control and was reduced by BoNT/A treatment. This suggests that BoNT/A attenuates LL-37-mediated upregulation of rosacea biomarkers.

BoNT/A recovered disturbance in the epidermal barrier

BoNT/A reduced the abnormal epidermal thickening by 33.4% compared with that of the LL-37 group (**Fig. 3A and B**). In the LL-37 group, skin hydration decreased by 59.2% relative to the control group. The BoNT/A-treated group showed a 64.6% increase in skin hydration compared to the LL-37 group (**Fig. 3C**). Additionally, TEWL levels in the LL-37 group increased by 136.3% compared with the control group, whereas BoNT/A treatment

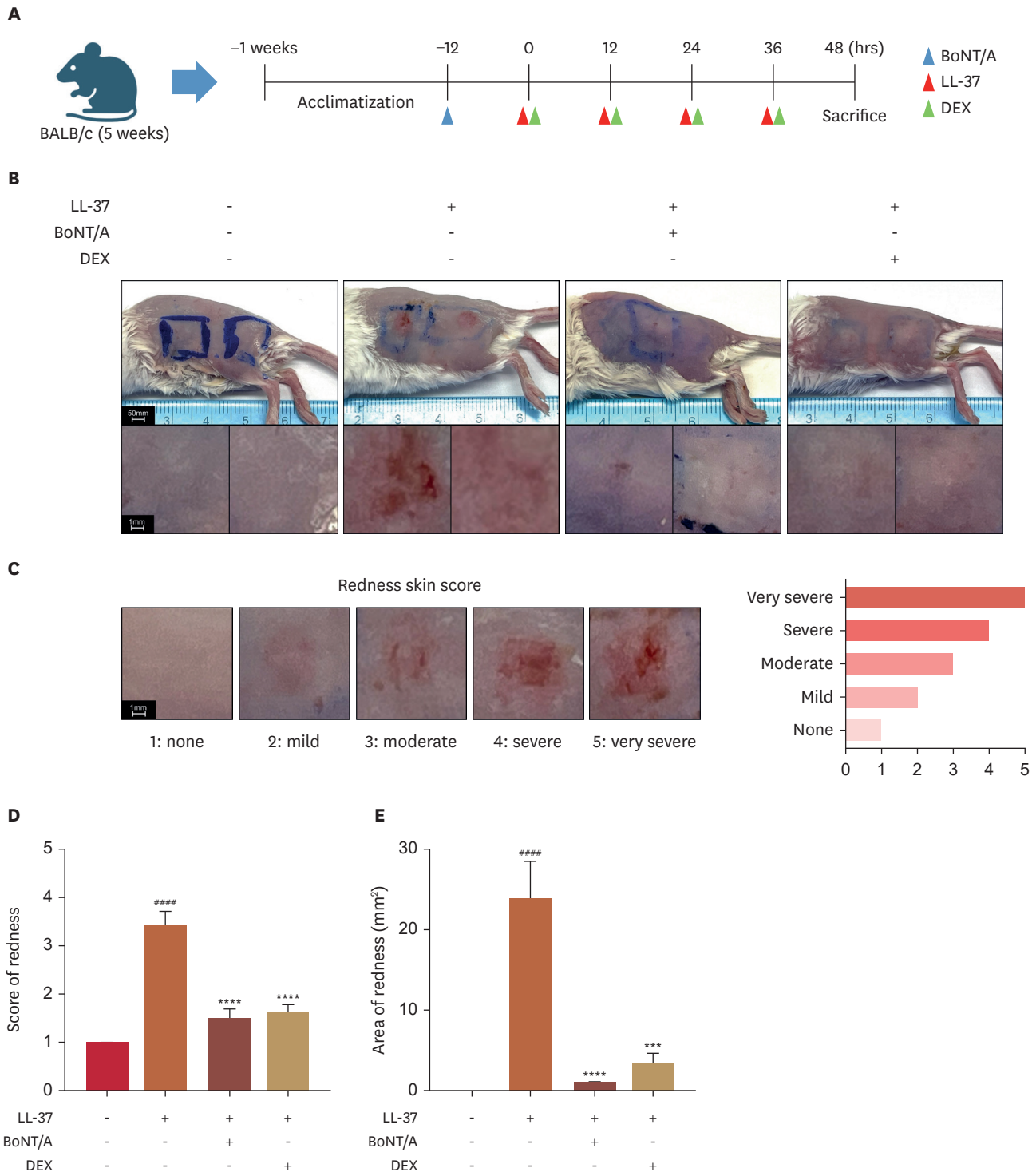


Fig. 1. *In vivo* effects of BoNT/A in an LL-37-induced rosacea mouse model. (A) Experimental design. (B) Representative images of mouse dorsal skin. (C) Standardized redness scoring criteria. (D) Redness score. (E) Redness area (n=4/group).

BoNT/A: botulinum neurotoxin A, LL-37: 37-amino acid C-terminal cathelicidin peptide, DEX: dexamethasone.

*** $p < 0.001$, **** $p < 0.0001$ vs. the LL-37 group; **** $p < 0.0001$ vs. the control group.

decreased TEWL by 43.7% compared with those of the LL-37 group (**Fig. 3D**). These findings suggest that BoNT/A protects against LL-37-mediated disruption of the skin barrier in rosacea-like skin lesions.

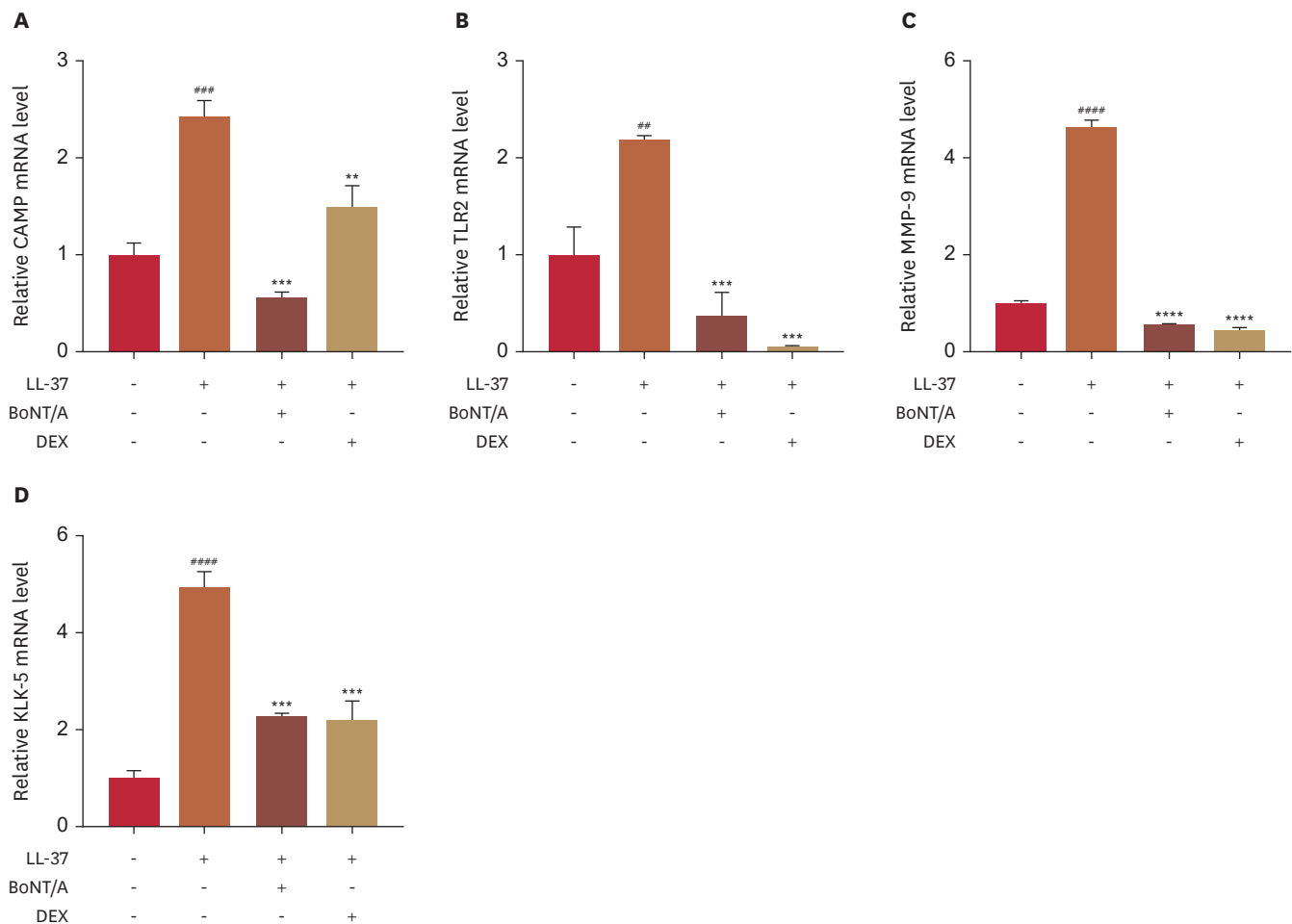


Fig. 2. Effects of BoNT/A on LL-37-related genes. mRNA levels of (A) CAMP, (B) TLR2, (C) MMP-9, and (D) KLK-5 in the mouse dorsal skin. Gene expression was quantified and presented relative to the glyceraldehyde-3-phosphate dehydrogenase mRNA levels (n=4/group).

BoNT/A: botulinum neurotoxin A, LL-37: 37-amino acid C-terminal cathelicidin peptide, CAMP: cathelicidin antimicrobial peptide, TLR2: Toll-like receptor 2, MMP-9: matrix metalloproteinase-9, KLK-5: kallikrein 5, DEX: dexamethasone.

p<0.01, *p<0.001, and ****p<0.0001 vs. the LL-37 group; ##p<0.01, ###p<0.001, and ####p<0.0001 vs. the control group.

BoNT/A inhibited the activity of MCs

To further evaluate the effects of BoNT/A, MC infiltration was analyzed using TB staining. LL-37 induced the highest levels of MC infiltration (7.75±0.96 cells/field), while BoNT/A and DEX treatments reduced cell infiltration compared to that of the LL-37 group (**Fig. 4A and B**). Additionally, mRNA levels of the pro-inflammatory cytokines such as IL-6 and tumor necrosis factor- α (TNF- α) were measured (**Fig. 4C and D**). Their expressions were highest in the LL-37 group. IL-6 and TNF- α were suppressed by 4.2-fold and 2.1-fold, respectively, indicating that BoNT/A suppresses LL-37-mediated MC activation.

BoNT/A alleviated NLRP3 inflammasome in LL-37-induced mice

The LL-37 group showed the highest mRNA expression of NLRP3 (1.6-fold), caspase-1 (2.4-fold), and IL-1 β (9.4-fold),

while BoNT/A treatment significantly reduced their expression by approximately 35%, 39%, and 69% for NLRP3, caspase-1, and IL-1 β , respectively (**Fig. 5A-C**). Similarly, the expression of these inflammasome markers in IHC staining was increased in the LL-37 group, while BoNT/A treatment suppressed their expression (**Fig. 5D-I**). These findings indicate that BoNT/A and DEX effectively suppress the NLRP3 inflammasome pathway associated with rosacea-like inflammation.

BoNT/A suppressed neurovascular-associated genes

CD31, a vascular endothelial cell marker, was examined using IHC staining. LL-37 treatment increased CD31 expression. In contrast, the staining intensity of CD31 in the BoNT/A and DEX groups was relatively weaker than that in the LL-37 group (**Fig. 6A and B**). Additionally, the mRNA expression levels of TRPV1, calcitonin-related polypeptide alpha (CALCA, encoding CGRP), vascular

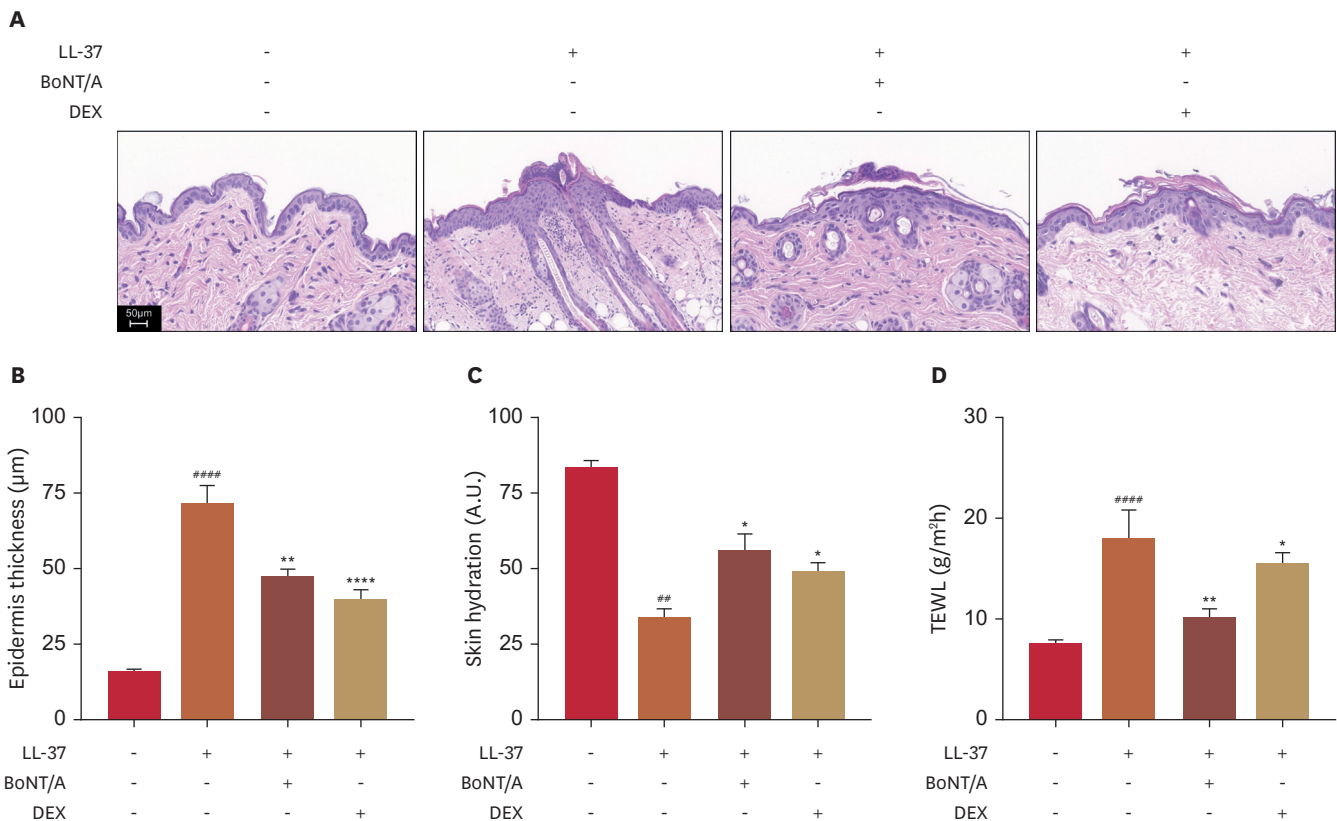


Fig. 3. Effects of BoNT/A on the epidermal barrier in an LL-37-induced mouse model. (A) Representative images of hematoxylin and eosin-stained images of mouse dorsal skin (scale bar: 50 μ m). (B) Epidermis thickness (μ m). (C) Skin hydration. (D) TEWL ($n=4$ /group). BoNT/A: botulinum neurotoxin A, LL-37: 37-amino acid C-terminal cathelicidin peptide, TEWL: transepidermal water loss, DEX: dexamethasone, A.U.: arbitrary units. * $p<0.05$, ** $p<0.01$, and **** $p<0.0001$ vs. the LL-37 group; ** $p<0.01$, and **** $p<0.0001$ vs. the control group.

endothelial growth factor (VEGF), chymase 1 (Cma1), and tryptase alpha/beta 1 (TPsab1), which engage in neurovascular response, were assessed (**Fig. 6C-G**). Notably, BoNT/A administration led to substantial reductions in Cma1 and CALCA expression, showing 24.3-fold and 17.3-fold decreases, respectively, compared with the LL-37 group. Other vascular mediators, such as TRPV1, VEGF, and TPSab1, were also significantly downregulated ranging from 1.8-fold to 3.6-fold reduction. Similar inhibitory effects were consistently observed in the DEX-treated positive control group. These findings indicate that BoNT/A and DEX effectively suppress neurovascular activation and angiogenesis associated with rosacea.

DISCUSSION

This study investigated the effects of BoNT/A on inflammatory and neurovascular responses using an LL-37-induced rosacea-like mouse model. LL-37 overexpression is known to promote cytokine secretion, angiogenesis, and erythema, thereby recapitulating key features of rosacea-like skin inflammation²⁹.

In this model, BoNT/A significantly reduced erythema severity,

MC infiltration, and the expression of proinflammatory cytokines. And no observable local or systemic adverse effects were noted during the experimental period. These findings indicate that BoNT/A attenuates the inflammatory responses associated with LL-37-induced skin lesions. Notably, the reduction in MC-related markers and neurovascular-associated genes, including TRPV1 and CALCA, suggests that BoNT/A may modulate neurogenic inflammation, which is considered a key driver of rosacea pathophysiology.

Skin barrier dysfunction is a key pathological feature of rosacea, caused by numerous factors, especially LL-37 overexpression and NLRP3 inflammasome activation. These processes lead to excessive inflammatory cytokine secretion, which weakens the skin barrier^{10,12}. In addition, skin barrier damage increases sensitivity to external stimuli and exacerbates TEWL, leading to increased skin dryness and redness³⁰. BoNT/A may help restore skin barrier function. In our study, BoNT/A enhanced skin hydration and barrier function.

The NLRP3 inflammasome plays a key role in rosacea pathogenesis. LL-37 overexpression is proposed as a critical factor inducing NLRP3 inflammasome activation, and it promotes

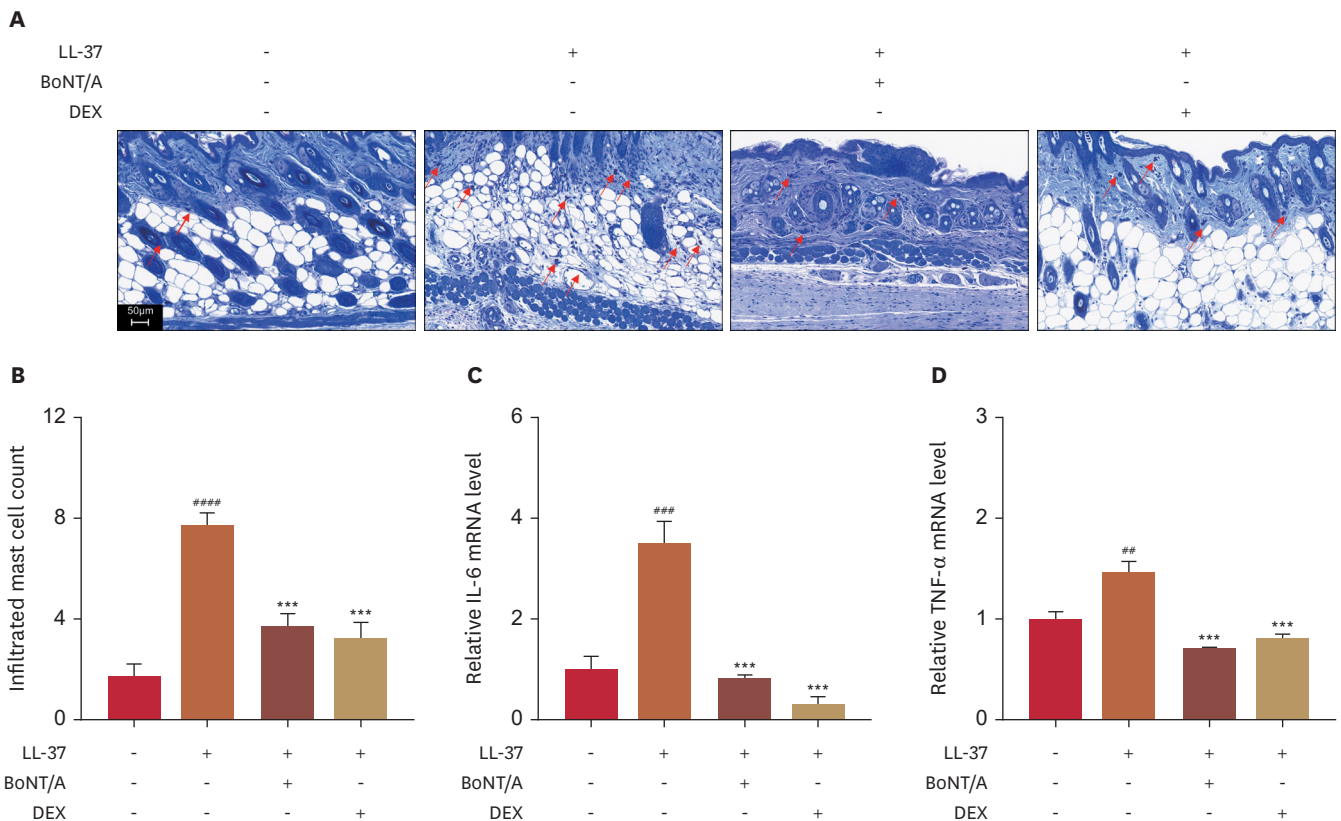


Fig. 4. Effects of BoNT/A on inflammation activation. (A) Representative images of toluidine blue-stained images of mouse dorsal skin (scale bar: 50 μ m). Infiltrated mast cells are indicated with red arrows. (B) Infiltrated mast cell count. mRNA levels of rosacea-associated inflammatory biomarkers (C) IL-6 and (D) TNF- α in mouse dorsal skin (n=4/group). Gene expression was quantified relative to glyceraldehyde-3-phosphate dehydrogenase RNA levels. BoNT/A: botulinum neurotoxin A, IL-6: interleukin-6, TNF- α : tumor necrosis factor- α , LL-37: 37-amino acid C-terminal cathelicidin peptide, DEX: dexamethasone. *** p <0.001 vs. the LL-37 group; ** p <0.01, *** p <0.001, and **** p <0.0001 vs. the control group.

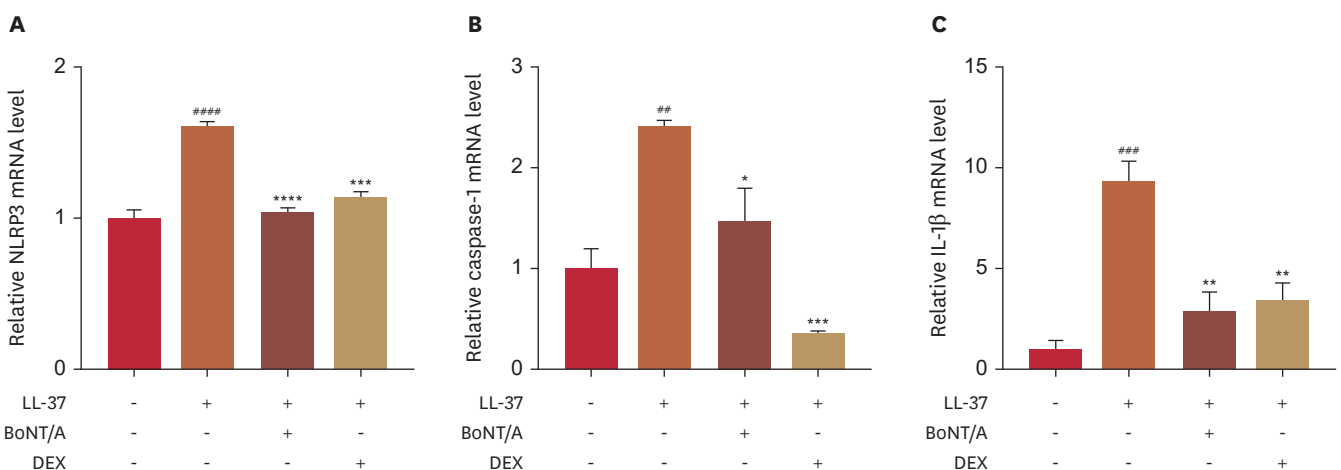


Fig. 5. Alleviation effects of BoNT/A on NLRP3 inflammasome in LL-37-induced rosacea mouse model. mRNA levels of (A) NLRP3, (B) caspase-1, and (C) IL-1 β in mouse dorsal skin were examined using reverse transcription-quantitative polymerase chain reaction analysis. Gene expression was quantified relative to the glyceraldehyde-3-phosphate dehydrogenase mRNA levels. (D) NLRP3, (E) caspase-1, and (F) IL-1 β were analyzed using IHC (scale bars: 50 μ m). Quantification of IHC staining intensity (A.U.) for (G) NLRP3, (H) caspase-1, and (I) IL-1 β (n=4/group). BoNT/A: botulinum neurotoxin A, NLRP3: NOD-, leucine-rich repeat, and pyrin domain-containing protein 3, LL-37: 37-amino acid C-terminal cathelicidin peptide, IL-1 β : interleukin-1 beta, IHC: immunohistochemistry, A.U.: arbitrary units, DEX: dexamethasone. * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001 vs. the LL-37 group; ** p <0.01, *** p <0.001, and **** p <0.0001 vs. the control group. (continued to the next page)

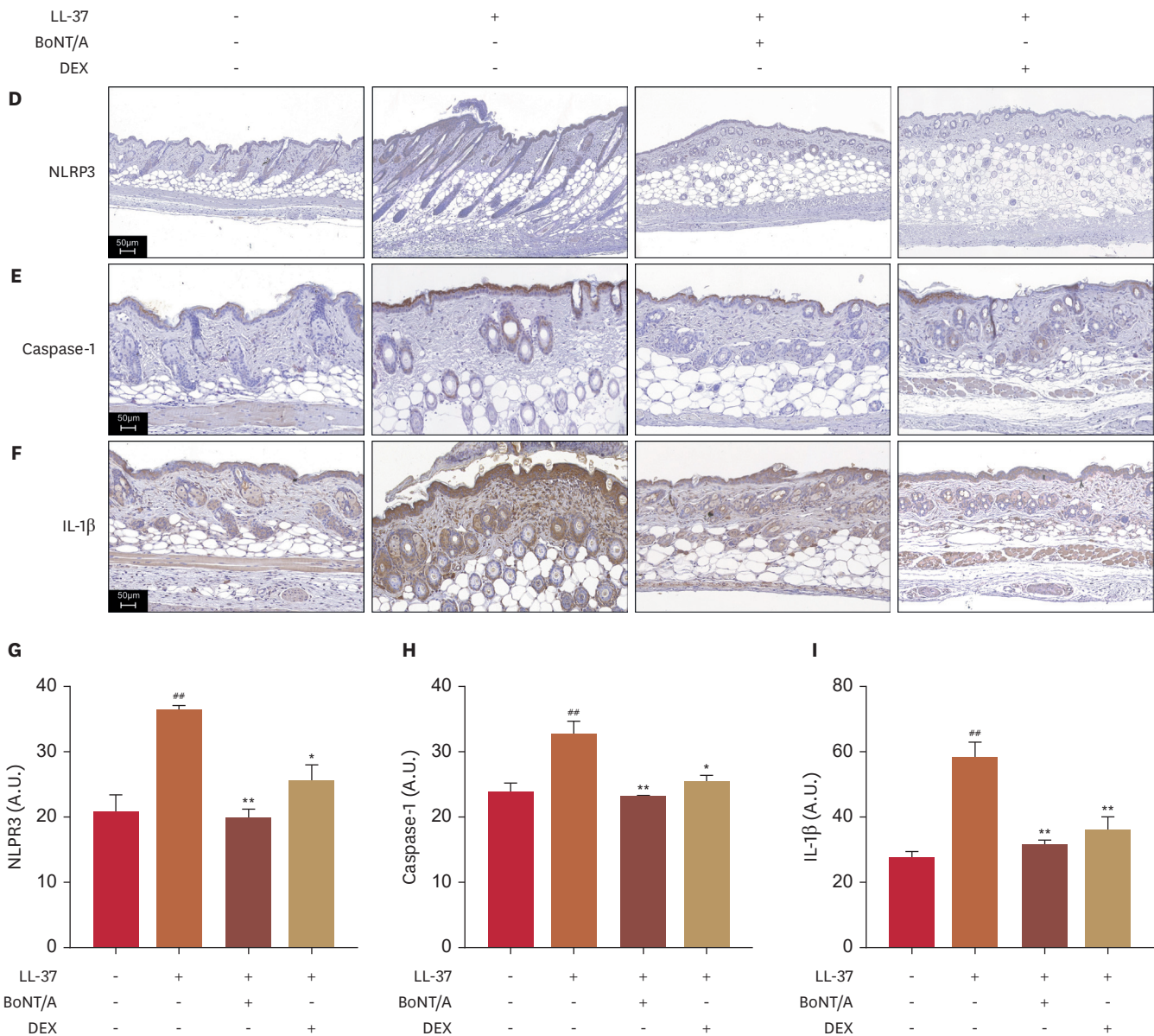


Fig. 5. (Continued) Alleviation effects of BoNT/A on NLRP3 inflammasome in LL-37-induced rosacea mouse model. mRNA levels of (A) NLRP3, (B) caspase-1, and (C) IL-1 β in mouse dorsal skin were examined using reverse transcription-quantitative polymerase chain reaction analysis. Gene expression was quantified relative to the glyceraldehyde-3-phosphate dehydrogenase mRNA levels. (D) NLRP3, (E) caspase-1, and (F) IL-1 β were analyzed using IHC (scale bars: 50 μ m). Quantification of IHC staining intensity (A.U.) for (G) NLRP3, (H) caspase-1, and (I) IL-1 β (n=4/group). BoNT/A: botulinum neurotoxin A, NLRP3: NOD-, leucine-rich repeat, and pyrin domain-containing protein 3, LL-37: 37-amino acid C-terminal cathelicidin peptide, IL-1 β : interleukin-1 beta, IHC: immunohistochemistry, A.U.: arbitrary units, DEX: dexamethasone.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. the LL-37 group; ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. the control group.

inflammatory cytokine release and exacerbates flushing and inflammation¹³. Additionally, MC activation in the inflammatory response of rosacea promotes the secretion of IL-6 and TNF- α , known as inflammatory cytokines. This forms a positive feedback loop that sustains inflammation and aggravates disease severity³¹.

Facial redness in patients with rosacea primarily results from vasodilation. Rosacea is characterized by the dilation of facial capillaries due to vascular hypersensitivity, which leads to flushing

and erythema³². TRPV1 contributes to neurovascular dysregulation and promotes inflammatory mediators such as CGRP and VEGF to increase blood flow, exacerbating symptoms³³. CD31, a protein located at the junction between endothelial cells, regulates vascular permeability³⁴, and plays a key role in angiogenesis. Elevated CD31 expression may be observed in patients with rosacea. Cma1 and TPsb1, enzymes secreted by MCs, induce angiogenesis by increasing the VEGF expression and promoting vascular

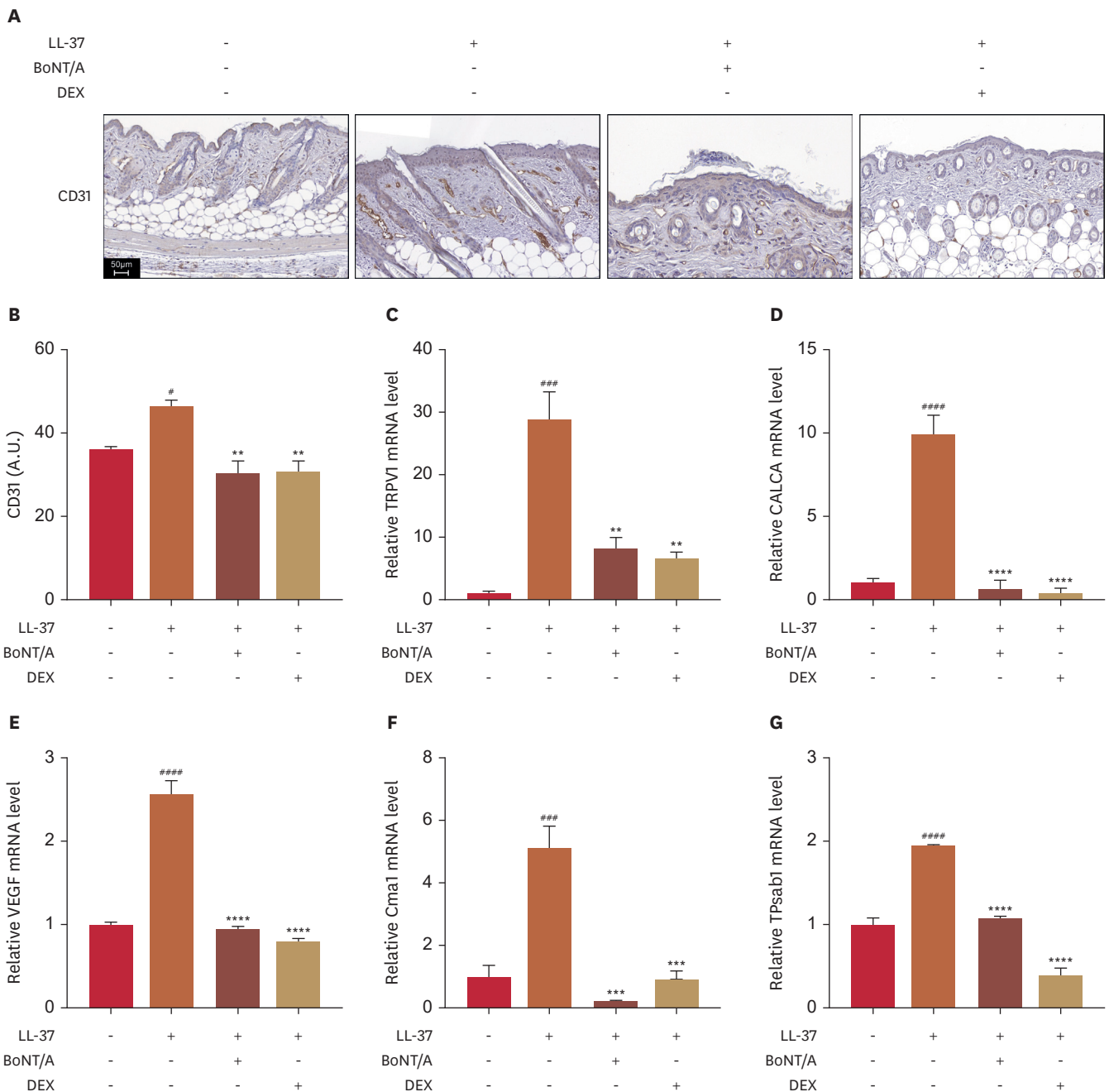


Fig. 6. Expression levels of neurovascular-associated genes after BoNT/A treatment in LL-37-induced rosacea mouse model. (A) CD31 expression was analyzed using IHC (scale bar: 50 µm). (B) Quantification of IHC staining intensity (A.U.) for CD31. mRNA levels of neurovascular cytokines (C) TRPV1, (D) CALCA, (E) VEGF, (F) Cma1, and (G) TPsb1 in mouse dorsal skin (n=4/group). Gene expression was quantified relative to the GAPDH mRNA levels.

BoNT/A: botulinum neurotoxin A, LL-37: 37-amino acid C-terminal cathelicidin peptide, IHC: immunohistochemistry, A.U.: arbitrary units, TRPV1: transient receptor potential vanilloid 1, CALCA: calcitonin related polypeptide alpha, VEGF: vascular endothelial growth factor, Cma1: chymase 1, TPsb1: tryptase alpha/beta 1, DEX: dexamethasone.

** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. the LL-37 group; * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$ vs. the control group.

endothelial cell proliferation³⁵.

Our findings show that BoNT/A treatment reduces the number of MCs and suppresses IL-6 and TNF- α expression. Moreover, vascular-related markers, including CGRP, VEGF, and CD31, as well as

MC-related enzymes, including Cma1 and TPsb1, were decreased in the BoNT/A-treated group relative to those in the LL-37 group, suggesting that BoNT/A simultaneously targets the neurovascular and immune pathogenesis by regulating MC-angiogenesis-related

inflammatory cytokines and neurovascular-related responses in rosacea. Furthermore, BoNT/A contributes to vasodilation control and prevention of flushing and erythema.

Overall, BoNT/A treatment was associated with a significant reduction in MC infiltration and the expression of neurovascular-associated genes. However, it remains to be elucidated whether BoNT/A exerts a direct inhibitory effect on the NLRP3 inflammasome or if the observed downregulation is a secondary phenomenon arising from the suppression of upstream triggers, such as neuropeptide release and MC degranulation.

While the preventive administration of BoNT/A successfully attenuated the inflammatory cascade, the current *in vivo* results do not exclude the possibility that certain molecular changes are epiphenomena of reduced cutaneous erythema and enhanced barrier integrity. Consequently, these findings should be interpreted as an integrated protective effect of BoNT/A on the rosacea-like microenvironment. Further mechanistic studies focusing on the direct interaction between BoNT/A and specific immune cell populations are required to delineate the exact signaling pathways involved.

Despite these findings, several limitations should be acknowledged. Because the sample size in this study was relatively small ($n=4$ per group), statistical power may be limited and variability may be increased. Therefore, the results of this study should be interpreted as exploratory rather than definitive. Future studies with a larger cohort are needed to verify these results. In addition, since the present study did not evaluate the therapeutic effects of BoNT/A or the possibility of symptom recurrence through long-term experiments, further studies are warranted. Moreover, as intradermal injections are invasive and may cause discomfort or reluctance in patients, investigating topical BoNT/A treatment strategy is also essential.

In summary, our findings support that BoNT/A exhibits a preventive effect on inflammatory responses, erythema, and angiogenesis in the LL-37-induced rosacea mouse model. We propose that BoNT/A prevents MC activation, blocks neurovascular activity, and alleviates NLRP3 inflammasome.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

DATA SHARING STATEMENT

The analyzed data sets generated during the present study are available from the corresponding author upon reasonable request.

SUPPLEMENTARY MATERIAL

Supplementary Table 1

Mouse primer sequences used in reverse transcription quantitative polymerase chain reaction

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