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ORIGINAL RESEARCH

UVB Upregulates Inflammatory Cytokines in Rosacea Cell Model by Promoting the Expression of TRPVs and TLR2

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Background: The expression of transient receptor potential vanilloid (TRPV) and Toll-like receptor 2 (TLR2) in the lesion sites of patients with rosacea is increased. Ultraviolet B (UVB) radiation can induce inflammation of keratinocytes in patients with rosacea, but the relationships between UVB and TRPVs/TLR2 in rosacea remain unclear.

Methods: RT-qPCR and Western Blot were applied to determine the expression levels of TRPV1, TRPV2, TRPV3, TRPV4 and TLR2 in the rosacea keratinocyte model after ultraviolet irradiation (n=3). After treating the rosacea cell model with TRPV1, TRPV4 and TLR2 inhibitors, ELISA was used to detect the expressions of rosacea-associated inflammatory cytokines in rosacea cells exposed to UVB, including IL-1β, IL-6, IL-8 and TNF-α (n=6). The changes in DNA methylation levels of TRPV1, TRPV4 and TLR2 promoters were detected by MeDIP, and the levels of histone modifications H3K4me3, H3K9me3 and H3K27me3 were determined by ChIP (n=3).

Results: UVB exposure increases the secretion levels of rosacea-associated inflammatory cytokines in HaCaT cells and LL-37-induced rosacea keratinocyte models, and promotes the expression of TRPV1, TRPV4 and TLR2 at mRNA and protein levels ($P < 0.05$). In the LL-37-induced HaCaT cell model, TRPV1 and TLR2 antagonists reduced the secretion levels of IL-1β and IL-8, and TRPV4 antagonists reduced IL-6 secretion level ($P < 0.05$). Under UVB exposure, TRPV4 and TLR2 antagonists respectively reduced the concentrations of IL-1β and TNF-α in the HaCaT cells stimulated by LL-37 ($P < 0.05$). In addition, UVB exposure increased the H3K4me3 level of the TRPV1 promoter region in HaCaT cells treated by LL-37 ($P < 0.05$).

Conclusion: Our results indicate that UVB induces inflammatory cytokines in rosacea keratinocyte models through upregulation of TRPV1, TRPV4, and TLR2 expression, potentially mediated by epigenetic mechanisms.

Keywords: Rosacea, ultraviolet, TRPV1, TRPV4, TLR2

Introduction

Rosacea is a prevalent, long-term inflammatory skin disease that commonly affects the center of the face. It has various skin manifestations, such as erythema, paroxysmal flushing, telangiectasia, papules, and pustules.[1] Patients often feel itchy, burning or stinging, and may also have ocular manifestations such as dilated capillaries at the eyelid margin, blepharitis, keratitis and conjunctivitis.[2] Although rosacea is not a fatal disease, facial skin lesions affect the patient's appearance, often causing psychological anxiety in patients and reducing their quality of life.[3] The pathogenesis of rosacea is not yet fully understood. According to existing research, the pathogenesis of rosacea is associated with six aspects: congenital immune dysfunction, skin barrier function impairment, neuroimmune communication network disorder, neurovascular regulatory dysfunction, microbial infection, and genetic factors.[4]

Ultraviolets may affect the occurrence and development of rosacea. Rosacea is more common in people with fair skin,[5] and the distribution of skin lesions in patients with rosacea mainly occurs in areas exposed to sunlight.[6,7] Under the induction of ultraviolet B (UVB), the dermal matrix in the lesion area of rosacea degradation and the loss of structural integrity around blood vessels occur in the skin, which subsequently leads to capillary dilation and the appearance of

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erythema.[8] UVB can induce innate immune responses and lead to the release of pro-inflammatory cytokines such as interleukin-1 β (IL-1β), IL-6, and IL-8 in HaCaT cells and primary normal human epidermal keratinocytes (NHEKs) in a dosage-dependent manner,[9] and activate the production of tumor necrosis factor-alpha (TNF-α) in primary keratinocytes.[10] The antimicrobial peptide cathelicidin is abundantly present in the skin lesions of rosacea patients. Under the action of its processing enzyme kallikrein 5 (KLK5), cathelicidin generates the product LL-37, causing the occurrence of rosacea inflammation.[11]

Persistent abnormal innate immune responses cause dysregulation of the neuroimmune communication network that controls vascular responses, thereby resulting in vasodilation in rosacea skin.[12] However, the current research on the complex networks of primary induction and secondary responses of neuroimmune communication has not been fully clarified. The transient receptor potential vanilloid (TRPV) may contribute significantly to the disruption of the neuroimmune communication network in rosacea. The mRNA expressions of TRPV1, TRPV2 and TRPV3 in the whole skin of rosacea patients were upregulated.[13] Additionally, immunofluorescence findings revealed elevated expression of TRPV4 in the epidermis of the affected lesions.[14] The expression of TRPV1 mRNA level and the density of TRPV1 nerve fibers in the whole skin are both increased in rosacea patients who were mainly manifested as erythema and telangiectasis.[15] Rosacea patients that mainly manifested as papules and pustules has the higher immune activities of TRPV2 and TRPV4 in dermis.[13] In patients with rosacea mainly presenting with rhinophyma, the staining intensity of dermal TRPV3 and TRPV4 increases, and the mRNA expressions of TRPV1 and TRPV3 were higher in whole skin.[13] Local application of selective TRPV4 inhibitors in mice can reduce skin pain and epidermal tissue damage induced by ultraviolet exposure.[16] Zhou et al demonstrated that TRPV4, functioning as a temperature-sensitive receptor, may contribute to pruritus in rosacea symptoms under the influence of local skin temperature changes.[14] The elevated expression of TRPVs in rosacea-affected skin tissue enhances the influx of Ca^{2+} ions at the synapses of sensory nerve endings, triggering the release of neuropeptide substances and consequently resulting in abnormal neurovascular regulation.[17] Moreover, the overexpression of TRPVs can stimulate immune cells located in proximity to cutaneous sensory nerve terminals, leading to the secretion of inflammatory cytokines and chemokines, and thereby inducing symptoms such as facial flushing and edema.[4]

Toll-like receptors (TLRs), functioning as key pattern recognition receptors, are essential in triggering inflammatory responses.[18] The skin can recognize TLRs via innate immune mechanisms, enabling it to detect potential threats and initiate defensive responses.[19,20] Within the Toll-like receptor family, TLR2 may mediate the pro-inflammatory effects induced by ultraviolet radiation. Compared with wild-type mice, TLR2 knockout mice have milder inflammatory responses such as erythema and scales induced by ultraviolets, and the induced expression of IL-1β is also decreased.[21] TLR2 is also implicated in the pathogenesis of rosacea. Kyoto Encyclopedia of Genes and Genomes (KEGG) and pathway analysis combined with gene set enrichment analysis revealed that the TLR2 gene in the full skin lesion samples of rosacea patients was enriched compared with that in healthy controls,[22] and the results of RT-PCR and immunohistochemistry indicated that the mRNA and protein of TLR2 were highly expressed in both the epidermis and dermis of the lesion sites.[23] Immunohistochemical analysis revealed co-localization of TLR2 and KLK5 in both the epidermis and dermis of the lesion sites in rosacea patients. In human epidermal keratinocytes, TLR2 does not significantly affect the transcription or protein synthesis of KLK5 but promotes its release in a calcium-dependent manner, thereby mediating the inflammatory process associated with rosacea.[23]

In a MAS-related G protein-coupled receptor X2-dependent manner, LL-37 upregulates TRPV4 expression via the Gi/o protein signaling pathway, thereby exacerbating inflammatory responses.[24] In LL-37-induced mouse and HaCaT cell models, LL-37 promotes overexpression of TLR2.[25] Injecting LL-37 into the skin of mice induced an inflammatory resembling the phenotype observed in individuals with rosacea.[26] In vitro, LL-37 can induce inflammatory responses from keratinocytes,[25] macrophages,[27] etc., facilitate degranulation of mast cells,[28] and simulate the inflammatory environment of rosacea. Therefore, LL-37 is often used for modeling rosacea in vivo and in vitro.[27,29] The HaCaT cell line is a keratinocyte cell line, and LL-37-induced HaCaT cells are widely recognized in vitro keratinocyte models of rosacea. By stimulating HaCaT cells with exogenous LL-37, the above pathological features can be stably reproduced, and the expected response to known therapeutic drugs is presented, confirming the pathological simulation and experimental reliability of this model.[29] In this study, we used it to simulate the epidermal keratinocytes of rosacea in vitro.

Epigenetics denotes inheritable modifications in gene activity and chromatin architecture caused by chemical modification when the DNA sequence remains unchanged.[30,31] These modifications include DNA methylation, histone modification, chromatin remodeling, and non-coding RNA regulation, among others.[32] In studies focusing on chronic visceral pain in rats, it was found that the acetylation of histone H3 within the TRPV1 promoter region, which mediates visceral pain in DRG neurons, was elevated, and an increased level of histone acetyltransferase EP300 was observed. Moreover, its combination with the TRPV1 promoter region was associated with the heightened TRPV1 expression detected in rat neurons after stress treatment.[33] In the studies on periodontitis [34] and type 2 diabetes,[35] it was observed that the methylation level in the promoter region of the TLR2 gene was significantly decreased. This epigenetic regulation leads to inflammatory responses and is contributes to the progression of the corresponding diseases. We hypothesized that UVB promotes inflammatory cytokine expression in rosacea keratinocytes through TRPVs or TLR2 modulation, potentially involving epigenetic mechanisms.

This study found that UVB radiation can promote the release of downstream pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α by up-regulating the expressions of TRPV1, TRPV4 and TLR2 in LL-37-induced rosacea keratinocytes. Pharmacological inhibition experiments further indicated that in vitro experimental model for rosacea, the antagonism of TRPV1 and TLR2 could specifically reduce the levels of IL-1β and IL-8, while the inhibition of TRPV4 mainly decreased IL-6. Under the stimulation of UVB, blocking TRPV4 or TLR2 can also respectively weaken the production of IL-1β and TNF-α. Mechanistically, UVB exposure can significantly increase the H3K4me3 modification level in the TRPV1 promoter region of rosacea cells, suggesting that its expression is regulated by epigenetics. Collectively, these results deepen our comprehension of the molecular pathways involved in UVB-induced rosacea pathogenesis and offer novel insights for the prevention and therapeutic management of rosacea.

Materials and Methods

Cell Culture and Treatment

Human HaCaT cells were obtained from the National Collection of Authenticated Cell Cultures (China). The HaCaT cells used for the experiment were all in 5–10 generations. HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, America) supplemented with 10% FBS, which is incubated in 37°C with 5% CO_2. The rosacea model of HaCaT cells was induced through treatment with LL-37 (Selleck, China). Adjust the position of the ultraviolet lamp (PHILIPS, Netherlands, irradiation wavelength: 297 nm) so that the irradiation intensity detected by the ultraviolet irradiometer (Beijing Shida Photoelectric Technology Limited Company, China) is 200 μW/cm². The UVB irradiation time (125s, 250s, 375s, 500s) was calculated based on different irradiation doses (25 mJ/cm², 50 mJ/cm², 75 mJ/cm², 100 mJ/cm²). When irradiating the cells in the culture dishes, the lid of the dish needed to be opened. The TRPV1 receptor antagonist capsazepine (10 μM, Selleck, China),[36] TRPV4 receptor antagonist RN-1734 (20 μM, Selleck, China)[37] and TLR2 receptor antagonist TLR2-IN-C29 (50 μM, Selleck, China)[38] were used to treat HaCaT cells stimulated by LL-37. All the above concentrations have been confirmed not to affect cell activity.[39–41] Cells were collected after 24 hours of culture following UVB irradiation or drug treatment.

Cytotoxicity Assay

HaCaT cells were grown in 96-well plates, and spent culture medium was removed when the cells were in logarithmic phase. 100 μL of media containing 10% CCK-8 reagent was introduced into each well, followed by incubated at 37°C in a 5% CO_2 environment for 1h. The absorbance at 450nm was subsequently determined using an automatic enzyme-linked immunoassay (BioTek, America).

Enzyme-Linked Immunosorbent Assay (ELISA)

The supernatant from the cell culture was harvested, and the levels of IL-1β (R&D, America), IL-6 (R&D, America), IL-8 (R&D, America) and TNF-α (R&D, America) were measured by enzyme-linked adsorption assay kits. The absorbance was measured by automatic enzyme-linked immunoassay (BioTek, America), and the data were analyzed by Excel software.

Quantitative Real-Time Polymerase Chain Reaction Analysis (qRT-PCR)

RNA was extracted employing Trizol reagent (Accurate Biology, China). Following this, the isolated RNA was converted into cDNA with Evo M-MLV RT Mix Kit with gDNA Clean for qPCR (Accurate Biology, China). Subsequently, qRT-PCR conducted employing the SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology, China) on a 96-well fluorescence quantitative PCR system (Roche, America). The primer sequences for RT-qPCR are shown in Table 1.

Western Blot

Cells were extracted using RIPA (Biyuntian, China) lysis buffer. The concentration of the extracted protein samples was determined by the BCA protein assay kit (Thermo Fisher Scientific, America). Following extraction, proteins were resolved via SDS-PAGE and subsequently transferred onto PVDF membranes. The membranes were subsequently closed in 5% skimmed milk at ambient temperature. Primary antibodies used for detection included: mouse anti-GAPDH (1:5000, Proteintech, America), mouse anti-TRPV1 (1:1000, Proteintech, America), rabbit anti-TRPV2 (1:1000, Abcam, UK), rabbit anti-TRPV3 (1:1000, Cell Signaling Technology, America), rabbit anti-TRPV4 (1:1000, Cell Signaling Technology, America), mouse anti-TLR2 (1:1000, Proteintech, America). The membranes were rinsed and then treated with HRP- labeled goat anti-mouse antibodies (Abiowell, China) or goat anti-rabbit antibodies (Abiowell, China). Immunoreactions were visualized using SuperSignalTM West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, America). Selecting the right exposure conditions, the ImageQuant LAS 4000mini instrument was used for development. Grayscale analysis of the target protein bands was conducted with the ImageJ software. Taking GAPDH as the internal reference, the ratio of the gray values of the target gene band to those of the internal reference gene band was the relative expression level of the target protein.

Methylated DNA Immunoprecipitation (MeDIP)

MeDIP was carried out using the EpiQuik™ Methylated DNA Immunoprecipitation Kit (EPIGENTEK, America). The primer pairs used in this experiment are shown in Table 2.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed using the EZ ChIP™ Chromatin Immunoprecipitation Kit (MERCK, Germany). Histone H3K4me3, H3K9me3 and H3K27me3 antibodies (ACTIVE MOTIF, America) were used for IP response.

Table 1 Sequences of Primers for RT-qPCR

Gene	Forward (5′-3′)	Reverse (5′-3′)
RPLP0	TGGTCATCCAGCAGGTGTTCGA	CAGACACTGGCAACATTGCGG
TRPV1	CTGCCGACCATCACAGTC	CTGCGATCATAGAGCCTGAGG
TRPV2	AGTCAACCTCAACTACCGAAAGG	CCGCATTGAAGAGCCGATCT
TRPV3	TGGGCAGGTTCATCAACGC	CCTTCGTGTTGGTACTTGGGG
TRPV4	GATGGGCGACCAAATCTGC	GAGGACTCATATAGGGTGGACTC
TLR2	CTCTCGGTGTCGGAATGTC	AGGATCAGCAGGAACAGAGC

Notes: RPLP0 was used as an internal reference control for gene expression normalization. The qRT-PCR results were processed using the $2^{-\Delta\Delta Ct}$ method.

Table 2 Sequences of Primers for MeDIP-PCR

Gene	Forward (5′-3′)	Reverse (5′-3′)
TRPV1	GTAGCACGCAGACCCCTAATC	GAAGTAGAAGATGCGCTTGACA
TRPV4	CTACGGCACCTATCGTCACC	TTAGGCGTTTCTTGTGGGTCA
TLR2	GCTGCTCGGCGTTCTCTCAGG	TGTCCAGTGCTTCAACCCACAACT

Statistical Analysis

Data analysis was performed with the aid of IBM SPSS Statistics 27.0.1 software, and statistical charts were drawn with GraphPad Prism 8.0.2 software. Data are expressed as mean ± standard deviation. The comparison of the mean of two independent samples was conducted using unpaired *T*-test, and when the data failed to conform to a normal distribution or exhibited heterogeneous variance, the non-parametric test of two independent samples (Mann–Whitney *U*-test) was employed. For multiple comparisons among independent sample means, the SNK-q test was applied for pairwise comparisons under conditions of equal variances, while the Tamhane's T2 test was applied in cases of unequal variances. A P value <0.05 was statistically significant.

Results

UVB Irradiation Affects Keratinocyte Proliferation and Cell Morphology

The proliferative activity of HaCaT cells following exposure to varying doses of UVB was assessed using the CCK-8 method. At an irradiation dose of 25 mJ/cm², the average cell survival rate was 77.17%, which showed a statistically significantly difference compared to the negative control group. The cell viability in the 50 mJ/cm² group decreased by 58.33% relative than control group. In the 75 mJ/cm² and 100 mJ/cm² groups, cell growth was markedly inhibited, with cell activity reduced to 46.00% and 32.83%, correspondingly (Figure 1A).

HaCaT cells cultured without UVB irradiation exhibited normal morphology, appearing flat and polygonal, and forming a cobblestone-like structure. After UVB irradiation at different doses, the morphological changes in the 25 mJ/cm² group were minimal. However, in the 50 mJ/cm² group, some cells became rounded, and a few detached or fragmented cells were observed. In the 75 mJ/cm² group, most cells lost their normal shape, and a considerable quantity of detached cells were observed. No intact cells were observed in the 100 mJ/cm² group, as the majority were severely damaged and disintegrated (Figure 1B). According to these results, an irradiation dose of 25 mJ/cm² was chosen for follow-up experiments to minimize the inhibitory impact of UVB on HaCaT cell activity.

UVB Irradiation Induced Increased Secretion of Inflammatory Cytokines in Rosacea Cell Model

The proliferation activity of HaCaT cells stimulated by different concentrations of LL-37 was evaluated by the CCK-8 method. When the treatment concentrations of LL-37 were 0 μM, 2 μM, 4 μM, 6 μM, and 8 μM, the proliferation activities of HaCaT cells were 100.0%, 99.31%, 115.0%, 83.77%, and 94.90% respectively (Figure 2A). No obvious changes were observed in the cell morphology under the microscope in each group (Figure 2B). After stimulating HaCaT cells with varying concentrations of LL-37 for 24h, the supernatant of the cell culture medium was collected. The levels of rosacea-associated inflammatory factors secreted by HaCaT cells in the medium supernatant, including IL-1β, IL-6,

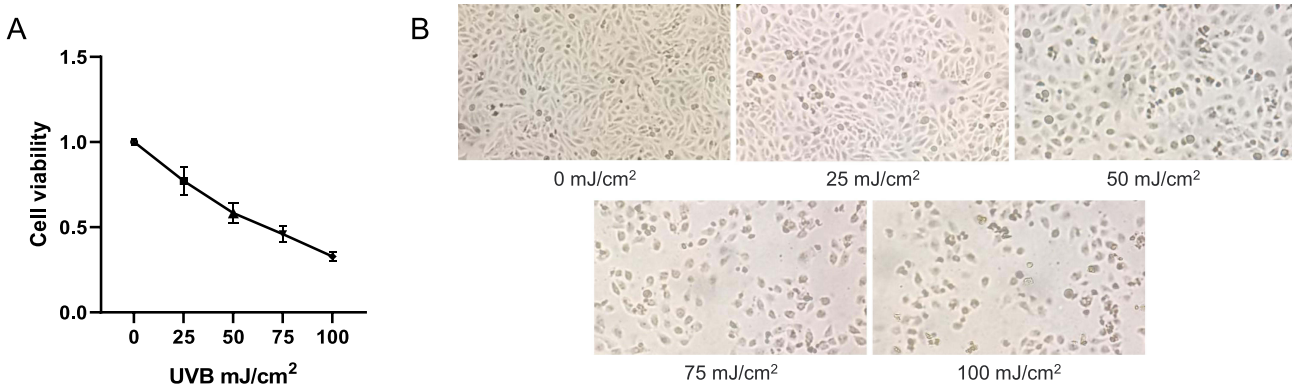

Figure 1 The effect of UVB irradiation on the growth activity and cell morphology of HaCaT cells. (**A**) CCK-8 was carried out for the proliferation activity of HaCaT cells after irradiation with 0 mJ/cm², 25 mJ/cm², 50 mJ/cm², 75 mJ/cm², 100 mJ/cm² UVB (n = 6). (**B**) The microscopic morphology of HaCaT cells after irradiation with 0 mJ/cm², 25 mJ/cm², 50 mJ/cm², 75 mJ/cm², 100 mJ/cm² UVB under a 100× microscope.

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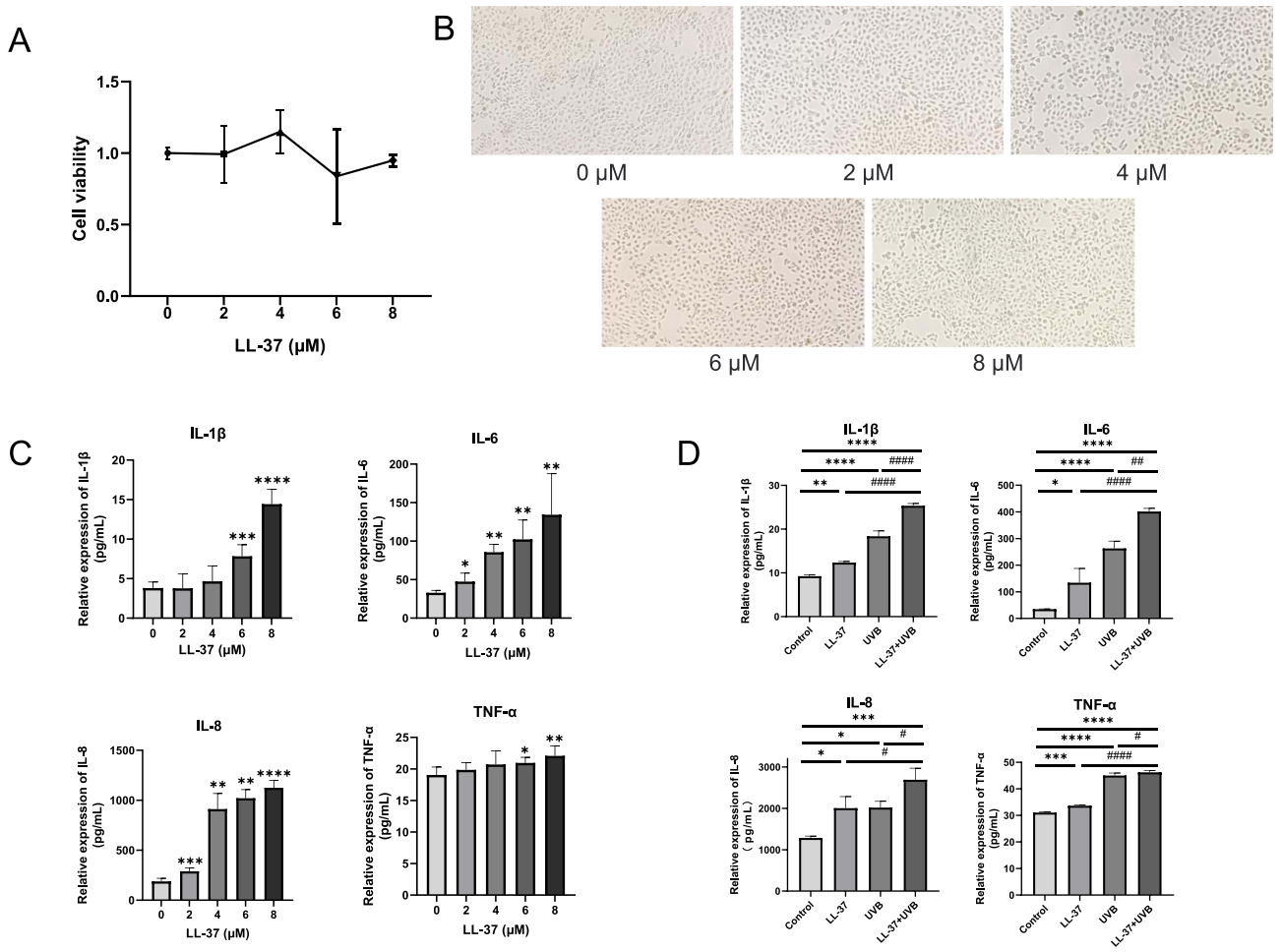

Figure 2 The effect of UVB irradiation on the secretion of inflammatory cytokines in the rosacea keratinocyte model. (**A**) The proliferation activity of HaCaT cells at 0 μM, 2 μM, 4 μM, 6 μM, and 8 μM LL-37 was detected by the CCK-8 method (n = 6). (**B**) The microscopic morphology of HaCaT cells at 0 μM, 2 μM, 4 μM, 6 μM, and 8 μM LL-37 under a 40× microscope. (**C**) The secretion levels of inflammatory factors in HaCaT cells stimulated by 0 μM, 2 μM, 4 μM, 6 μM, and 8 μM LL-37 were detected by ELISA (n = 6, *P < 0.05, *P < 0.01, ***P < 0.001, ****P < 0.0001). (**D**) The secretion levels of rosacea-related inflammatory factors in HaCaT cells after induction by 8 μM LL-37 and/or 25 mJ/cm² UVB were detected by ELISA (n = 3, compared with the control group, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; compared with the LL-37 + UVB group, #P < 0.05, ### P < 0.001, ##### P < 0.0001).

IL-8, and TNF-α, were measured employing ELISA methodology (Figure 2C). In comparison with the control group, when the LL-37 stimulation concentration reached 6 μM, the concentrations of IL-1β and TNF-α increased by 1.06 and 0.10 times respectively. When the stimulation concentration of LL-37 was 2 μM, the release of IL-6 and IL-8 increased by 0.44 and 0.53 times respectively. LL-37 stimulation enhanced the expression of rosacea-related inflammatory factors in HaCaT cells, enabling these cells to mimic the inflammatory characteristics of rosacea epidermal keratinocytes in vitro. When the LL-37 stimulation concentration was 8 μM, the concentration of these four cytokines in HaCaT cells was significantly elevated. In subsequent experiments, HaCaT cells stimulated with 8 μM LL-37 were used as an in vitro experimental model for rosacea.

Compared with the control group, LL-37 markedly triggered the upregulation of inflammatory cytokines linked to rosacea pathology, including IL-1β, IL-6, IL-8, and TNF-α in HaCaT cells, and their expressions were 1.34, 3.89, 1.56 and 1.08 times that of the control group, respectively. Compared with the control group, UVB irradiation markedly increased the secretion of IL-1β, IL-6, IL-8, and TNF-α in HaCaT cells, and the expression levels were 1.99-, 7.62-, 1.57-, and 1.45-fold higher than those in the control group. In the LL-37+UVB group, the levels of IL-1β, IL-6, IL-8, and TNF-α increased by 1.74, 10.63, 1.09 and 0.49 times respectively compared with the control group. Furthermore, in contrast to the LL-37-induced group, UVB irradiation in the LL-37+UVB group significantly upregulated the expression of IL-1β, IL-6, IL-8, and TNF-α in HaCaT cells, with expression levels of 2.05, 2.99, 1.34 and 1.37 times, respectively. Additionally, the secretion

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levels of IL-1β, IL-6, IL-8, and TNF-α in the LL-37-induced rosacea keratinocyte model in the LL-37+UVB group were markedly elevated by 0.38, 0.53, 0.33 and 0.03 time respectively, compared to those in the UVB-treated cells (Figure 2D).

UVB Irradiation Induced Increased Expression of TRPV1, TRPV2, TRPV3, TRPV4 and TLR2 in Rosacea Keratinocyte Model

qRT-PCR was employed to examine the effects of UVB on the mRNA expression levels of TRPV1, TRPV2, TRPV3, TRPV4 and TLR2 in HaCaT cells and HaCaT cells stimulated by LL-37 (Figure 3A). Compared with control group, the mRNA expression levels of TRPV1, TRPV4 and TLR2 in HaCaT cells treated with LL-37 were 1.22 times, 2.23 times and 1.41 times that of the control group, respectively, while the mRNA expressions of TRPV2 and TRPV3 had no significant changes. The mRNA expressions of TRPV1, TRPV4 and TLR2 in UVB irradiation group were 1.385, 2.073 and 2.486 times that of the control group, respectively. And the mRNA expressions of TRPV2 and TRPV3 exhibited an increasing tendency, variations did not reach statistical significance. The mRNA expression levels of TRPV1, TRPV4 and TLR2 in the LL-37+UVB group increased by 0.99, 2.83 and 2.06 times respectively in comparison with the control group. TRPV1, TRPV4 and TLR2 mRNA levels in LL-37+UVB group increased significantly by 0.63, 0.72 and 1.17 times respectively compared to LL-37 group. The

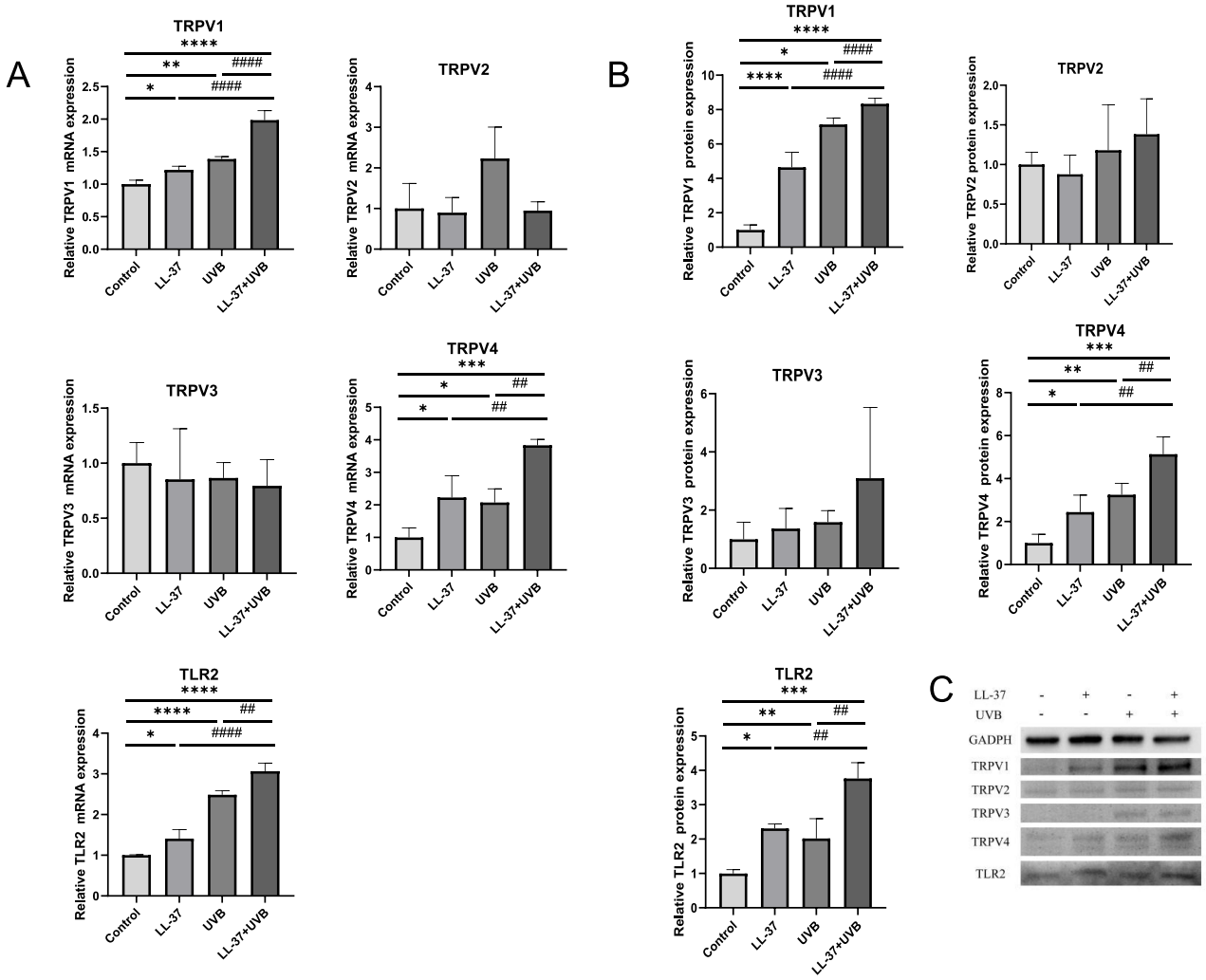

Figure 3 The influence of UVB on the mRNA and protein expressions of TRPV1, TRPV2, TRPV3, TRPV4 and TLR2 in the rosacea keratinocyte model. (**A**) The expression levels of TRPV1, TRPV2, TRPV3, TRPV4 and TLR2 mRNA in HaCaT cells and HaCaT cells treated by 8 μM LL-37 after 25 mJ/cm² UVB exposure were detected by qRT-PCR. (**B** and **C**) The protein expression levels of TRPV1, TRPV2, TRPV3, TRPV4 and TLR2 in HaCaT cells and HaCaT cells stimulated by 8 μM LL-37 after 25 mJ/cm² UVB irradiation were measured by Western blot. (n = 3, compared with the control group, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; compared with the LL-37 + UVB group, ##P < 0.01, #### P < 0.0001.).

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expression levels of TRPV1, TRPV4 and TLR2 mRNA were 1.43 times, 1.85 times, and 1.23 times that of the UVB group, respectively. However, TRPV2 and TRPV3 mRNA expression levels in LL-37+UVB group showed no statistically significant differences relative to those in LL-37 group or UVB group.

Under the induction of LL-37, the protein expression levels of TRPV1, TRPV4, and TLR2 in HaCaT cells increased by 3.65, 1.44 and 2.31 times compared with the control group, respectively. Similarly, UVB irradiation also led to the protein expressions of TRPV1, TRPV4 and TLR2 in HaCaT cells increasing by 7.14, 3.25 and 2.02 times, respectively, relative to the control group. In the LL-37+UVB group, the protein expression levels of TRPV1, TRPV4, and TLR2 were 8.341 times, 5.135 times and 3.765 times that of the control group, respectively. Furthermore, in comparison to the LL-37 induction group alone, the protein expression intensities of TRPV1, TRPV4, and TLR2 in the LL-37+UVB group increased by 0.79, 1.10 and 0.63 times. Additionally, the protein expression levels of TRPV1, TRPV4, and TLR2 in the LL-37+UVB group increased by 0.17 times, 0.58 times and 0.87 times respectively compared to the UVB irradiation group alone. Nevertheless, no statistically significant alterations were detected in TRPV2 expression across all four groups. In comparison to the control group, LL-37 induction did not result in significant alterations in TRPV3 protein expression, whereas UVB irradiation induced an upward trend in TRPV3 protein expression without statistical significance. Moreover, no statistically significant difference was observed in TRPV3 protein expression between the UVB irradiation group and the LL-37+UVB group (Figure 3B and C). It may suggest that in the LL-37-induced HaCaT cell model, UVB exposure did not cause significant changes in TRPV2 and TRPV3 at the mRNA and protein levels.

TRPV1, TRPV4 and TLR2 Receptor Antagonists Inhibited UVB-Induced Inflammatory Factor Expression in Rosacea Cell Model

ELISA was employed to quantify the concentrations of rosacea-associated inflammatory factors in the supernatant of cell culture media. Our results demonstrated that capsazepine, a TRPV1 antagonist, reduced the expressions of IL-1β and IL-8 induced by LL-37 to 0.87 times and 0.72 times that of the control group, respectively. Additionally, the concentrations of IL-6 and TNF-α induced by LL-37 exhibited a decreasing tendency, though the variations did not reach statistically significant (Figure 4A). RN-1734, an antagonist of TRPV4, decreased the secretion level of IL-6 induced by LL-37 to 0.80 times that of the control group. There was a decreasing trend in the secretion levels of IL-1β and IL-8, but the difference was not statistically significant. In addition, it had no inhibitory effect on TNF-α generation (Figure 4B). Treatment with TLR2-in-C29, an antagonist of TLR2, inhibited the secretion of IL-1β and IL-8 induced by LL-37 to 0.52 times and 0.68 times that of the control group, and reduced the LL-37-induced secretion of IL-6 and TNF-α but the results demonstrated no significant difference (Figure 4C).

UVB irradiation up-regulated the expression of these cytokines induced by LL-37, while capsazepine showed a decreased trend in the proinflammatory effect of UVB on HaCaT cells treated by LL-37, but the outcomes did not reach statistical significance (Figure 4D). RN-1734 inhibited the proinflammatory effect of UVB on rosacea cell model, significantly inhibited the secretion of IL-1β by 0.21 times compared with the control group, and reduced the secretion levels of IL-6, IL-8, and TNF-α, but the results were not statistically different (Figure 4E). Compared with UVB irradiated LL-37 induced keratinocyte model group, TLR2-IN-C29 could significantly inhibit the promoting effect of UVB on the concentration of TNF-α in rosacea cell model to 0.92 times that of the control group, and reduce the expression levels of IL-6 and IL-8, but no statistically significant difference was observed. However, it had no inhibitory ampact on the secretion of IL-1β (Figure 4F). It may suggest that the inhibitors of these pathways only have a relatively obvious inhibitory effect on some inflammatory factors.

Epigenetic Changes of TRPV1, TRPV4 and TLR2 Promoters in Rosacea

We speculate whether UVB could cause epigenetic changes in TRPV1, TRPV4 and TLR2 promoter regions in rosacea keratinocyte models, and then affect the expression levels of TRPV1, TRPV4 and TLR2. We used MeDIP method to investigate whether UVB irradiation could change the DNA methylation levels of promoters of TRPV1, TRPV4 and TLR2 genes in the keratinocyte model of rosacea. Compared with LL-37 induction group, the relative levels of TRPV1, TRPV4 and TLR2 promoter methylation in the rosacea model of HaCaT cells after UVB irradiation showed a decreased trend, although the findings did not achieve statistical significance (Figure 5A).

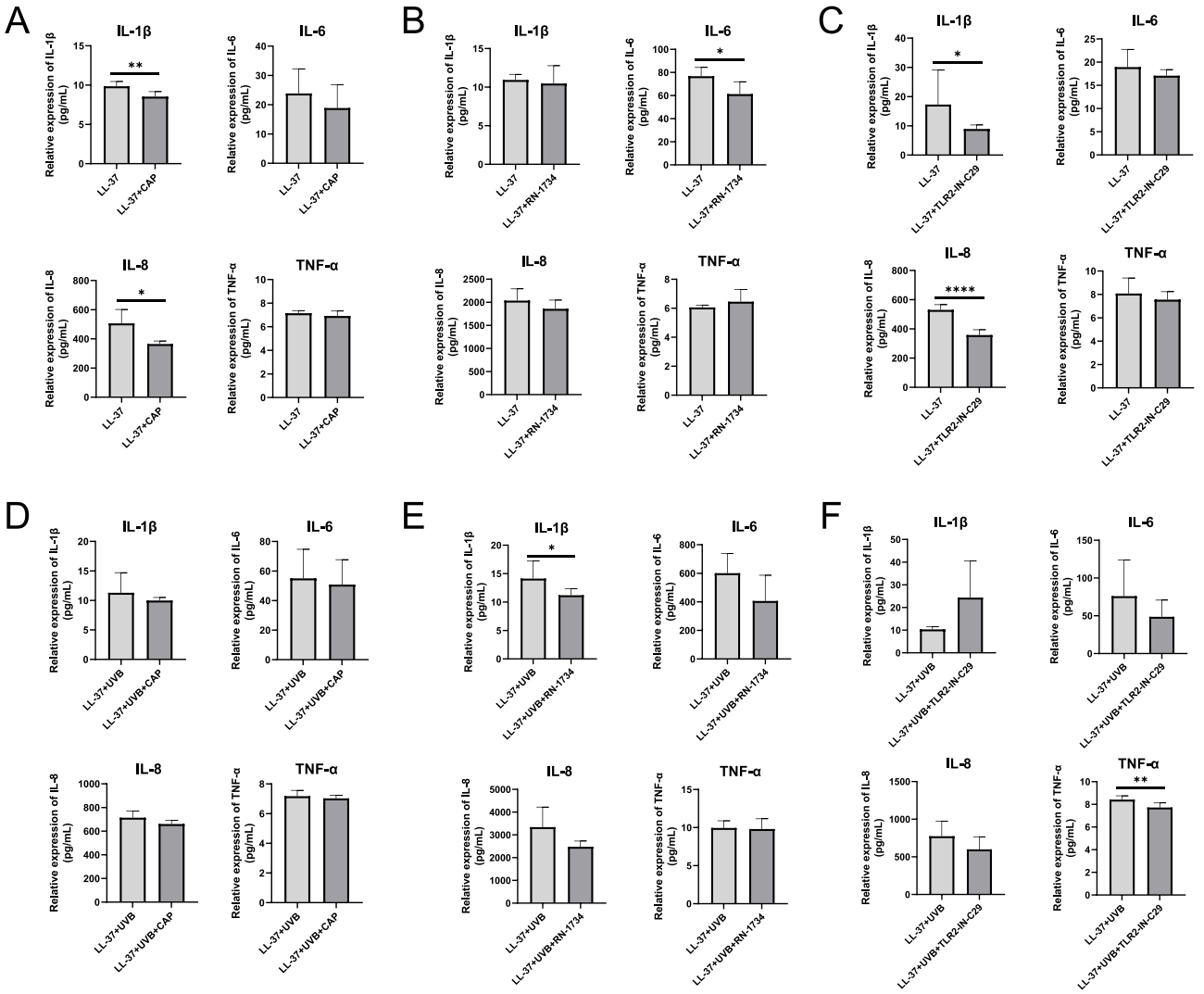

Figure 4 The effects of TRPV1, TRPV4 and TLR2 receptor antagonists on the expression of inflammatory factors in vitro experimental model for rosacea that underwent or did not undergo UVB irradiation. (**A-C**) The levels of inflammatory factors in the rosacea keratinocyte model were determined by ELISA after the treatment with 10 μM CAP, 20 μM RN-1734 and 50 μM TLR-IN-C29. (**D-F**) The levels of inflammatory factors in the rosacea keratinocyte model were detected by ELISA after 25 mJ/cm² UVB irradiation and treatment with 10 μM CAP, 20 μM RN-1734, and 50 μM TLR-IN-C29. (n=6, *P < 0.05, **P < 0.01, ****P < 0.0001.) CAP: capsazepine.

The three most common histone methylation modification modes of H3K4me3, H3K9me3 and H3K27me3 were selected as research objects, and the levels of H3K4me3, H3K9me3 and H3K27me3 in the promoters of TRPV1, TRPV4 and TLR2 genes were investigated by ChIP method. Compared with LL-37 group, UVB caused an elevation in the H3K4me3 modification level of TRPV1, with a fold-change of 3.05. However, the increasing trend of the H3K9me3 and H3K27me3 modification levels of TRPV1 and the H3K4me3 and H3K9me3 modification levels of TRPV4 had no significant difference. UVB irradiation decreased the H3K27me3 modification levels of TRPV4 and the H3K4me3, H3K9me3 and H3K27me3 modification levels of TLR2, but the variations were not statistically significant (Figure 5B). These results may suggest that UVB regulates the expression of TRPV4 and TLR2 through pathways other than these epigenetic modifications.

Discussion

The results of our study demonstrate that UVB radiation up-regulates TRPV1 expression in keratinocytes, potentially through increased H3K4me3 levels at the TRPV1 gene promoter. In contrast, UVB-induced up-regulation of TRPV4 and TLR2 occurs independently of this epigenetic mechanism. In vitro experimental model for rosacea, the coordinated overexpression of these genes synergistically enhances the inflammatory response, suggesting their collective

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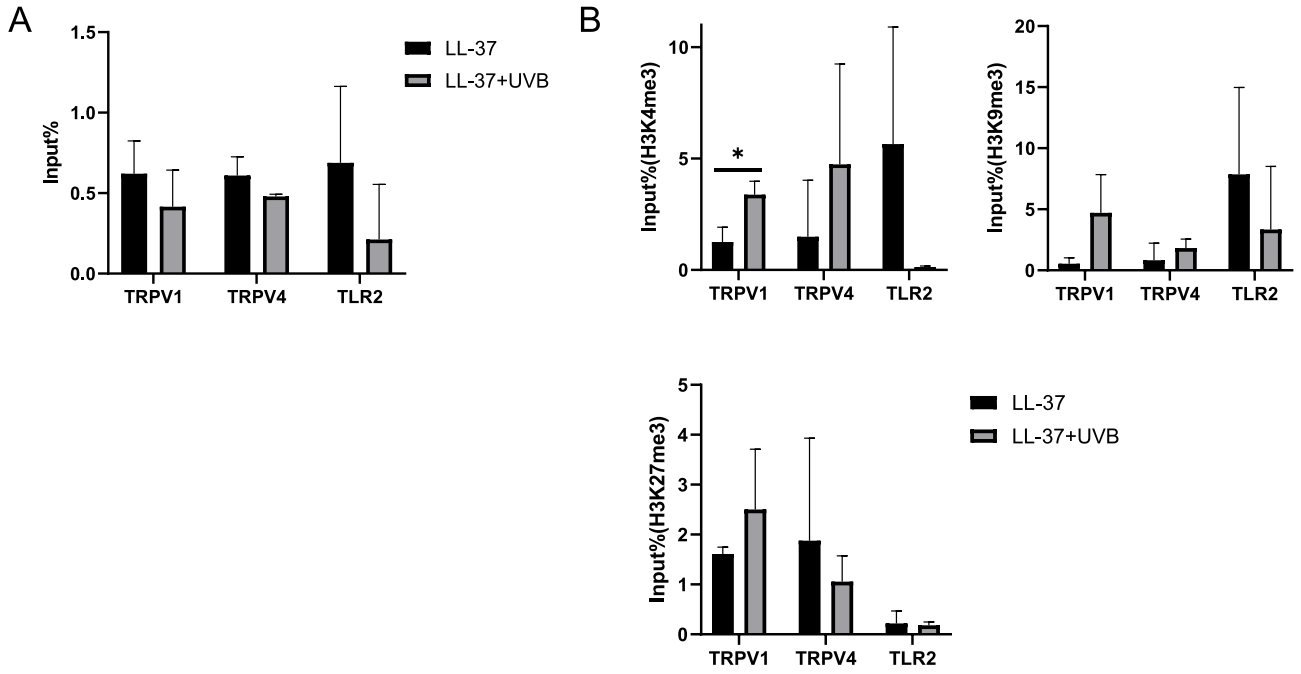

Figure 5 The epigenetic alterations of TRPV1, TRPV4 and TLR2 promoters in rosacea cell model. (**A**) MeDIP was used to assess the relative methylation modification levels of the promoters of TRPV1, TRPV4 and TLR2 genes in the rosacea keratinocyte model after 25 mJ/cm² UVB irradiation. (**B**) ChIP was applied to assess the modification levels of H3K4me3, H3K9me3 and H3K27me3 in the promoters of TRPV1, TRPV4 and TLR2 genes in the rosacea keratinocyte model after 25 mJ/cm² UVB irradiation. (n = 3, *P < 0.05.).

contribution to disease pathogenesis. Keratinocytes exhibit sensitivity to ultraviolet irradiation, and UVB exposure has been shown to induce apoptosis and necrosis in HaCaT cells,[42] as well as cause DNA damage, lipid oxidation, and protein degradation within cells.[43] Our findings demonstrated that UVB irradiation reduced the proliferation of HaCaT cells in a way that was dependent on the dosage and progressively induced cellular degeneration, fragmentation, and death as the irradiation dose increased.

LL-37 is prominently elevated in facial lesions of individuals with rosacea and contributes to the pathological process of rosacea via multiple mechanisms, including triggering of the NLRP3 inflammasome in macrophages,[44] elevation in the levels of inflammatory factors such as IL-1β in keratinocytes,[45] and induction of mast cell degranulation.[24] Consequently, LL-37 is frequently employed in the establishment of in vivo and in vitro experimental models for rosacea.[26,29,46] In patients with rosacea, elevated expression levels of IL-1β, IL-8,[47] and TNF-α [48] in the whole skin,[49] and IL-6 in plasma,[50] have been observed. These cytokines trigger inflammatory responses in the skin, leading to characteristic symptoms such as facial flushing. Our experimental findings demonstrate that LL-37 stimulation induces HaCaT cells to produce rosacea-associated inflammatory factors. The secretion levels of these inflammatory factors increase progressively with up-regulated concentrations of LL-37, consistent with previous reports from in vitro rosacea models.[51,52] To simulate the pathological state of rosacea keratinocytes in vitro, we stimulated HaCaT cells with LL-37.

UVB radiation is related to the innate immune response of the skin and can induce the production of cytokines such as IL-1β,[53] IL-6,[54] IL-8 and TNF-α [55] in the skin of healthy people in a time- and dose-dependent manner, triggering inflammatory responses in the skin. Our findings indicate that, compared to normal controls, UVB irradiation significantly increases the secretion levels of IL-1β, IL-6, IL-8, and TNF-α in HaCaT cells. Furthermore, when comparing with the LL-37-treated group or the UVB-only group with the HaCaT cells treated by LL-37, UVB irradiation increased the secretions of the four cytokines in the HaCaT cells stimulated by LL-37, suggesting that UVB irradiation promotes the release of inflammatory factors in both HaCaT cells and the LL-37-induced HaCaT cell model; UVB and LL-37 have a synergistic effect, after UVB irradiation, the LL-37-induced rosacea keratinocyte model will generate more pronounced inflammatory responses than the LL-37-treated HaCaT cell model that was not exposed to UVB and the HaCaT cells that also undergo irradiation.

The expressions of TRPV1, TRPV2, TRPV3, and TRPV4 are upregulated in the skin of rosacea patients,[13] with an increased density of TRPV1 nerve fibers in the epidermis and dermis observed.[15] TRPVs are Ca^{2+} ion channel proteins, and their activation induces intracellular Ca^{2+} influx and alterations in membrane potential. Elevated TRPV expression may enhance Ca^{2+} flux within sensory nerve endings, leading to the secretion of neuropeptides such as substance P and the subsequent activation of immune cells surrounding these nerve endings. This cascade triggers the secretion of pro-inflammatory mediators, including cytokines and chemokines, thereby contributing to symptoms such as facial flushing and edema. Additionally, TLR2 is highly expressed in the whole skin of rosacea patients, co-localizing with KLK5 at lesion sites. TLR2 promotes KLK5 release in human epidermal keratinocytes in a calcium-dependent manner, mediating the inflammatory process of rosacea.[23] Our findings demonstrate that, compared to the normal control group, both LL-37 induction alone and UVB irradiation significantly increase the mRNA and protein expression of TRPV1, TRPV4, and TLR2 in HaCaT cells. Furthermore, in the LL-37-induced HaCaT cell model, UVB irradiation leads to higher mRNA and protein expression levels of TRPV1, TRPV4, and TLR2 compared to either LL-37 induction or UVB irradiation alone. These results indicate that UVB exerts its pro-inflammatory effects by upregulating TRPV1, TRPV4, and TLR2 expression, with this effect being more pronounced in rosacea patients. However, no significant differences were detected in the mRNA or protein expression levels of TRPV2 and TRPV3 across all groups, these mean LL-37 and UVB do not influence the productions of TRPV2 and TRPV3 in normal HaCaT cells and rosacea keratinocyte model.

We found that both TRPV1 antagonist capsazepine and TLR2 antagonist TLR2-in-C29 could significantly inhibit IL-1β and IL-8 in the HaCaT cells stimulated by LL-37, and TRPV4 antagonist RN-1734 could greatly inhibit IL-6 in LL-37-induced HaCaT cell model. These results indicated that inhibiting TRPV1 or TLR2 alone can reduce the levels of IL-1β and IL-8 in the LL-37-induced rosacea keratinocyte model. In addition, inhibiting TRPV4 alone reduce IL-6. RN-1734 and TLR2-IN-C29 also respectively lowered the concentrations of IL-1β and TNF-α in the HaCaT cells treated by LL-37 irradiated by UVB. It was suggested that UVB increases the secretion of IL-1β and TNF-α in the HaCaT cells stimulated by LL-37 through TRPV4 and TLR2 respectively.

DNA methylation and histone modification are key components of the epigenetic regulatory mechanism. DNA methylation is essential for various physiological processes, including embryonic development and gametogenesis.[56] In pathological contexts, aberrant DNA methylation can alter gene expression levels and contribute to disease pathogenesis.[57] Similarly, histone modifications also regulate gene expression and mediate both physiological and pathological processes. Notably, histone methylation marks such as H3K4me3, H3K9me3, and H3K27me3 have been extensively associated with cancer development.[58–62] In this study, we examined the DNA methylation levels of the promoters of TRPV1, TRPV4, and TLR2 genes, as well as the levels of histone H3K4me3, H3K9me3, and H3K27me3 modifications in a UVB-irradiated rosacea keratinocyte model, for exploring the reasons for the increase of TRPV1, TRPV4 and TLR2 in the rosacea keratinocyte model caused by UVB from the perspective of epigenetics.

Compared with the LL-37 induction group, the relative methylation levels of the TRPV1, TRPV4, and TLR2 promoters in the HaCaT cells treated by LL-37 after UVB irradiation exhibited a decreasing trend, however, these changes were not statistically significant. DNA methylation can recruit specific proteins to suppress gene expression or inhibit the binding of DNA to transcription factors, leading to gene silencing.[63] UVB irradiation may induce hypo-methylation of the TRPV1, TRPV4, and TLR2 promoters in the HaCaT cells stimulated by LL-37, thereby promoting their overexpression. Nevertheless, this hypothesis requires further validation in future studies.

In comparison with the LL-37 induction group, UVB irradiation significantly increased the H3K4me3 modification level of the TRPV1 promoter in the LL-37-induced HaCaT cell model. H3K4me3 is generally recognized as a histone mark that recruit chromatin-modifying enzymes and activates transcriptional mechanisms.[64] Our findings suggest that UVB irradiation may enhance TRPV1 transcription in the rosacea keratinocyte model by increasing the H3K4me3 level at the promoter, thereby significantly upregulating TRPV1 expression. However, UVB irradiation had no notable effect on H3K4me3 modification levels at the TRPV4 and TLR2 promoters in the LL-37-induced rosacea keratinocyte model, indicating that UVB may upregulate TRPV4 and TLR2 expression through alternative pathways. Additionally, UVB irradiation did not cause significant alterations in the H3K9me3 and H3K27me3 modification levels of TRPV1, TRPV4, and TLR2, suggesting that UVB-mediated regulation of these genes may be independent of H3K9me3 and H3K27me3. The precise regulatory mechanisms warrant further investigation.

Based on the results of this study, we propose an integrated mechanism model: In rosacea keratinocytes, UVB radiation synergistically upregulates the expression of TRPV1, TRPV4 and TLR2 through epigenetic mechanisms (such as increasing the H3K4me3 modification of the TRPV1 promoter) and non-DNA demethylation-dependent pathways. The overexpression of these receptors may amplify inflammation through the following pathways: (1) Calcium signaling cascade: The activation of TRPV1/TRPV4 triggers significant intracellular calcium ion (Ca^{2+}) influx. Ca^{2+}, as an important second messenger, can not only directly activate downstream effector molecules such as calmodulin, but also may promote TLR2-mediated signal transduction, thereby forming a positive feedback loop of pro-inflammatory signals. (2) Neuroimmune interaction: The TRPV channels expressed in keratinocytes can be directly activated by physical stimuli such as UVB or inflammatory mediators, leading to the release of neuropeptides (such as substance P, calcitonin gene-related peptide). These neuropeptides act on local immune cells (such as mast cells and macrophages) and vascular endothelial cells, driving neurogenic inflammation, which may be an important basis for clinical phenotypes such as flushing, erythema, and burning sensation. Therefore, the coordinated upregulations of TRPV1/TRPV4 and TLR2 in keratinocytes constitute the core molecular hub connecting environmental stimuli (UVB), epidermal innate immune abnormalities and neurovascular dysfunction.

Relying solely on the the keratinocyte model of rosacea is insufficient to fully represent the pathology of rosacea, fails to fully simulate the complex neuroimmune microenvironment in vivo, and lacks direct functional rescue experiments on calcium signaling or neuropeptide release to fully verify the above hypothesis. Furthermore, this study lacks rescue experiments to confirm the role of H3K4me3 modification in the TRPV1 promoter region in inflammation. In future research, it is necessary to verify this epigenetic-inflammatory axis in animal models. By using techniques such as calcium imaging and specific enzyme inhibitors/activators, the dynamics of calcium signals after the activation of TRPV channels and their interaction with the TLR2 pathway will be deeply analyzed. As H3K4me3 modification is a dynamic process, it is necessary to explore the regulatory effect of histone lysine methyltransferase or lysine demethylase on H3K4me3 in the future. The function of H3K4me3 modification in the promoter region of TRPV1 can be verified by regulating the action of catalytic enzymes. Meanwhile, analyzing the correlation between H3K4me3 modification levels and TRPV1 expression as well as the severity of the disease in patient tissue samples will greatly promote the clinical transformation of these fundamental findings and lay a theoretical foundation for the development of novel therapies targeting environmental inducing factors and epigenetic regulation.

The results of this study offer certain hints for understanding the environmental induction mechanism of rosacea and developing new intervention strategies. As a definite disease-exacerbating factor, this study found that UVB can enhance the sensitivity and response intensity of keratinocytes to inflammatory stimuli through epigenetic initiation mechanisms. Targeting this reversible epigenetic target may offer new therapeutic strategies for existing treatments, such as developing local H3K4me3 modification modulators rather than merely inhibiting downstream inflammatory mediators. In addition, TRPV1, TRPV4 and TLR2 are jointly upregulated in the rosacea model exposed to UVB and form a pro-inflammatory network, suggesting that combined blocking of this receptor network or the use of multi-target inhibitors may produce broader and more durable therapeutic effects than single receptor antagonism.

Conclusion

To sum up, our findings suggest that UVB exposure likely upregulates TRPV1 in keratinocytes by increasing H3K4me3 modification at the TRPV1 promoter. Additionally, UVB boosts TRPV4 and TLR2 expression in these cells via pathways independent of DNA demethylation, as well as the H3K4me3, H3K9me3, and H3K27me3 epigenetic mechanisms. Subsequently, the overexpressions of TRPV1, TRPV4, and TLR2 drive the inflammatory response in the rosacea cell model. Our achievements provide new ideas for the research on the pathogenesis of rosacea in the direction of epigenetics. Meanwhile, our results also emphasize the crucial involvement of ultraviolet rays in the development of rosacea and propose the significance of strengthening ultraviolet protection for the prevention and treatment of rosacea. In future research, perhaps we can detect the effects of epigenetic modulators or ultraviolet protection interventions on the prevention and treatment of rosacea in vivo experiments.

Abbreviations

ChIP, chromatin immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-1 β; KEGG, Kyoto Encyclopedia of Genes and Genomes; KLK5, kallikrein 5; MeDIP, methylated DNA immunoprecipitation; NHEKs, normal human epidermal keratinocytes; qRT-PCR, Quantitative real-time polymerase chain reaction analysis; TLR2, Toll-like receptors 2; TNF-α, tumor necrosis factor-alpha; TRPV, transient receptor potential ion channel protein vanilloid; UVB, Ultraviolet B.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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