

PKC ζ as a promising therapeutic target for TNF α -induced inflammatory disorders in chronic cutaneous wounds

JIAN ZHANG*, XUEKANG YANG*, HONGTAO WANG*, BIN ZHAO, XUE WU, LINLIN SU,
SONGTAO XIE, YUNCHUAN WANG, JUN LI, JIAQI LIU, MENG Dong LIU, FU HAN,
TING HE, WEI ZHANG, KE TAO and DAHAI HU

Department of Burns and Cutaneous Surgery, Xijing Hospital,
The Fourth Military Medical University, Xi'an, Shaanxi 710032, P.R. China

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Abstract. Protein kinase C ζ (PKC ζ) is a member of the atypical protein kinase C family. Its roles in macrophages or skin-resident keratinocytes have not been fully evaluated. In this study, we provide evidence that PKC ζ mediates lipopolysaccharide (LPS)-induced tumor necrosis factor α (TNF α) gene expression in the mouse macrophage cell line, RAW264.7. TNF α has been proven to be one of the main culprits of chronic wounds and impaired acute wounds, which are characterized by excessive inflammation, enhanced proteolysis and reduced matrix deposition. Among the multiple effects of TNF α on keratinocytes, the induction of chemokines which are indispensable factors involved in the massive infiltration of various inflammatory cells into skin lesions serves as a crucial mechanism. In the present study, we found that PKC ζ inhibitor or its specific siRNA inhibited the TNF α -induced upregulation in the levels of the chemokines, interleukin (IL)-8, monocyte chemoattractant protein-1 (MCP-1) and intercellular cell adhesion molecule-1 (ICAM-1) in HaCaT keratinocytes. Moreover,

under a disrupted inflammatory environment, activated keratinocytes can synthesize large amounts of matrix metalloproteinases (MMP), which has a negative effect on tissue remodeling. We discovered that TNF α promoted the expression of MMP9 in a PKC ζ -dependent manner. Further experiments revealed that nuclear factor- κ B (NF- κ B) was a key downstream molecule of PKC ζ . In addition, as shown *in vitro*, PKC ζ was not involved in the TNF α -induced decrease in HaCaT cell migration and proliferation. *In vivo* experiments demonstrated that TNF α -induced wound closure impairment and inflammatory disorders were significantly attenuated in the PKC ζ inhibitor group. On the whole, our findings suggest that PKC ζ is a crucial regulator in LPS- or TNF α -induced inflammatory responses in RAW264.7 cells and HaCaT keratinocytes, and that PKC ζ /NF- κ B signaling may be a potential target for interventional therapy for TNF α -induced skin inflammatory injury.

Introduction

Chronic wounds or impaired acute wounds severely affect the physical and mental health of patients. This non-healing wound is generally caused by bacterial infection or diabetes, as well as subsequent sustained inflammatory responses, defective matrix remodelling and impaired re-epithelialization (1). Lipopolysaccharide (LPS), a main ingredient of the Gram-negative bacterial cell wall, can trigger immune responses by stimulating macrophages to secrete inflammatory cytokines, such as interleukin (IL)-1 β and tumor necrosis factor α (TNF α). TNF α is considered to be a central pathogenic factor in chronic wounds, and anti-TNF α therapy has shown promising effects in improving these injuries (2,3).

Skin-resident cells, particularly keratinocytes play an active role in cutaneous inflammation by producing various cytokines, chemokines and adhesion molecules (4). Chemokines, such as monocyte chemoattractant protein-1 (MCP-1), regulated upon activation normal T cell expressed and secreted factor (RANTES), IL-8, interferon- γ -inducible protein-10 (IP-10) and intercellular adhesion molecule intercellular cell adhesion molecule-1 (ICAM-1) promote the recruitment of excessive immunocytes into skin lesions, which is a key mechanism in the deterioration of chronic wounds (3,5,6). In addition, the persistent deficiency in cell-cell and cell-matrix adhesion initiated by hyperinflammation can

Correspondence to: Professor Dahai Huor or Professor Ke Tao, Department of Burns and Cutaneous Surgery, Xijing Hospital, The Fourth Military Medical University, 127 Changle West Road, Xi'an, Shaanxi 710032, P.R. China
E-mail: xjburnsur@163.com
E-mail: melody896@163.com

*Contributed equally

Abbreviations: PKC ζ , protein kinase C ζ ; IL-1 β , interleukin-1 β ; TNF α , tumor necrosis factor α ; LPS, lipopolysaccharide; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated upon activation normal T cell expressed and secreted factor; IP-10, interferon- γ -inducible protein-10; ICAM-1, intercellular cell adhesion molecule-1; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9; NF- κ B, nuclear factor- κ B

Key words: protein kinase C ζ , lipopolysaccharide, macrophage, tumor necrosis factor α , keratinocytes, chemokines, intercellular cell adhesion molecule-1, matrix metalloproteinases-9, nuclear factor- κ B, chronic wounds

result in defects in tissue remodeling. Matrix metalloproteinases (MMPs) secreted by keratinocytes, fibroblasts and immune cells contribute to the degradation of extracellular matrix (ECM), and are responsible for this defect (7). Excessive MMP9 production has been strongly linked to various inflammatory skin diseases (8). Keratinocyte migration and proliferation play fundamental roles in re-epithelialization. Previous studies have reported that the capacities of migration and proliferation of keratinocytes are impaired in chronic wounds (9-12); however, the roles of TNF α are not yet completely clear.

Atypical protein kinase C (aPKC), a subfamily of atypical serine/threonine protein kinase C, requires neither Ca²⁺ nor diacylglycerol for optimal activity compared to conventional PKC and novel PKC subfamilies (13). PKC ζ , a member of the aPKC family, is an emerging regulator of various biological processes, such as cell polarity, tumorigenesis and chemotaxis (14-19). In several cell types, PKC ζ has been implicated in nuclear factor- κ B (NF- κ B) activation (20-23). The functions of PKC ζ in LPS-stimulated macrophages and TNF α -stimulated keratinocytes have not yet been clearly examined.

The present study confirmed that PKC ζ is crucial in LPS-induced TNF α expression in macrophages. Moreover, importantly, to the best of our knowledge, we demonstrate for the first time that in human HaCaT keratinocytes PKC ζ regulates the TNF α -induced expression of the inflammatory related factors, IL-8, MCP-1, ICAM-1 and MMP9, which to a large degree is mediated via NF- κ B activation. In addition, this study also demonstrates that TNF α at pathological concentrations (10 or 100 ng/ml) inhibits HaCaT cell migration and proliferation *in vitro*; however, PKC ζ was not found to be involved in these processes. *In vivo* experiments revealed that TNF α administration to the surrounding wound tissue at the late phase of wound healing resulted in the delay of wound closure and inopportune upregulations in the levels of IL-8, MCP-1, ICAM-1 and MMP9; however, PKC ζ inhibitor attenuated TNF α -initiated wound closure impairment and inflammatory disorders. Therefore, our findings provide novel insight into the prevention of the aberrant activity of skin keratinocytes induced by TNF α in chronic wounds.

Materials and methods

Cell culture. The immortalized mouse macrophage cell line, RAW264.7, and the human keratinocyte cell line, HaCaT, were obtained from the American Type Culture Collection (Manassas, Va, USA) and the China Center for Type Culture Collection (Wuhan, Hubei, China), respectively. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). The cells were incubated in an incubator at 37°C, 5% CO₂.

Cell cytotoxicity assay. The cell counting kit-8 (CCK-8) (7Sea Biotech, Shanghai, China) was used to examine the cytotoxicity according to the instructions provide by the manufacturer. The RAW264.7 cells were seeded in a 96-well plate at a density of 5.0x10³ cells/well and incubated at 37°C for 12 h. The cells were then treated with fresh medium alone or with LPS (100 ng/ml; Sigma, Santa Clara, CA, USA) with or without PKC ζ -specific pseudosubstrate inhibitor (1, 5 and

10 μ M; Invitrogen, Carlsbad, CA, USA) for 48 h. Subsequently, 10 μ l CCK-8 reagent were added to each well and the cells were cultured for a further 2 h at 37°C, 5% CO₂. The absorbance was measured at 450 nm using a Tecan Infinite M200 Pro Nano Quant microplate reader (Tecan, Männedorf, Switzerland).

RNA interference. The PKC ζ -specific siRNA duplexes (PKC ζ -siRNA) and scramble control siRNA duplexes (scramble-siRNA) were purchased from GenePharma Co. (Shanghai, China). The sequences of these siRNAs are listed in Table I. The HaCaT cells were transfected with either PKC ζ -siRNA (100 nM) or scramble-siRNA (100 nM) using Lipofectamine 2000 transfection reagent (Invitrogen) in accordance with the instructions of the manufacturer. After 6 h, the transfection mixture was removed and supplemented with fresh complete medium. The transfected cells were incubated in an incubator at 37°C, 5% CO₂ for a further 20 or 40 h, and the efficiency of siRNA interference was examined by reverse transcription-quantitative PCR (RT-qPCR) or western blot analysis.

RT-qPCR. The RAW264.7 cells or HaCaT cells were lysed in TRIzol reagent (Takara, Shiga, Japan) and total RNA was extracted in accordance with the instructions of the manufacturer. Total RNA (500 ng) was reverse transcribed into cDNA on the basis of the instructions of Prime Script™ RT reagent kit (Takara). The obtained cDNA was subjected to amplification using the SYBR® Premix Ex Taq™ kit (Takara) and Bio-Rad IQ5 Real-Time system (Bio-Rad, Hercules, CA, USA). The primers sequences are listed in Table II. The PCR amplification condition 4 stages: i) initial denaturation, 95°C for 30 sec; ii) denaturation, 95°C for 30 sec; iii) annealing, 58°C or 60°C for 10 sec; and iv) elongation, 72°C for 15 sec for total 38 cycles. The results were normalized against the mean Cq-values of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), namely Δ Cq, and the fold increases were calculated as 2^{- $\Delta\Delta$ Cq}.

Western blot analysis. The cells were lysed in RIPA buffer (Heart Biological Technology Co., Ltd., Xi'an, China) containing 1% aprotinin, 1% activated Na₃VO₄ and 1% PMSF on ice. The protein concentration was detected according to the instructions of the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Total proteins (50 μ g) were loaded onto a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, separated by electrophoresis and transferred onto PVDF membranes (Millipore, Boston, MA, USA). The membranes were blocked with 5% skim milk at room temperature for 2 h and then incubated with rabbit anti-human PKC ζ (1:1,000, #sc-216; Santa Cruz Biotechnology, Inc., CA, USA), rabbit anti-human MMP2 (1:1,000, #10373-2-AP), MMP9 (1:500, #10375-2-AP), mouse anti-human β -actin (1:1,000, #60008-1-Ig) (both from China Branch of Proteintech Co., Hubei, China) and rabbit anti-human phospho-PKC ζ antibodies (1:500, #9378; Cell Signaling Technology, Boston, MA, USA) at 4°C overnight. The following day, the membranes were sequentially incubated with HRP-conjugated goat anti-rabbit IgG (1:3,000, #A0208) or HRP-conjugated goat anti-mouse IgG (1:3,000, #A0216) (Beyotime, Shanghai, China) at 37°C for 1 h. Immunoreactive proteins were detected using ECL reagent (Millipore) and the FluorChem FC system (Alpha Innotech, San Leandro, CA, USA). Bands were quantitated using ImageJ software.

Table I. Sequences of siRNA used in this study.

siRNA	Sense (5'→3')	Antisense (5'→3')
PKC ζ	GACACAACGAGCACUUUCUTT	AGAAAGUGCUCGUUGUGUCTT
Scramble	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
PKC ζ , protein kinase C ζ .		

Table II. Sequences of primers used in this study.

Primers	Forward primer (5'→3')	Reverse primer (5'→3')
Mouse-TNF α	CTATCTCCAGGTTCTCTTCAA	GCAGAGAGGAGGTTGACTTTC
Mouse-IL-1 β	GCTCATCTGGGATCCTCTCC	CCTGCCTGAAGCTCTTGTG
Mouse-GAPDH	ATTGTCAGCAATGCATCCTG	ATGGACTGTGGtCATGAGCC
Human-PKC ζ	TGCTTACATTTCTCATCCC	TCATTCTTCTTCAACCGCAC
Human-MMP2	TGAAGTATGGGAACGCCGAT	CGTACTTGCCATCCTTCTCA
Human-MMP9	TCTTCCCTTCACTTTCCTG	GCCACGAGGAACAACTGTA
Human-IL-8	TACTCCAAACCTTTCCACCC	AACTTCTCCACAACCCTCTG
Human-MCP-1	GCAATCAATGCCCCAGTCA	TGCTGCTGGTGATTCTTCTATAGCT
Human-ICAM-1	CACAGTCACCTATGGCAACGA	TGGCTTCGTCAGAATCACGTT
Human-GAPDH	CTCCTCCACCTTTGACGCTG	TCCTCTTGCTCTTGCTGG
Mouse-IL-8	TTGCCTTGACCCTGAAGCCCC	GGCACATCAGGTACGATCCAGGC
Mouse-MCP-1	CATCCACGTGTTGGCTCA	GATCATCTTGCTGGTGAATGAGT
Mouse-ICAM-1	GGCACCCAGCAGAAGTTGTT	CCTCAGTCACCTCTACCAAG
Mouse-MMP9	TCTGTATGGTCGTGGCTCTA	CCTGTAATGGGCTTCTCTATG

TNF α , tumor necrosis factor α ; IL-1 β , interleukin-1 β ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PKC ζ , protein kinase C ζ ; MMP, matrix metalloproteinase; MCP-1, monocyte chemotactic protein-1; ICAM-1, intercellular cell adhesion molecule-1.

Nuclear and cytoplasmic protein extraction. The cells were seeded in a 6-well plate and incubated at 37°C for 24 h. The cells were pre-treated with or without the NF- κ B inhibitor, BAY11-7082 (5 μ M; Selleck, Houston, TX, USA), for 2 h or PKC ζ I (5 μ M; Invitrogen) for 3 h and then stimulated with TNF α (rhTNF α , 10 ng/ml; Shanghai Bioleaf Biotech Co., Ltd., Shanghai, China) for 2 h in the presence of BAY11-7082 or PKC ζ I. The nuclear proteins were isolated on the basis of the instructions of a commercial nuclear and cytoplasmic protein extraction kit (Beyotime). Separated nuclear proteins were analyzed by immunoblotting. The internal reference protein of nuclear protein was histone H3. The primary antibodies included rabbit anti-human NF- κ B-p65 antibody (1:500, #sc-372) and goat anti-human histone H3 antibody (1:500, #sc-8654) (Santa Cruz Biotechnology, Inc.). The secondary antibodies included HRP-conjugated goat anti-rabbit IgG (1:1,000, #A0208) and donkey anti-goat IgG (1:1,000, #A0108) (Beyotime).

NF- κ B immunofluorescence analysis. All operations were executed in accordance with the instructions of the NF- κ B activation, Nuclear Translocation assay kit (Beyotime). Cells growing on coverslip were fixed with 4% paraformaldehyde for 20 min, and then incubated with 5% BSA for 30 min at room temperature to suppress non-specific binding of IgG. The

cells were then incubated with rabbit anti-human NF- κ B-p65 antibody overnight at 4°C. The following day, the NF- κ B-p65 antibody was removed and the cells were sequentially incubated with anti-rabbit Cy3 IgG at room temperature for 1 h. The nucleus was stained with DAPI. Images were captured using a FSX100 microscope (Olympus, Tokyo, Japan).

Cell proliferation assay. Cell proliferation was evaluated by CCK-8 assay (7Sea Biotech) according to the instructions provided by the manufacturer. Briefly, the cells were seeded in a 96-well plate at a density of 4.0x10³ cells/well and incubated at 37°C for 24 h. The cells were then treated with or without PKC ζ -specific pseudosubstrate inhibitor (10 μ M; Invitrogen). After 3 h, the inhibitor mixture was removed and replaced with fresh medium alone or with TNF α (rhTNF α , 10 or 100 ng/ml; Shanghai Bioleaf Biotech Co., Ltd.) for 24, 48 and 72 h. At the indicated time point, the medium was removed and supplemented with the medium containing CCK-8 reagent (10 μ l/well) and the cells were cultured for a further 1-1.5 h at 37°C, 5% CO₂. Subsequently, the absorbance was measured at 450 nm using a Tecan Infinite M200 Pro Nano Quant microplate reader (Tecan).

Scratch wound healing assay. The HaCaT cells were seeded in 35-mm culture dishes in DMEM supplemented with

10% FBS (both from Gibco) at 37°C, 5% CO₂ for 24 h, until full confluence. Subsequently, 10 μ g/ml mitomycin C (Invitrogen) was added for 1 h to completely inhibit cell proliferation. A scratch of 500 μ m in width was created on the confluent cells with a 10 μ l pipette tip. The cells were washed 3 times with phosphate-buffered saline (PBS) and pre-treated with or without PKC ζ inhibitor (10 μ M; Invitrogen) for 3 h, then incubated in serum-free DMEM alone (control group), or serum-free DMEM containing TNF α (10 or 100 ng/ml; Bioleaf) at 37°C, 5% CO₂ for 24 h. The cells were photographed under a microscope and the gap width of scratch was measured using Image Pro Plus software. The cell migration distance was the difference value between the gap width at 0 and 24 h. The migration distance at control group was set as 1.

Detection of the activity of PKC ζ in vivo. Male 6-week old BALB/c mice (n=40) were obtained from The Center of Experimental Animal, the Fourth Military Medical University. The experiment was repeated 3 times. All animal experiments were performed in accordance with the guidelines from the Administration of Animal Experiments for Medical Research Purposes issued by the Ministry of Health of China, which is in accordance with the principle of ARRIVE. The protocol was approved by the Animal Experiment Administration Committee of the Fourth Military Medical University. The mice were randomly divided into 2 groups (n=8) as follows: the PBS control group and TNF α group. To examine the differences in the phosphorylation level of PKC ζ between the PBS- and TNF α -treated wounds, a 1.0x1.0 cm full-thickness wound was created on the back of each BALB/c mouse following anesthetization by an intraperitoneal injection of 1% pentobarbital sodium (40 mg/kg). Subsequently, 100 μ l PBS or 100 μ l TNF α (200 ng, dissolved in PBS) was injected into the tissue around the wound, and after 2 h, the samples of the wound-edge skin (WES) and normal skin (NS) in each mouse were collected for the examination of the levels of PKC ζ and phosphorylated PKC ζ by western blot analysis.

In vivo wound closure assay. The BALB/c mice were randomly divided into 3 groups (n=8) as follows: the PBS control group, TNF α group and TNF α + PKC ζ inhibitor group. Following anesthetization, a 1.0x1.0 cm full-thickness wound was created on the back of each BALB/c mouse. Subsequently, 100 μ l PBS, 100 μ l TNF α solution (rmTNF α , 50 ng, dissolved in PBS; Bioleaf) or 100 μ l TNF α solution + PKC ζ inhibitor (20 μ M) was injected into the skin tissue around the wound each day from day 6 post-incision (days 6, 7, 8, 9, 10, 11 and 12 post-incision). The wound area of each mouse was digitally photographed and measured using Photoshop software or the skin tissue samples of the wound edges in each mouse were collected for mRNA extraction and RT-qPCR analysis on days 7, 9 and 13 post-wounding.

Statistical analysis. All experiments were repeated at least 3 times, and the results are the means \pm SD. The analysis of statistically significant differences between groups was performed by one-way analysis of variance (ANOVA) with the Student's t-test or the Mann-Whitney U test using SPSS 17.0 software. A value of p<0.05 was considered to indicate a statistically significant difference.

Results

PKC ζ mediates LPS-induced inflammatory responses in RAW264.7 cells. A previous study reported that PKC ζ activity is required for LPS-induced IL-1 β production in RAW264.7 cells (22); however, whether PKC ζ mediates the expression of another important inflammatory factor, namely TNF α , has not yet been examined, at least to the best of our knowledge. PKC ζ can be activated through phosphorylating its threonine 410 site. In the present study, we validated that stimulation with LPS (100 ng/ml) resulted in an increase in the phosphorylation level of PKC ζ in the RAW264.7 cells (Fig. 1A). To measure the cytotoxicity of LPS or PKC ζ -specific pseudosubstrate inhibitor (PKC ζ I) in the RAW264.7 cells, CCK-8 assay was performed. The results revealed that both LPS at a concentration of 100 ng/ml and PKC ζ I up to 10 μ M did not damage the viability of the RAW264.7 cells (Fig. 1B). Pre-treatment of the cells with PKC ζ I inhibited the LPS-induced phosphorylation of PKC ζ (Fig. 1C) and suppressed the LPS-induced increase in the mRNA expression levels of IL-1 β and TNF α (Fig. 1D and E).

TNF α promotes the activation of PKC ζ in HaCaT keratinocytes. To examine whether PKC ζ is involved in the effects of TNF α on HaCaT keratinocytes, we firstly examined whether TNF α stimulates the activation of PKC ζ . Following treatment with TNF α (10 ng/ml) and at the indicated time points (0, 5, 15, 30 and 60 min), the HaCaT cells were lysed and the lysates were assessed by western blot analysis. The results revealed that exposure to TNF α resulted in increased phosphorylation levels of PKC ζ , which was firstly observed at 15 min and reached peak levels at 60 min (Fig. 2A). We then found that pre-treatment of the cells with PKC ζ I (10 μ M) for 3 h distinctly suppressed the TNF α -induced PKC ζ phosphorylation (Fig. 2B). In addition, the effects of PKC ζ -specific small interfering RNA (PKC ζ -siRNA) were examined by RT-qPCR and western blot analysis. The results revealed that the mRNA and protein expression levels of PKC ζ were all effectively suppressed (Fig. 2C and D).

PKC ζ is involved in TNF α -induced inflammatory responses in HaCaT cells. It is known that the chemokines, MCP-1 and IL-8, and the adhesion molecule, ICAM-1, are crucial in the pathogenesis of inflammatory disorders in chronic wounds. Thus, to determine whether PKC ζ is involved in the TNF α -induced inflammatory responses in HaCaT cells, we examined the effects of PKC ζ I on the mRNA expression levels of IL-8, MCP-1 and ICAM-1. Exposure to TNF α led to an increase in the mRNA expression levels of IL-8, MCP-1 and ICAM-1 over time which respectively peaked at 6, 12 and 6 h post-TNF α treatment, and then gradually declined, but remained at significantly high levels at 48 h (Fig. 3A-C). The cells were pre-treated with PKC ζ I for 3 h in advance, and then stimulated with TNF α for 12 h, which largely inhibited the TNF α -induced upregulation of IL-8, MCP-1 and ICAM-1. The silencing of PKC ζ by transfection with siRNA for 6 h also led to similar results (Fig. 3D-F).

PKC ζ mediates the TNF α -induced upregulation of MMP9 in HaCaT cells. MMPs, are a crucial class of protease, and

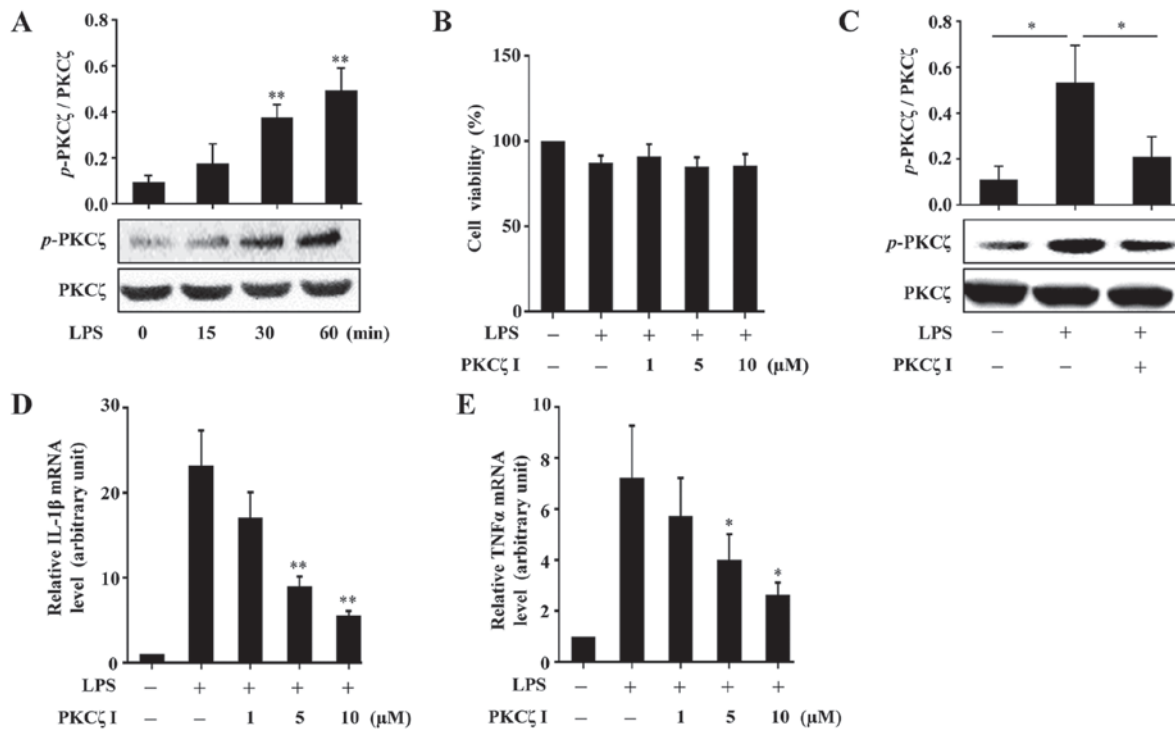


Figure 1. The roles of protein kinase C ζ (PKC ζ) in lipopolysaccharide (LPS)-induced inflammatory responses in RAW264.7 macrophages. (A) Representative immunoblots assessing the changes in the levels of phosphorylated PKC ζ in RAW264.7 cells following treatment with 100 ng/ml LPS at indicated time points (0, 15, 30 and 60 min). (B) CCK-8 assay assessing the effects of LPS (100 ng/ml) alone or with PKC ζ specific pseudosubstrate inhibitor (PKC ζ I) (1, 5 or 10 μ M) on the viability of RAW264.7 cells. Data represent means \pm SD of 3 independent experiments, the value in LPS-free group arbitrarily set as 100%. (C) Representative immunoblots showing the effects of PKC ζ I (10 μ M) on LPS-induced PKC ζ phosphorylation. (D and E) RT-qPCR analysis showing the effects of PKC ζ I on LPS-induced mRNA level changes of interleukin-1 β (IL-1 β) or tumor necrosis factor α (TNF α). Each bar represent the means \pm SD of 3 independent experiments, and normalized to corresponding total PKC ζ protein level (A and C) or GAPDH mRNA level with the value in the LPS-free group set as 1 arbitrarily (D and E); * p <0.05 and ** p <0.01 vs. the value at 0 min (A) or the value in corresponding LPS alone group (C-E).

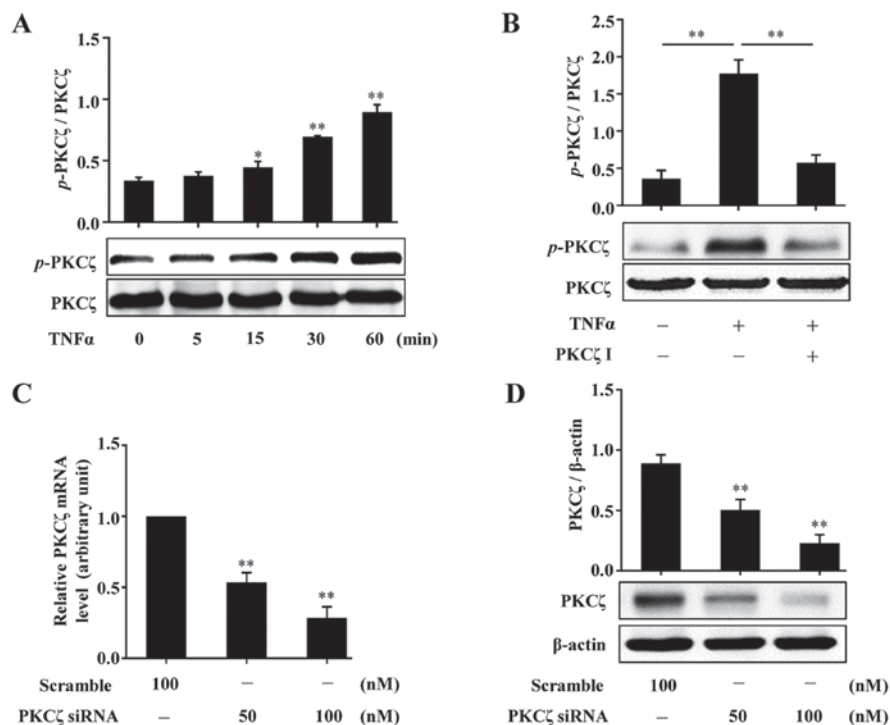


Figure 2. Effects of tumor necrosis factor α (TNF α) on the activation of protein kinase C ζ (PKC ζ) in HaCaT cells. (A) Representative immunoblots showing the changes in the phosphorylation levels of PKC ζ in HaCaT cells stimulated with 10 ng/ml TNF α at indicated time points (0, 5, 15, 30 and 60 min), respectively. (B) Representative immunoblots showing the effects of PKC ζ I pre-treatment on TNF α -induced PKC ζ phosphorylation. (C and D) RT-qPCR (C) or immunoblotting (D) assessing the effects of PKC ζ specific interfering RNA (PKC ζ -siRNA) on the knockdown of PKC ζ at the mRNA and protein levels. Bars represent the means \pm SD of 3 independent experiments, and normalized to corresponding total PKC ζ protein level (A and B), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level with the value in the scramble group set as 1 arbitrarily (C) or β -actin protein level (D). * p <0.05 and ** p <0.01 vs. the value at 0 min (A), in TNF α alone group (B), or in scramble group (C and D).

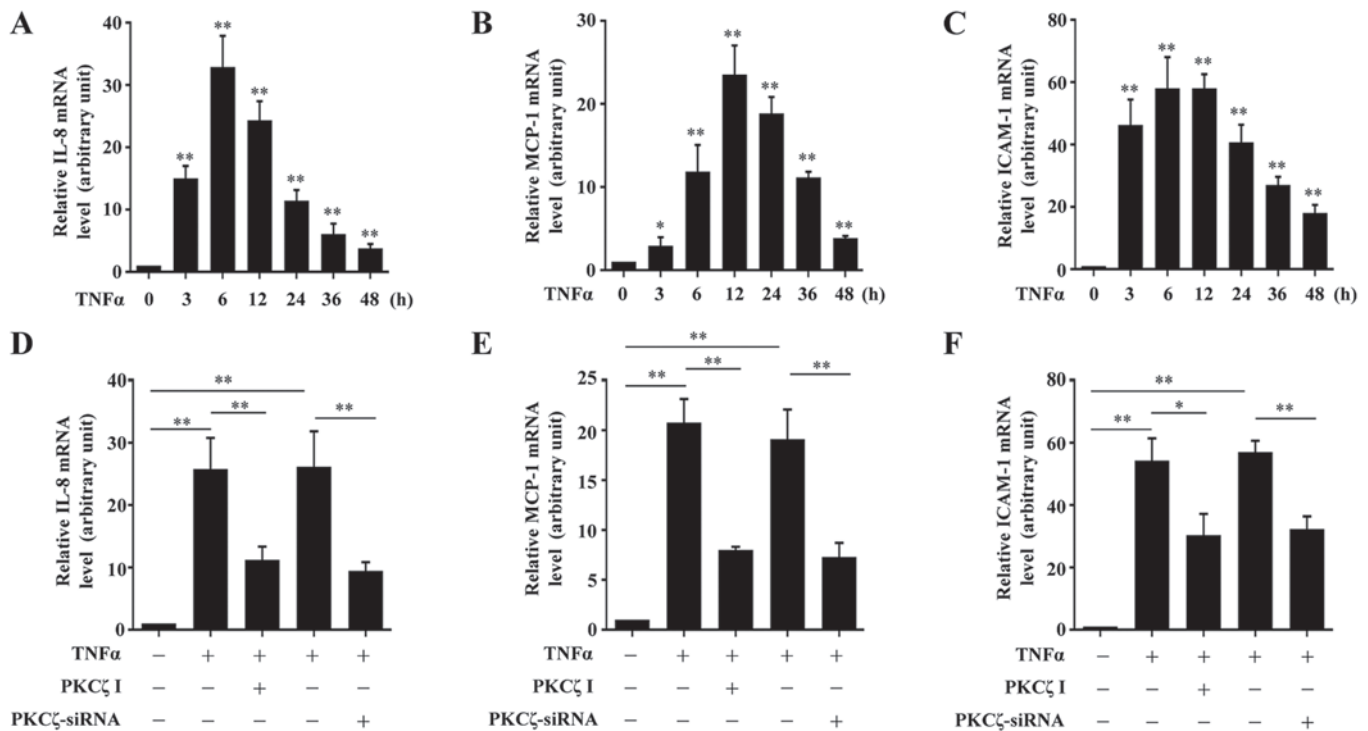


Figure 3. The roles of protein kinase C ζ (PKC ζ) in tumor necrosis factor α (TNF α)-induced expression of interleukin-1 β (IL-8), monocyte chemoattractant protein-1 (MCP-1) and adhesion molecule intercellular cell adhesion molecule-1 (ICAM-1) in HaCaT cells. (A-C) The mRNA levels of (A) IL-8, (B) MCP-1 and (C) ICAM-1 in HaCaT cells exposed to TNF α (10 ng/ml) at indicated time points (0, 3, 6, 12, 24, 36 and 48 h) were assessed by RT-qPCR assays. (D-F) RT-qPCR assays showing the effects of PKC ζ I (10 μ M) or PKC ζ -siRNA (100 nM) on the TNF α -induced upregulation of (D) IL-8, (E) MCP-1 and (F) ICAM-1. Bars represent the means \pm SD of 3 independent experiments, and normalized to the GAPDH mRNA level with the value in the TNF α -free treatment group set as 1 arbitrarily. * p <0.05 and ** p <0.01 vs. the value at 0 h (A-C).

have been reported to be abnormally upregulated in impaired wound healing (7,8). We firstly examined the effects of TNF α on the expressions of MMP2 and MMP9 in HaCaT cells. The cells were treated with TNF α for various periods of time (0, 12, 24 and 48 h), and the cells were lysed and the lysates were assessed by western blot analysis. The results revealed that TNF α had no significant effect on the protein expression of MMP2; however, MMP9 protein expression was indeed elevated by exposure to TNF α (Fig. 4A). The analysis of MMP2 and MMP9 mRNA expression levels by RT-qPCR also revealed similar results (Fig. 4B and C). To explore the role of PKC ζ in the TNF α -induced changes in the levels of MMP9 in the HaCaT cells, we used PKC ζ I or PKC ζ siRNA to disrupt the normal function of PKC ζ . The results revealed that the TNF α -induced upregulation of MMP9 at both the protein and mRNA level tightly depended on the activity of PKC ζ , as treatment with PKC ζ I or PKC ζ siRNA decreased MMP9 expression (Fig. 4D-E).

Inhibition of the activity of PKC ζ impairs the TNF α -induced nuclear translocation of NF- κ B-p65 that is required for the upregulation of MCP-1, IL-8, ICAM-1 and MMP9 following stimulation with TNF α . From the above-mentioned results, we found that PKC ζ regulated the expression of MCP-1, IL-8, ICAM-1 and MMP9 beginning from the transcriptional level. Moreover, several studies have suggested that PKC ζ is likely to be an upstream regulator of NF- κ B, which is an important transcription factor involved in the regulation of many biological processes (20-23). Thus, we hypothesized that the

PKC ζ -mediated inflammatory responses and MMP9 expression in HaCaT cells may depend on the activation of NF- κ B. The cells were pre-treated with the NF- κ B inhibitor, BAY11-7082, for 2 h or PKC ζ I for 3 h, and then stimulated with TNF α for 2 h in the presence of BAY11-7082 or PKC ζ I. The nuclear transport of p65, a key subunit of NF- κ B, was assessed by western blot analysis or immunofluorescence. The results revealed that following TNF α stimulation, there was a distinct nuclear translocation of p65; however, this nuclear import was prevented by PKC ζ I, which was in accordance with the function of BAY11-7082 (Fig. 5). Further experiments confirmed the requirement of NF- κ B in the TNF α -induced upregulation of MCP-1, IL-8, ICAM-1 and MMP9 by inhibiting the function of NF- κ B with BAY11-7082. The use of BAY11-7082 decreased the levels of MCP-1, IL-8, ICAM-1 and MMP9 induced by TNF α (Fig. 6).

PKC ζ does not significantly affect TNF α -weakened HaCaT keratinocyte migration and proliferation in vitro. Keratinocyte migration and proliferation are important to the rate of wound closure. We thus examined the effect of TNF α and PKC ζ I on HaCaT cell migration using a scratch wound healing model. The cells were treated with 10 μ g/ml mitomycin C for 1 h to inhibit cell proliferation. A scratch was made on fully confluent cells. Subsequently, following pre-treatment with or without PKC ζ I for 3 h, the cells were stimulated with TNF α at pathological concentrations (10 or 100 ng/ml) for 24 h. The results revealed that exposure to TNF α markedly decreased the rate of wound healing and the inhibition of the function of PKC ζ

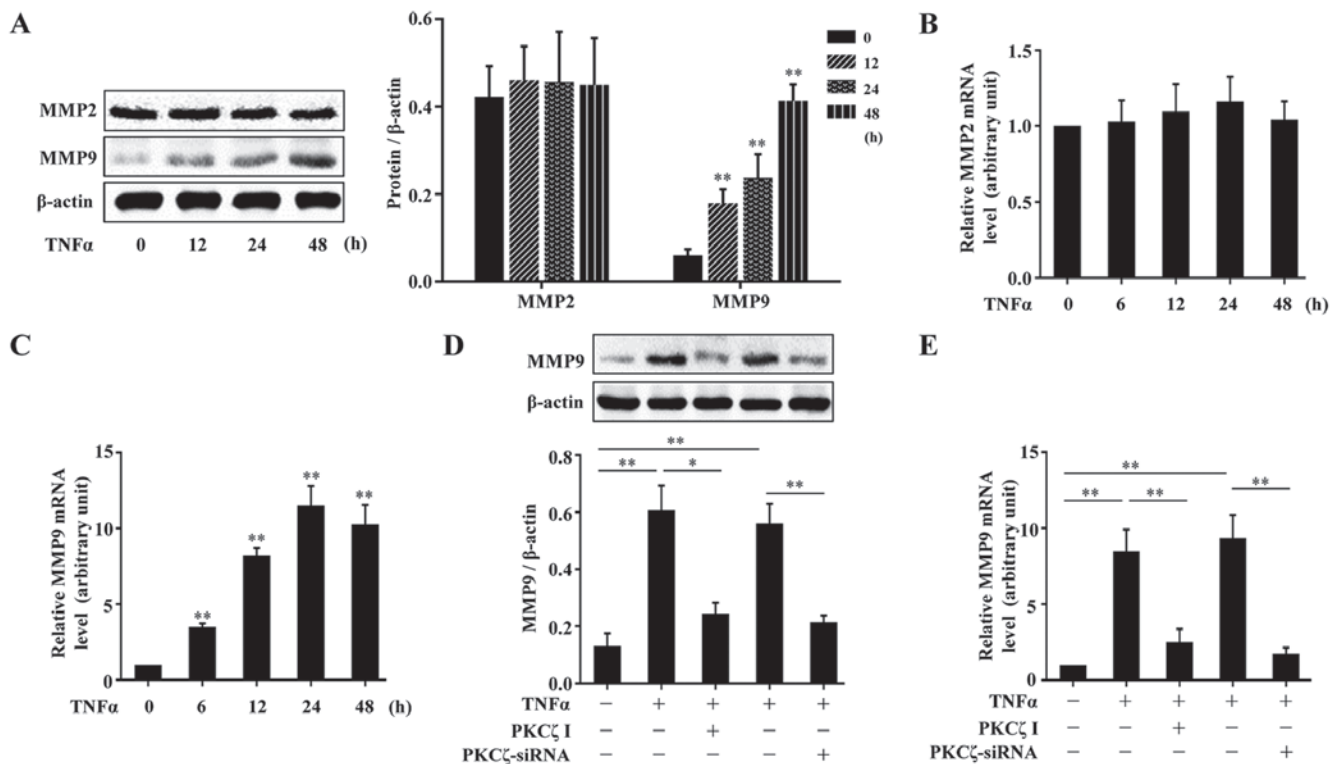


Figure 4. Effect of tumor necrosis factor α (TNF α) on the expression of matrix metalloproteinase in HaCaT cells and the role of protein kinase C ζ (PKC ζ). (A) Representative immunoblots showing the protein level changes of matrix metalloproteinase (MMP)2 and MMP9 in HaCaT cells exposed to TNF α (10 ng/ml). (B and C) RT-qPCR analysis showing the changes in the mRNA levels of MMP2 and MMP9 in HaCaT cells stimulated with TNF α . (D and E) Immunoblotting (D) or RT-qPCR (E) assessing the effect of PKC ζ I or PKC ζ -siRNA on the TNF α -induced upregulation of MMP9 at protein or mRNA level. Bars represent the means \pm SD of 3 independent experiments, and normalized to the β -actin protein level (A and D) or GAPDH mRNA level with the value in TNF α -free group set as 1 arbitrarily (B, C and E). * $p < 0.05$ and ** $p < 0.01$ vs. the value at 0 h (A-C).

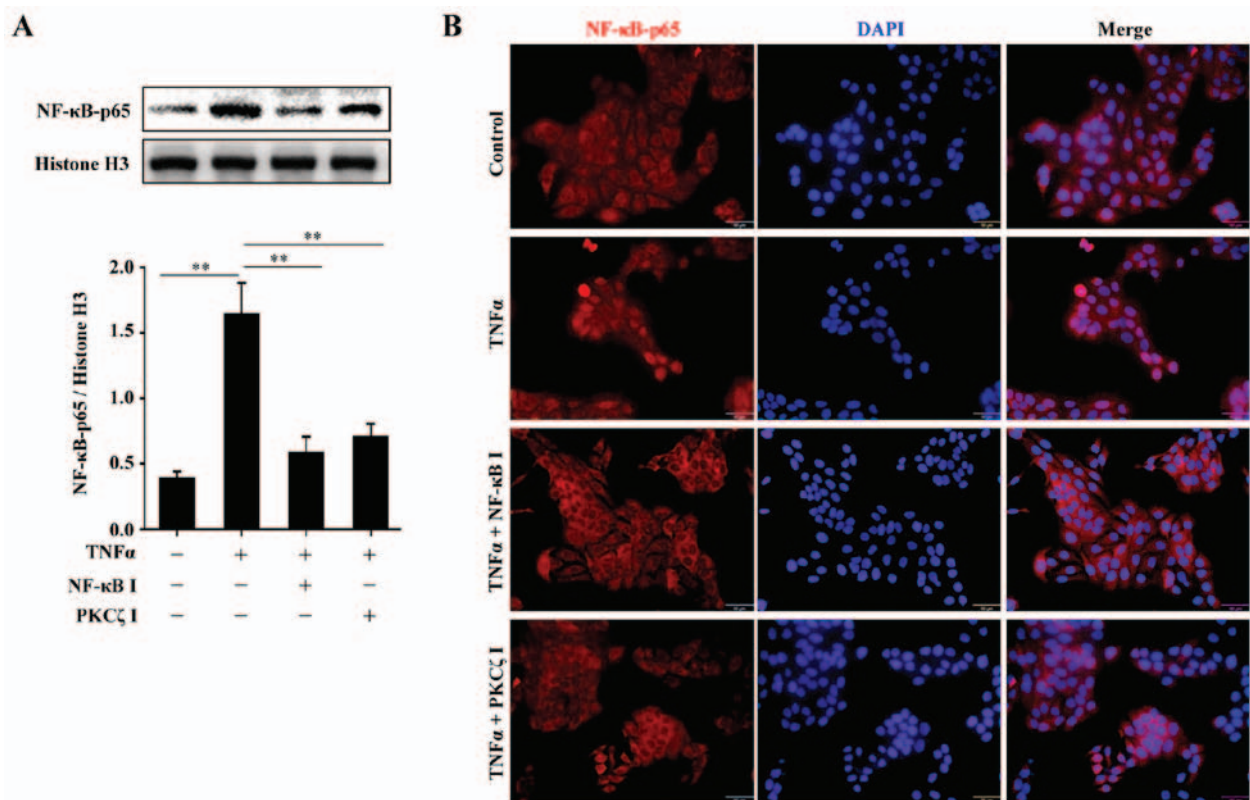


Figure 5. Involvement of protein kinase C ζ (PKC ζ) in tumor necrosis factor α (TNF α)-induced NF- κ B-p65 nuclear translocation. (A) Representative immunoblots showing the effects of NF- κ B inhibitor BAY11-7082 and PKC ζ I on TNF α -induced NF- κ B-p65 nuclear import. (B) The localization of NF- κ B-p65 in HaCaT cells was analyzed by immunofluorescence. Bars represent the means \pm SD of 3 independent experiments, and normalized to histone H3 protein level. ** $p < 0.01$. Scale bar, 50 μ m.

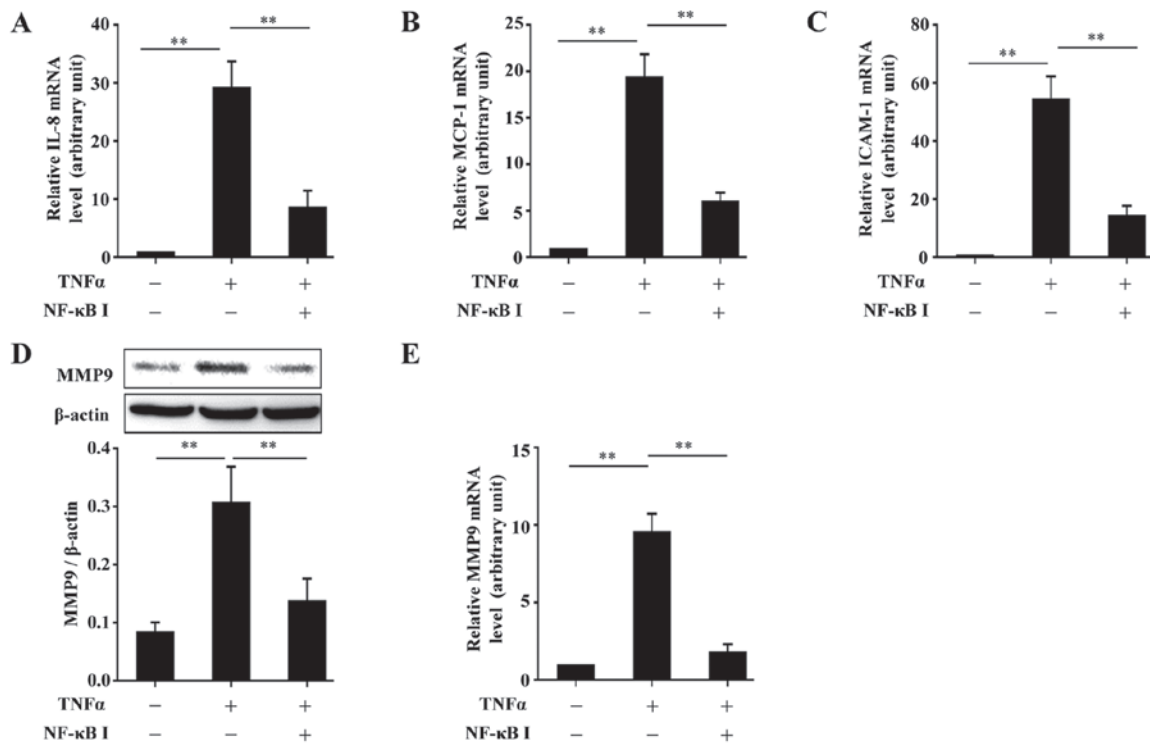


Figure 6. The role of NF- κ B in tumor necrosis factor α (TNF α)-induced expression of interleukin-1 β (IL-8), monocyte chemotactic protein-1 (MCP-1), intercellular cell adhesion molecule-1 (ICAM-1) and MMP9 in HaCaT cells. (A-C) RT-qPCR analysis showing the effects of BAY11-7082 on the TNF α -induced changes in the mRNA levels of (A) IL-8, (B) MCP-1 and (C) ICAM-1 in HaCaT cells. (D and E) Immunoblotting (D) or RT-qPCR (E) assays showing the effect of BAY11-7082 on the TNF α -induced expression of MMP9 at protein or mRNA level in HaCaT cells. All the bars represent the means \pm SD of 3 independent experiments, and normalized to the GAPDH mRNA level with the value in the TNF α -free group set as 1 arbitrarily (A-C and E) or to β -actin level (D). ** p <0.01.

by PKC ζ I did not significantly attenuate the TNF α -induced decrease in keratinocyte migration (Fig. 7A and B). CCK-8 assay was then used to examine the effect of TNF α and PKC ζ on HaCaT cell proliferation. The absorbance at 450 nm was directly proportional to the cell proliferation capacity. The results revealed that pathological concentrations of TNF α caused a small degree of inhibition on cell proliferation at 48 h; however, this difference was not statistically significant. Until 72 h, exposure to 100 ng/ml TNF α inhibited cell proliferation by approximately 11.6%, which was statistically significant (Fig. 7C). We also found that PKC ζ I pre-treatment did not significantly affect the TNF α -induced impairment of keratinocyte proliferation (Fig. 7C).

PKC ζ inhibition attenuates the TNF α -induced wound closure delay and inflammatory disorders in vivo. Previous studies have reported that from the 5th day after normal acute wounding, the mRNA levels of 2 pro-inflammatory cytokines, IL-1 β and TNF α , and the chemokine, MCP-1, in wound tissues are declined and cannot be detected on day 13 post-wounding; however, in the wounds of diabetic mice, the mRNA levels of IL-1 β , TNF α and MCP-1 are still strongly elevated and sustained during the late phase (days 7-13 after wounding) of the wound repair process (5,24). It has become a consensus that sustained inflammation triggers the prolonged infiltration of immune cells at the late stage of cutaneous wound repair and can cause defects in wound healing. Furthermore, a number of studies have confirmed that the local injection of potent pro-inflammatory cytokines, IL-1 α or TNF α or IFN- γ , in the skin of various

animals, such as mice, dogs, rats, rabbits and human volunteers can cause significant inflammatory responses and the rapid infiltration of immunocytes (25-30). In the present study, we locally injected PBS or TNF α into the wound edges of BALB/c mice, and after 2 h we found that compared with the normal cutaneous tissue, the phosphorylation of PKC ζ was increased in the wound tissue treated with TNF α (Fig. 8A). To explore whether prolonged TNF α exposure can result in wound closure delays in mice and whether PKC ζ is involved in this process, 100 μ l PBS, 100 μ l TNF α solution (50 ng, dissolved in PBS) or 100 μ l TNF α solution + PKC ζ inhibitor (20 μ M) were injected into the skin tissue around the wound each day on days 6-12 post-wounding. The results revealed that the TNF α injection at the late phase of wound closure caused a delay in wound repair; however, the administration of PKC ζ inhibitor led to a favourable improvement, attenuating this impairment (Fig. 8B and C). Our *in vitro* experiments revealed that PKC ζ played an indispensable role in TNF α -induced inflammatory responses in keratinocytes. Total RNA was extracted from the wound tissues of the PBS, TNF α and TNF α + PKC ζ I-treated mice at different intervals (days 7, 9 and 13 after wounding) for RT-qPCR analysis. We observed a gradual decrease in the levels of IL-8, MCP-1, ICAM-1 and MMP9 to very low levels on day 13 post-wounding in the PBS control mice. However, in the TNF α -treated mice, until day 13 post-wounding, the expression levels of these molecules remained at markedly high levels, which may be one of the causes that resulted in defects in wound closure. However, as expected, PKC ζ inhibition markedly inhibited the TNF α -induced upregulation in the levels of IL-8, MCP-1, ICAM-1 and MMP9 (Fig. 9).

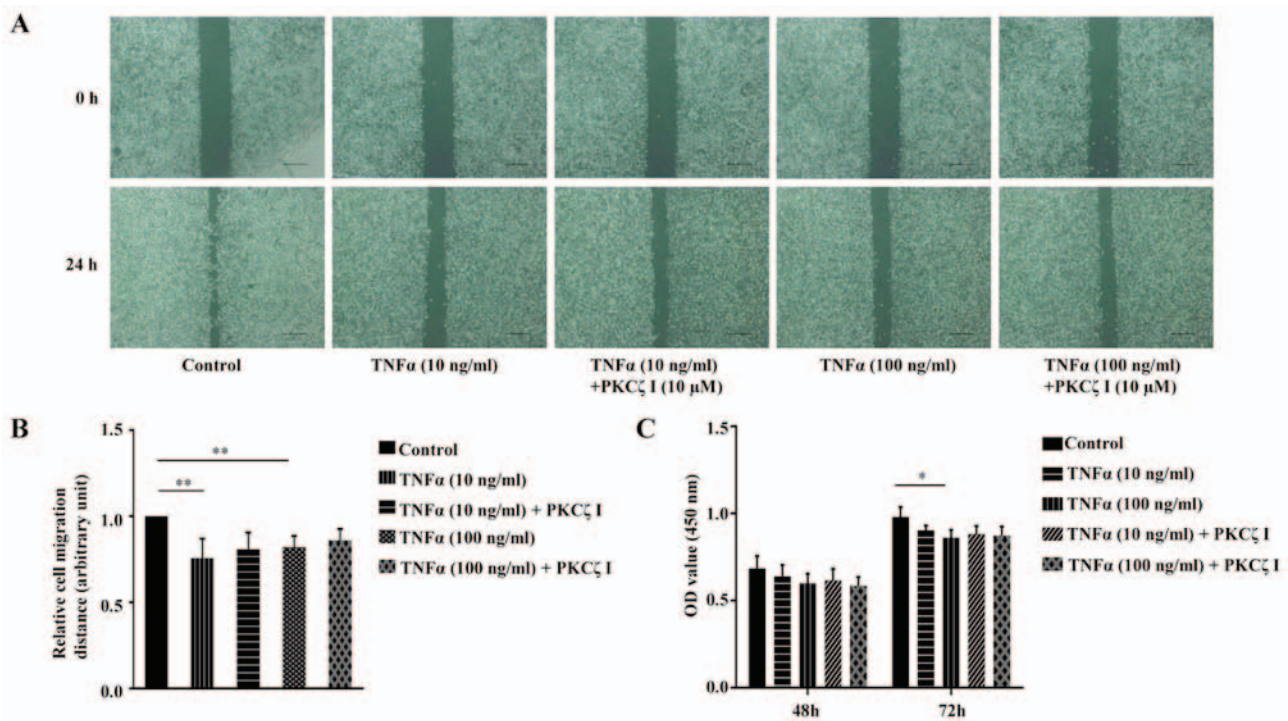


Figure 7. Effects of tumor necrosis factor α (TNF α) on the ability of migration and proliferation of HaCaT cells *in vitro* and the role of protein kinase C ζ (PKC ζ) in these processes. (A) Representative images of the gap areas at 0 or 24 h after TNF α (10 or 100 ng/ml) treatment in the absence or presence of PKC ζ I in scratch wound healing assay. Scale bar, 200 μ m. (B) Histogram showing the relative cell migration distance. The migration distance was the difference value between the gap width at 0 and 24 h and the value at control group was set as 1 arbitrarily. (C) CCK-8 assay showing the proliferation of HaCaT cells after TNF α (10 or 100 ng/ml) treatment with or without PKC ζ I. The absorbance at 450 nm is in direct proportion to the cell proliferation ability. Bars represent the means \pm SD of 3 independent experiments. *p<0.05 and **p<0.01.

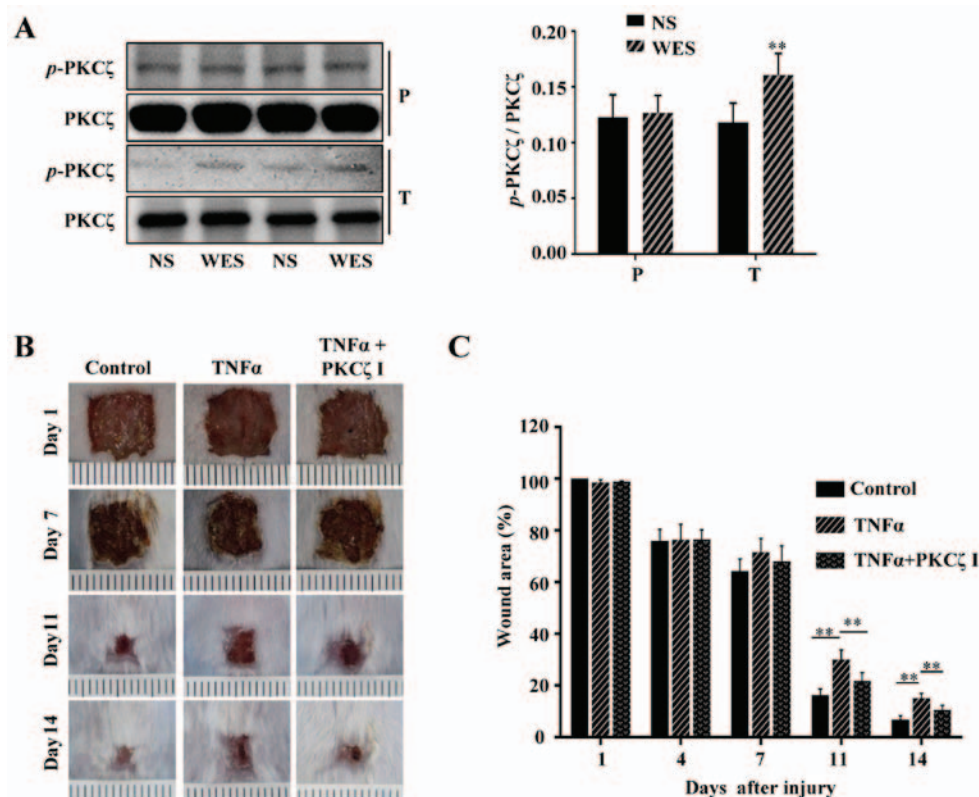


Figure 8. The activity of protein kinase C ζ (PKC ζ) in tumor necrosis factor α (TNF α)-treated wounds and effects of PKC ζ inhibitor on the attenuation of prolonged TNF α environment-induced skin wound closure delay. (A) Representative immunoblots showing the phosphorylation levels of PKC ζ in paired tissue samples of the normal skin (NS) and PBS or TNF α -treated wound edge skin (WES). P, PBS-treated mouse; T, TNF α -treated mouse. (B and C) Representative images of the wound areas in PBS group (control), TNF α group and TNF α +PKC ζ inhibitor (PKC ζ I) group on days 1, 7, 11 and 14 post-incision. The wound closure rate was the ratio of the remanent wound area on the indicated days and the original wound area on day 1 post-incision. Bars represent the means \pm SD of 3 independent experiments, n=8. **p<0.01.

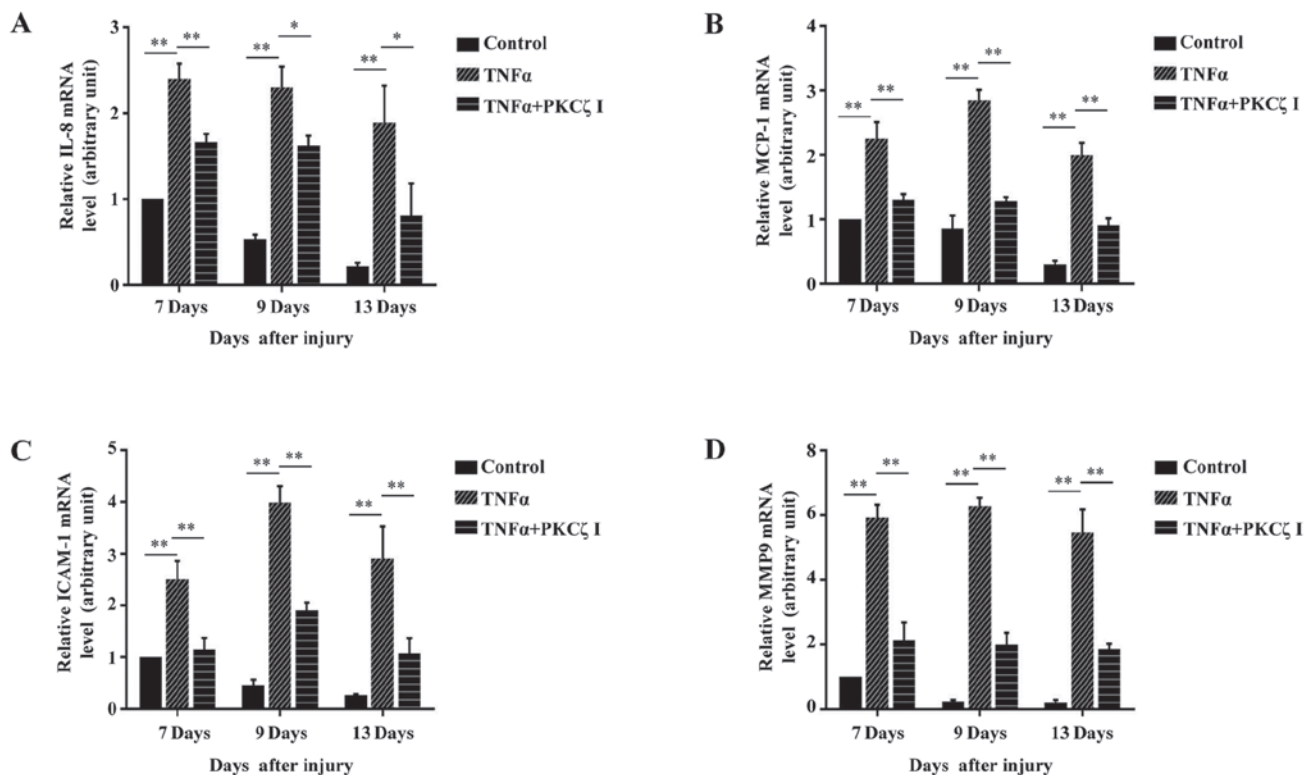


Figure 9. Roles of protein kinase C ζ (PKC ζ) in prolonged tumor necrosis factor α (TNF α) environment-induced upregulation of interleukin-1 β (IL-8), monocyte chemoattractant protein-1 (MCP-1), intercellular cell adhesion molecule-1 (ICAM-1) and MMP9. (A-D) RT-qPCR analysis showing the effects of PKC ζ inhibitor (PKC ζ I) on the TNF α -induced increased mRNA levels of (A) IL-8, (B) MCP-1 and (C) ICAM-1 and (D) MMP9 in wounds edges at different days of the late phase of wound healing (days 7, 9 and 13 after wounding). Bars represent the means \pm SD of 3 independent experiments, and normalized to the GAPDH mRNA level. The value on day 7 in control group was set as 1 arbitrarily. *p<0.05 and **p<0.01.

Discussion

A severely enhanced and prolonged TNF α environment in chronic wounds, such as diabetic foot ulcers can trigger abnormally excessive inflammatory responses, which leads to severe defects in tissue remodeling and re-epithelialization (1). This notoriously non-healing wound is a threat to human health and the improvement of this injury is challenging.

Previous studies have suggested that the roles of PKC ζ in different biological processes depend on cell types, milieu and specific binding partners (31,32). Bacterial LPS can powerfully activate the innate immune system of the body (33). It has been shown that PKC ζ activity is required for LPS-stimulated IL-1 β gene expression in RAW264.7 cells (22). This study demonstrated that not only IL-1 β , but also the expression of TNF α was regulated by PKC ζ activity in LPS-stimulated RAW264.7 cells (Fig. 1).

TNF α is considered to be one of the main culprits in non-healing wounds. Keratinocytes exposed to TNF α show inflammatory related functions other than maintaining epithelialization. In the inflammatory epidermis, the chemokines, IL-8 and MCP-1, and the intercellular adhesion molecule-1 (ICAM-1), play important roles in mediating the interaction between immune cells and keratinocytes (34-36). These infiltrating immune cells synthesize more and more inflammatory cytokines and chemokines to further lead to the amplification of inflammation, which contributes to the pathogenesis of chronic wounds. Therefore, the in-depth mechanisms of the effects of

TNF α on keratinocytes are worthy of exploration. Previous studies have indicated that PKC ζ is an important regulator of TNF α signaling via the NF- κ B and MAPK signaling pathways in retinal endothelial cells and human umbilical vein endothelial cells (23,37,38); however, the function of PKC ζ in human keratinocytes has not been fully investigated. Moreover, a previous study indicated that PKC ζ expression was relatively increased and that it was involved in the upregulation of CD1d in the lesions of psoriasis, which is another chronic inflammatory skin disorder characterized by abnormal TNF α stimulation (39). This study demonstrated that TNF α enhanced the phosphorylation level (Thr410) of PKC ζ in a time-dependent manner in HaCaT keratinocytes (Fig. 2A), which led us to explore whether PKC ζ plays a role in TNF α -induced inflammatory responses. PKC ζ -specific pseudosubstrate peptide inhibitor (PKC ζ I) or siRNA was used to block the activity of PKC ζ (Fig. 2B) or to silence its expression (Fig. 2C and D). The present study demonstrated that the mRNA expression levels of IL-8, MCP-1 and ICAM-1 in the HaCaT keratinocytes were all elevated by TNF α stimulation, and peaked respectively at 6, 12 and 6 h post-TNF α treatment and gradually reduced with time (Fig. 3A-C). Pre-treatment with PKC ζ I or PKC ζ -siRNA significantly prevented the TNF α -induced upregulation in the levels of IL-8, MCP-1 and ICAM-1 (Fig. 3D-F). These results provide new insight, indicating that PKC ζ I is a positive regulator of the pro-inflammatory effects of TNF α on keratinocytes.

Compared with MMP2, MMP9 is poorly expressed in normal skin (40). In acute wounds, MMP9 is rapidly increased

as an early response to injury, and then is rapidly decreased to the basic levels (29). The tight regulation and control of MMP9 are important since the persistent and high expression of MMP9 initiated by excessive inflammation can lead to a sustained deficiency in cell-cell and cell-matrix adhesion, which is the subsequent cause of non-healing wounds (8,41,42). Moreover, active MMP9 has been reported that to cleave and activate IL-8 (43,44), which may result in the further dysregulation of inflammation. Our results revealed that the exposure of HaCaT cells to TNF α incurred a time-dependent upregulation in the levels of MMP9; however, MMP2 was not affected (Fig. 4A-C). PKC ζ has been reported to be involved in the regulation of MMP9 in rabbit smooth muscle cells (45). Therefore, we speculated that PKC ζ may be an important mediator in the TNF α -induced expression of MMP9 in keratinocytes. As expected, inhibiting the function of PKC ζ using an inhibitor or siRNA significantly suppressed the increased expression of MMP9 stimulated by TNF α at both the mRNA and protein level (Fig. 4D and E).

In previous studies PKC ζ was reported to be involved in NF- κ B activation by affecting the Ser311 site phosphorylation or nuclear translocation of p65, a key subunit of the NF- κ B complex (20-23). However, the association between PKC ζ and NF- κ B has not been explored in human keratinocytes, at least to the best of our knowledge. Our results of western blot analysis of nucleoproteins demonstrated that in HaCaT cells, PKC ζ inhibitor exerted similar effects to the NF- κ B inhibitor, BAY11-7082, by partly preventing the TNF α -induced nuclear import of p65 (Fig. 5A), and the results from immunofluorescence assay also confirmed the above conclusion (Fig. 5B). Further experiments indicated that BAY11-7082 inhibited the TNF α -induced increase in the expression of IL-8, MCP-1, ICAM-1 and MMP9 (Fig. 6A-D). Therefore, our results indicated that in HaCaT keratinocytes, PKC ζ regulates the expression of IL-8, MCP-1, ICAM-1 and MMP9 by influencing NF- κ B activation.

Keratinocytes require the transition from a quiescent phenotype to an actively migratory and proliferative phenotype during wound healing. Accumulating evidence has indicated that keratinocytes at the diabetic wound edges have an impaired migration and proliferative activity (9,10). Our scratch wound assay demonstrated that TNF α at pathological concentrations significantly inhibited HaCaT cell migration (Fig. 7A and B), which was consistent with the results of a previous study by Zhang *et al* (10). PKC ζ inhibitor faintly improved this inhibition of cell migration; however, its effect was not significant (Fig. 7A and B). TNF α has been reported to actively participate in the promotion of apoptosis and inhibition of proliferation in many other epithelial cell types (46,47). Our CCK-8 proliferation assay revealed that exposure to 100 ng/ml TNF α for 72 h inhibited the proliferation of HaCaT cells; however, this inhibitory effect was not strong, and thus HaCaT cells were not very sensitive to the TNF α -induced inhibition of proliferation as other epithelial cell types (Fig. 7C). Of note, we found that the presence of PKC ζ I appeared to faintly increase this sensitivity (Fig. 7C). This may be due to the fact that TNF α /PKC ζ /NF- κ B can also induce the expression of some anti-apoptotic proteins (48).

In the present study, PBS or TNF α was locally administered to the wound edges of BALB/c mice, and we found that after 2 h, TNF α treatment effectively upregulated the phosphorylation

level of PKC ζ in wound tissue (Fig. 8A). Previous studies have shown that in chronic wounds, the expression of TNF α does not return to the basic level, but maintains a persistent high level at the late stage (5,24). Local injections of inflammatory cytokines in the skin can induce rapid inflammatory reaction (25-30), thus mimicking the environment of various inflammatory skin disease. To further examine whether the prolonged presence of TNF α at the late phase of wound closure influences the rate of wound healing, exogenous TNF α was injected into the tissue around the wound on day 6-12 post-wounding. We demonstrated that the administration of TNF α markedly inhibited wound closure (Fig. 8B and C) and stimulated the sustained high expression of IL-8, MCP-1, ICAM-1 and MMP9 in wounds (Fig. 9). The concomitant administration of PKC ζ I significantly attenuated this non-healing condition (Fig. 8B and C) and resulted in the attenuation of the TNF α -induced upregulation of the above-mentioned molecules (Fig. 9). These findings suggest that PKC ζ is involved in TNF α -triggered inflammatory disorders in unhealed wounds. In the future, in order to elucidate the mechanisms through which PKC ζ regulates chronic wound healing, further investigations are clearly warranted.

In conclusion, to the best of our knowledge, this study, for the first time indicates that PKC ζ effectively regulates TNF α -induced inflammatory responses and MMP9 expression *in vitro* and *in vivo*, which may provide a promising target for researching the interventional therapy of chronic inflammatory skin defects.

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