Original Article
Defects in dermal Vγ4 γ δ T cells result in delayed wound healing in diabetic mice

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Abstract: The skin serves as a physical and chemical barrier to provide an initial line of defense against environmental threats; however, this function is impaired in diabetes. Vγ4 γ δ T cells in the dermis are an important part of the resident cutaneous immunosurveillance program, but these cells have yet to be explored in the context of diabetes. In this study, we observed that the impaired maintenance of dermal Vγ4 γ δ T cells is caused by reduced production of IL-7 in the skin of diabetic mice, which was closely associated with weakened activation of the mTOR pathway in the epidermis of diabetic mice. Weakened CCL20/CCR6 chemokine signaling resulted in the impaired recruitment of dermal Vγ4 γ δ T cells following wounding in diabetic mice. Meanwhile, reduced levels of IL-23 and IL-1β in the dermis around the wounds of diabetic mice resulted in the impaired production of IL-17 by dermal Vγ4 γ δ T cells. Therefore, diminished dermal Vγ4 γ δ T cells and impaired IL-17 production by these cells were important factors in the markedly reduced IL-17 levels in the skin around the wounds of diabetic mice. Because reduced IL-17 levels at the wound edge have been closely associated with delayed wound closure in diabetic mice, defects in dermal Vγ4 γ δ T cells may be an important mechanism underlying delayed wound healing in diabetic mice.

Keywords: Diabetes, wound healing, dermal Vγ4 γ δ T cells

Introduction

Non-healing wounds are the most common chronic complication of diabetes and are difficult to cure, which severely harms the physical and mental health and quality of life of the patients and imposes a huge medical burden. Though we have made considerable progress in elucidating the molecular mechanism underlying non-healing wounds in diabetes, the detailed mechanism remains incompletely understood. Additionally, clinical practice lacks effective treatments for non-healing wounds. The treatment of diabetic wounds is currently a difficult problem facing health care providers [1].

The process of wound repair is mainly regulated by cytokines and growth factors [2]. Therefore, studies of wound repair will help to illuminate the mechanism of diabetic non-healing wounds and provide a new theoretical foundation for treatment by means of exploring the key cells and factors that regulate wound healing.

Interleukin 17 (IL-17) is a pleiotropic cytokine that acts on many cells associated with inflammation and wounds. IL-17 not only plays an important role in host defense at epithelial surfaces [3] but serves as one key factor contributing to keratinocyte proliferation [4], induction of angiogenesis [5, 6], modulation of mesenchymal stem cell and macrophage functions [7, 8] and recruitment of neutrophils [9] following wounding. Furthermore, IL-17a^{-/-} mice exhibit delayed wound healing, suggesting that IL-17 plays an essential role in wound repair and reestablishing the antimicrobial skin barrier [10]. However, the effects of IL-17 on diabetic delayed wound healing have not been investigated.

Dermal Vγ4 γ δ T cells are the predominant source of IL-17 in the skin [11]. Skin γ δ T cells serve as a distinct lymphocyte population with a unique and broad functional repertoire and play important roles in Ab responses, inflammation and tissue repair [12]. Recent studies have shown that epidermal γ δ T cells appear to contribute to wound repair by producing IGF-1 and KGF-1 [13, 14], and impaired homeostasis and activation of epidermal γ δ T cells have been shown in diabetic epidermis [15], which leads to the cells' inability to perform tissue repair functions. However, diabetic dermal Vγ4 γ δ T cells have not been evaluated, and little is known about whether dermal Vγ4 γ δ T cells are associated with diabetic wound defects.

The data presented here show that impaired maintenance of dermal Vγ4 γ δ T cells is caused by the reduced production of IL-7 in the skin of diabetic mice, which is closely associated with weakened activation of the mTOR pathway in the epidermis of diabetic mice. Weakened CCL20/CCR6 chemokine signaling leads to the impaired recruitment of dermal Vγ4 γ δ T cells following wounding in diabetic mice. Meanwhile, reduced levels of IL-23 and IL-1β in the dermis around the wounds of diabetic mice results in the impaired production of IL-17 by dermal Vγ4 γ δ T cells. Therefore, a reduction in the number of dermal Vγ4 γ δ T cells and impaired IL-17 production by these cells result in markedly reduced IL-17 levels in the skin around the wounds of diabetic mice. Because reduced IL-17 levels at the wound edge are closely associated with delayed wound closure in diabetic mice, defects in dermal Vγ4 γ δ T cells may be an important mechanism underlying delayed wound healing in diabetic mice.

Materials and methods

Animals

C57BL/6J (B6) mice were purchased from the Experimental Animal Department of the Third Military Medical University in Chongqing, China. All animals were maintained under specific pathogen-free conditions and used at 6 to 8 weeks of age.

STZ-induced diabetic animal model

C57BL/6J (B6) mice were injected i.p. with 150 µl of STZ (100 mg/kg, Sigma-Aldrich, USA) or the vehicle control for 6 consecutive days. Venous blood glucose levels were measured in non-fasted animals using a glucometer. The mice were evaluated every 2 days at 2:00 p.m. and were considered diabetic when the blood glucose levels were sustained above 250 mg/dL.

Wounding procedure

Wounding was performed on mice anesthetized with sodium pentobarbital. Briefly, the dorsal surface of the mouse was shaved, the back skin and panniculus carnosus were pulled up, and one or two sets of sterile full-thickness wounds were generated using a sterile 4-mm punch tool.

In some experiments, 200 ng of recombinant IL-17 (R&D Systems, USA) or control buffer alone was applied to each wound site immediately post-wounding and daily thereafter. In some experiments, 1 µg of IL-7, IL-1β or IL-23 (R&D Systems, USA) was intradermally injected daily on the back skin of the mice.

For rapamycin administration, mice were injected i.p. with 200 µl rapamycin (4 mg/kg) (Selleck Chemicals, Houston) in 0.2% carboxymethyl cellulose and 0.25% Tween 80 (Sigma-Aldrich, USA) in distilled H_2O or with vehicle control daily.

Isolation of epidermal sheet and dermal sheet

The skin harvested from STZ-induced diabetic and control mice was washed twice in sterile PBS. Next, the skin was cut into 5 mm × 5 mm pieces and washed again with PBS. The pieces were digested with 0.5 g/l of dispase II (Sigma, USA) at 37°C for 1-2 hours, and then the epidermis and dermis were separated carefully. Dermal sheets were incubated in 15 ml RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) containing 40 mg collagenase IV (Sigma-Aldrich, USA), 0.01% DNase I, and 20% FBS for 45 min at 37°C to release cells. The cells were suspended in RPMI 1640 Medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 mg/ml streptomycin, 100 U/mL penicillin and 2 mM glutamine (Hyclone, USA).

Isolation of primary keratinocytes

Primary keratinocytes were isolated from newborn B6 mice according to the protocol men-

tioned in the results. The isolated cells were re-suspended in serum-free keratinocyte medium (K-SFM, GIBCO, 17005) with human recombinant epidermal growth factor (0.1-0.2 ng/ml), bovine pituitary extract (20-30 mg/ml), mouse epidermal growth factor (10 ng/ml; BD, 354-001), cholera toxin (1 × 10^{-10} M; Sigma, C9903), calcium chloride (0.05 mM) and penicillin and streptomycin solution (100 IU/ml, GIBCO, 15140122). The cells were counted and cultured under 5% CO_2 at 37°C in an incubator. The culture medium was replenished every 2-3 days.

Antibodies and flow cytometry

PerCP Cy5.5-conjugated mAbs specific for γ δ TCR (GL3 Tianjin Sungene Biotech Co. Ltd), FITC-conjugated mAbs specific for Vγ2 TCR (BD Biosciences) and BV421-conjugated mAbs specific for CD196 (BD Biosciences) and IL-17 (BD Biosciences) were purchased. A Cytofix/Cytoperm kit (BD Biosciences) was used for intracellular cytokine staining. Flow cytometry data acquisition was performed on an Attune Acoustic Focusing Cytometer (Applied Biosystems, Life Technologies, CA, USA), and the data were analyzed using FlowJo software (Tree Star Incorporation, USA). Experiments were repeated at least three times using the same conditions and settings.

Western blot analysis

Proteins were extracted from cells or epidermal tissue of mice using lysis kits (KeyGEN BioTECH, CA) that contained 1% protease inhibitor cocktail, 5% phenymethylsulphonyl fluoride and 5% phosphatase inhibitor cocktail according to the manufacturer's protocol. The lysed cellular samples were scraped, collected and agitated for 20 minutes followed by centrifugation at 14,000 × g for 15 minutes at 4°C. The supernatant was collected as total cellular protein, and the protein concentrations were determined by a BCA protein assay (Thermo Scientific, Rockford, USA). Equal protein (20 μg) from each sample was loaded onto 10% SDS-PAGE gels for electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Immobilon, USA). The membrane was blocked with Tris-buffered saline (TBS) containing 3% bull serum albumin (BIOSHARP, CA) for 2 hours at room temperature and then incubated with primary rabbit antibodies to p-S6K (Thr389), S6K, p-AKT (Ser473), AKT, p-4E-BP1 (Thr37/46), 4E-BP1 (1:1000, Cell Signaling Technologies, Beverly, MA), rabbit antibodies to IL-7 and IL-17 (1:200, Santa Cruz Biotechnology, USA), rabbit antibodies to IL-1β, IL-23 and CCL20 (1:1000, Abcam, UK) and a mouse antibody to GAPDH (1:5000, KANGCHEN BIO-TECH, CA) at 4°C overnight. The membranes were subsequently washed 5 times with TBS containing 0.1% Tween 20 and then incubated with HRP-labeled goat anti-rabbit/mouse secondary antibody (1:5000, ZSGB-BIO, CA) for 1 hour at room temperature. Finally, the membranes were washed 5 times with TBS containing 0.1% Tween 20 and visualized using enhanced chemiluminescence (Pierce, USA) according to the manufacturer's instructions. The bound antibodies were detected using the ChemiDoc™ XRS western blot detection system (Bio-Rad, USA).

Quantitative real-time RT-PCR

The cultured cells and skin tissues were washed with PBS, and the total RNA was extracted with an RNeasy® Mini kit (QIAGEN, GER) according to the manufacturer's instructions. Total RNA was extracted from epidermal sheets of mouse back skin around wounds using an RNeasy extraction kit (QIAGEN) according to the manufacturer's instructions. The RNA concentrations and qualities were measured by DU800 UV/Vis Spectrophotometer (Beckman Coulter, USA). The mRNA was reverse transcribed with a First Strand cDNA Synthesis kit (TOYOBO, JPN). Real-time PCR was performed using SYBR Green PCR Master Mix (TOYOBO, JPN) under the following conditions: 95°C for 2 minutes, followed by 50 repetitive cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 32 seconds. The primers used in this study were as follows:

	Forward primer	Backward primer
IL-17	5-CTCCAGAAGGCCCTC-AGACTAC-3	5-AGCTTTCCCTC-CGC-ATTGACACAG-3
IL-7	5-GCCTGTCACATCATC-TGAGTGCC-3	5-CAGGAGGCATCCAG-GAACTTCTG-3
IL-1β	5-ATCTCGCAGCAGCAC-ATC-3	5-ATCTCGCAGCAGCA-CATC-3
IL-23	5-CTGTGCCTAGGAGTA-GCAGTC-3	5-AGTCTCTTCATCCTC-TTCTTCTCT-3
CCL20	5-ACAGTGTGGGAAGCA-AGTCC-3	5-CCGTGAACTCCTTTG-ACCAT-3
GAPDH	5-CGTGCCGCCTGGAGA-AAC-3	5-AGTGGGAGTTGCTGT-TGAAGTC-3

A

B

C
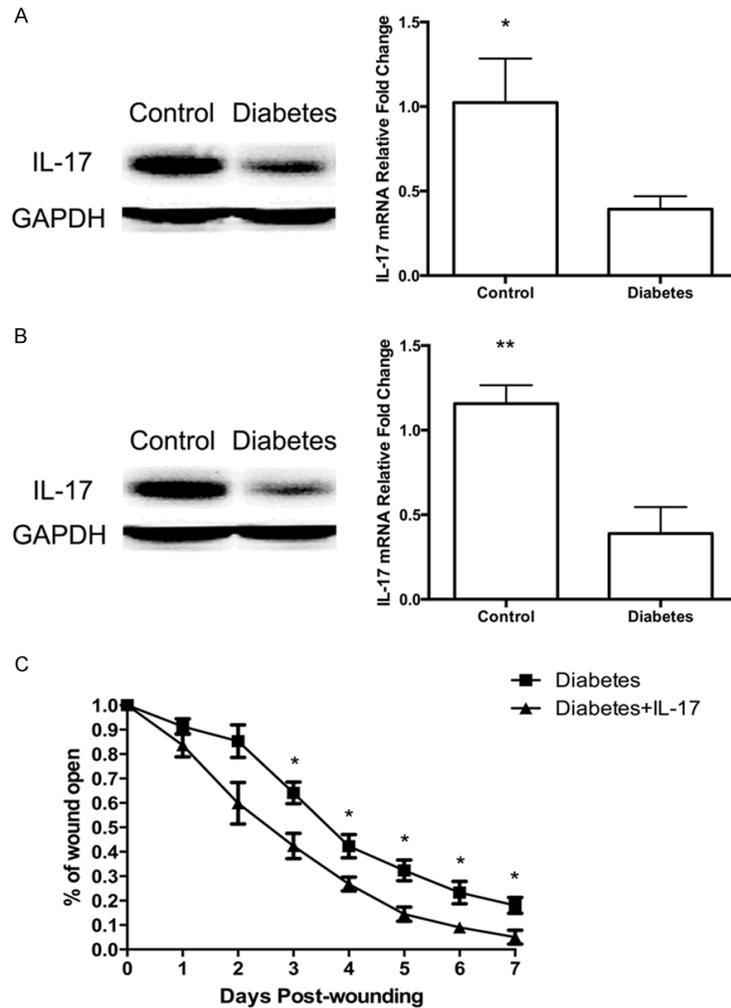
Figure 1. Reduced expression of IL-17 in diabetic skin resulted in abnormal wound healing in diabetic mice. Wild-type C57BL/6J mice were administered daily i.p. injections of STZ or vehicle control for 6 days and received full-thickness wounds in their back skin 4 weeks after STZ treatment. A. Reduced expression of IL-17 in the intact skin of diabetic mice at both the mRNA and protein level. B. Reduced expression of IL-17 in the skin around the wounds of diabetic mice at both the mRNA and protein level. On day 1 after wounding, the skin around the wounds of STZ-induced diabetic or control mice were obtained for detecting the expression of IL-17 by means of western blot and quantitative PCR. C. Application of IL-17 to wounds promotes diabetic wound healing. Mice were administered IL-17 (200 ng) or buffer control after wounding, and wound closure was measured over time. *p < 0.05 and **p < 0.005 vs vehicle control (two-tailed, unpaired Student's t-test).

Quantification of the mRNA levels was conducted in the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) following the manufacturer's protocols. The data were analyzed by the 2-ΔΔ threshold (Ct) method, and GAPDH served as an internal control.

Statistical comparisons were performed with Student's t-test. The data are presented as the mean ± standard deviation (SD). In all cases, a *P* value less than 0.05 was considered to be statistically significant.

Results

Reduced expression of IL-17 in diabetic skin results in abnormal wound healing in diabetic mice

Considering that IL-17 plays an essential role in wound repair and reestablishing the antimicrobial skin barrier, and IL-17a$^{-/-}$ mice exhibit wound healing defects [10], we investigated whether IL-17 was involved in the delayed wound repair of diabetic mice. Wild-type C57BL/6J mice were administered STZ or vehicle control daily for 6 days [16] and then received full-thickness wounds in their back skin [17] at 4 weeks after STZ treatment. Diabetic mice showed markedly weakened IL-17 in the intact skin and in the skin around the wounds (**Figure 1A** and **1B**) compared with wild-type controls. In addition, IL-17 administration promoted wound healing in diabetic mice (**Figure 1C**). Our data indicates that reduced IL-17 levels around the wound margin are closely associated with delayed wound closure in diabetic mice.

Dermal Vγ4 γ δ T cells are diminished in the skin of diabetic mice

Dermal Vγ4 γ δ T cells are the predominant source of IL-17 in the skin [11]. To investigate whether dermal Vγ4 γ δ T cells were involved in the reduced levels of IL-17 in the skin of diabetic mice, dermal Vγ4 γ δ T cells in diabetic
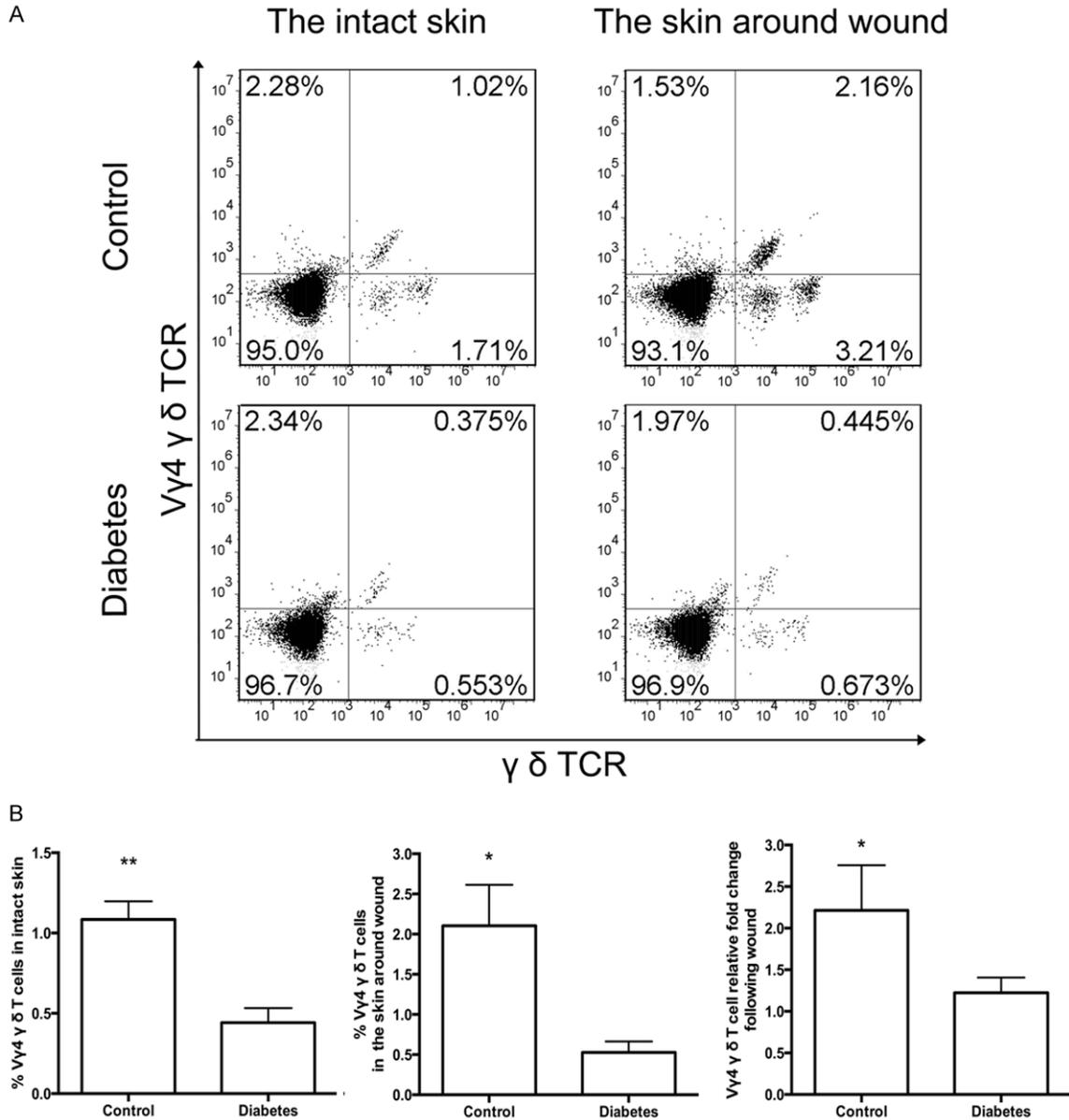
Figure 2. Dermal Vγ4 γ δ T cells were diminished in the skin of diabetic mice. Wild-type C57BL/6J mice were administered daily i.p. injections of STZ or vehicle control for 6 days and received full-thickness wounds in their back skin 4 weeks after STZ treatment. A. On day 1 after wounding, single-cell suspensions of intact dermis and the dermis around the wounds of STZ-induced diabetic or control mice were obtained and stained by γ δ TCR and Vγ2 TCR to examine the number of Vγ4 γ δ T cells in the dermis by using FACS. B. Reduced Vγ4 γ δ T cells in intact dermis and the dermis around the wounds of STZ-induced diabetic mice. Dermal Vγ4 γ δ T cells showed an obvious increase on day 1 after wounding in wild-type controls compared with diabetic mice. *p < 0.05 and **p < 0.005 vs vehicle control (two-tailed, unpaired Student's t-test).

intact and wound skin were examined. Diabetic mice showed a reduction of Vγ4 γ δ T cells in the intact and wound dermis (**Figure 2A** and **2B**) compared with the wild-type controls, suggesting that the impaired maintenance of Vγ4 γ δ T cells in the intact dermis of diabetic mice.

Because IL-17 in the skin is primarily produced by dermal Vγ4 γ δ T cells, the reduced number of dermal Vγ4 γ δ T cells is an important mechanism underlying the markedly reduced IL-17 levels in the skin around wounds in diabetic mice.
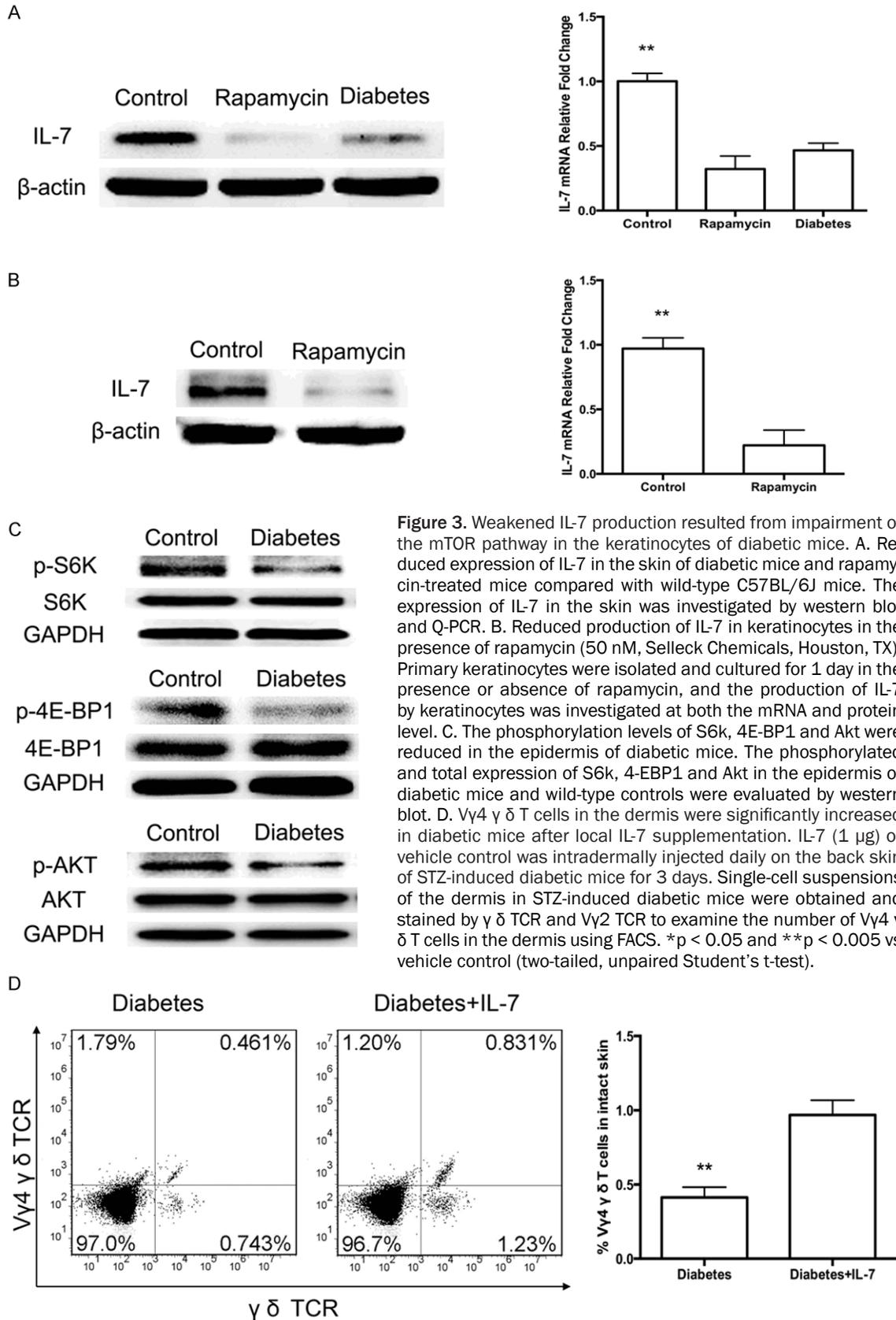
Figure 3. Weakened IL-7 production resulted from impairment of the mTOR pathway in the keratinocytes of diabetic mice. A. Reduced expression of IL-7 in the skin of diabetic mice and rapamycin-treated mice compared with wild-type C57BL/6J mice. The expression of IL-7 in the skin was investigated by western blot and Q-PCR. B. Reduced production of IL-7 in keratinocytes in the presence of rapamycin (50 nM, Selleck Chemicals, Houston, TX). Primary keratinocytes were isolated and cultured for 1 day in the presence or absence of rapamycin, and the production of IL-7 by keratinocytes was investigated at both the mRNA and protein level. C. The phosphorylation levels of S6k, 4E-BP1 and Akt were reduced in the epidermis of diabetic mice. The phosphorylated and total expression of S6k, 4-EBP1 and Akt in the epidermis of diabetic mice and wild-type controls were evaluated by western blot. D. Vγ4 γ δ T cells in the dermis were significantly increased in diabetic mice after local IL-7 supplementation. IL-7 (1 μg) or vehicle control was intradermally injected daily on the back skin of STZ-induced diabetic mice for 3 days. Single-cell suspensions of the dermis in STZ-induced diabetic mice were obtained and stained by γ δ TCR and Vγ2 TCR to examine the number of Vγ4 γ δ T cells in the dermis using FACS. *p < 0.05 and **p < 0.005 vs vehicle control (two-tailed, unpaired Student's t-test).
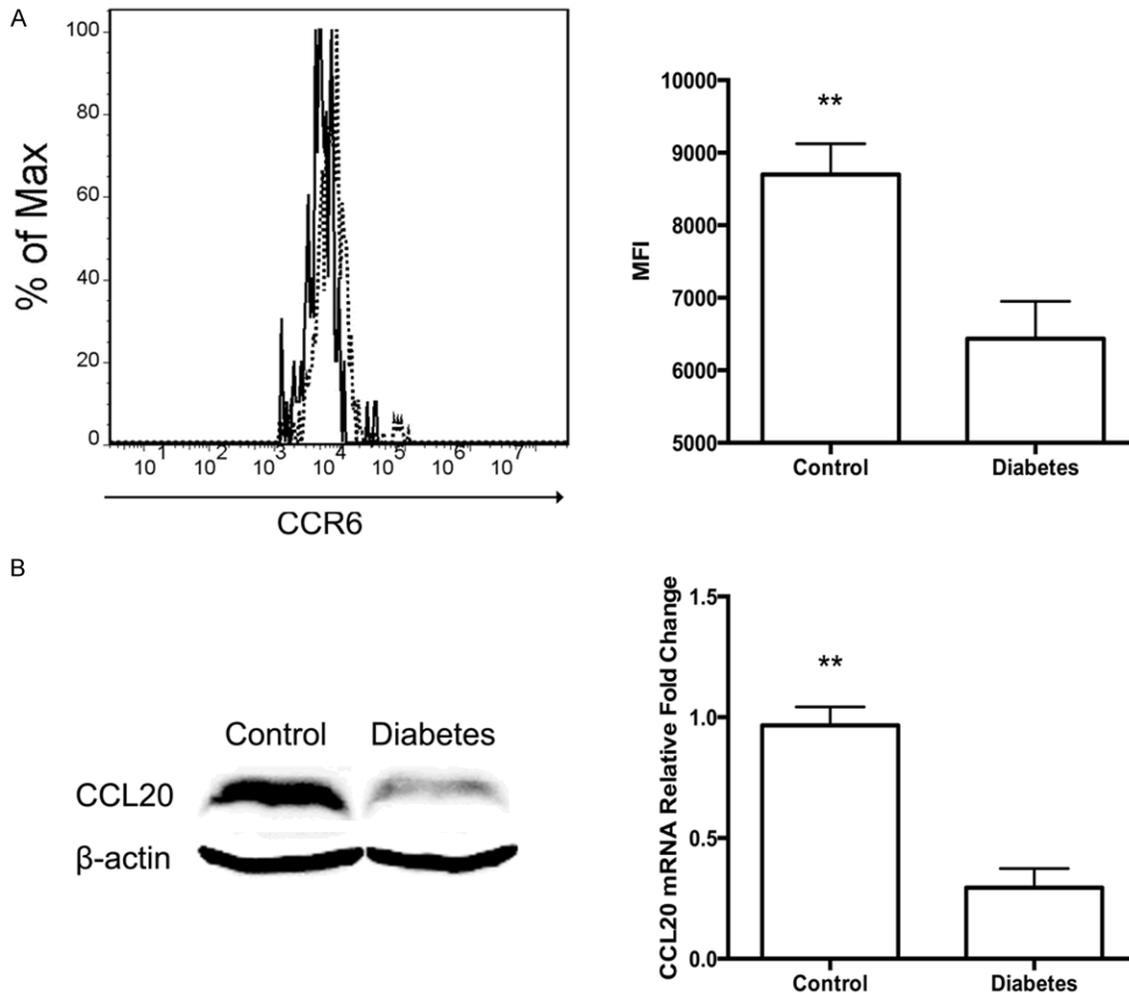
Figure 4. Impaired CCL20/CCR6 chemokine signaling in the wounded dermis of diabetic mice. Wild-type C57BL/6J mice were administered daily i.p. injections of STZ or vehicle control for 6 days and received full-thickness wounds in their back skin 4 weeks after STZ treatment. A. Vγ4 γ δ T cells in the dermis around the wounds of diabetic mice expressed reduced levels of CCR6 compared with wild-type controls. On day 1 after wounding, single-cell suspensions of the dermis around the wounds of STZ-induced diabetic or control mice were obtained and stained by CCR6 and Vγ2 TCR to examine the CCR6 levels on dermal Vγ4 γ δ T cells by FACS. The mean fluorescence intensity (MFI) of CCR6 expression by dermal Vγ4 γ δ T cells is presented in the right panel. B. Reduced expression of CCL20 in the dermis around the wounds of diabetic mice compared with wild-type C57BL/6J mice. On day 1 after wounding, the dermis around the wounds of STZ-induced diabetic or control mice was obtained to detect the expression of CCL20 by western blot and Q-PCR. *$p < 0.05$ and **$p < 0.005$ vs vehicle control (two-tailed, unpaired Student's t-test).

Reduced IL-7 production in the skin disrupts dermal Vγ4 γ δ T cell maintenance, which is closely associated with impairments in the mTOR pathway in the epidermis in diabetic mice

Dermal Vγ4 γ δ T cells are maintained through their local proliferation in steady-state conditions independent of circulating precursors [18]. Dermal Vγ4 γ δ T cells display a unique profile of cytokine dependence on IL-7, which controls their development and maintenance [18]. Thus, the expression of IL-7 in the skin of

diabetic mice was investigated. The expression of IL-7 in the skin was reduced in diabetic mice (**Figure 3A**). Meanwhile, we observed that dermal Vγ4 γ δ T cells were significantly increased in diabetic mice after local IL-7 supplementation (**Figure 3D**). These findings suggest that the reduction of IL-7 in the skin induces abnormal dermal Vγ4 γ δ T cell maintenance, leading to reduced numbers of Vγ4 γ δ T cells in the dermis of diabetic mice.

Previous studies have demonstrated that epidermal keratinocytes produce biologically rele-

vant amounts of IL-7 [19-21], and intestinal epithelial cell-derived IL-7 expression is regulated through the STAT1/IRF-1 and IRF-2 pathways [22]. However, the mechanism of regulation of IL-7 production in the skin remains unknown. The mTOR pathway has been shown to be the central regulator of metabolism that plays a pivotal role in the pathogenesis of diabetes [23-25]. Therefore, we determined whether keratinocyte-derived IL-7 is regulated through the mTOR pathway. To address this issue, primary keratinocytes were isolated from newborn C57 wild-type mice [26] and cultured for 1 day in the presence or absence of rapamycin. The results showed that the production of IL-7 by keratinocytes was reduced significantly following rapamycin treatment at both the mRNA and protein levels (**Figure 3B**). Meanwhile, we found that the expression of IL-7 in the skin was also reduced in rapamycin-treated mice (**Figure 3A**). The results indicate that the activation of the mTOR pathway is required for the production of IL-7 in keratinocytes, and impairments in the mTOR pathway result in a reduction in IL-7 production in the skin. Furthermore, western blotting was used to examine the phosphorylation of the mTOR pathway in the epidermis of diabetic mice, and the results showed that the activation of the mTOR pathway in the epidermis of diabetic mice was markedly inhibited compared to wild-type controls (**Figure 3C**). Therefore, we propose that in STZ-induced diabetic mice, reduced IL-7 production in the skin is closely associated with impairments in the mTOR pathway in the epidermis, leading to disrupted dermal Vγ4 γ δ T cell maintenance.

Impaired CCL20/CCR6 chemokine signaling contributes to the reduced recruitment of dermal Vγ4 γ δ T cells in the wound skin of diabetic mice

We observed that the quantity of DETCs was increased upon wounding in wild-type controls; however, the quantity of DETCs was only slightly increased in diabetic mice (**Figure 2A** and **2B**). We wondered whether the recruitment of dermal Vγ4 γ δ T cells following wounding was affected in diabetic mice. The chemokine receptor CCR6 is associated with the recruitment of γ δ T cells [27, 28], and dermal Vγ4 γ δ T cells constitutively express CCR6 [29]. Thus we determined whether the expression of CCR6 in dermal Vγ4 γ δ T cells was affected in dia-

betic mice. The results showed that Vγ4 γ δ T cells in the dermis around the wounds of diabetic mice expressed reduced levels of CCR6 compared with wild-type controls (**Figure 4A**).

CCL20 is a ligand of CCR6 [30], which has a dominant influence on γ δ T cell recruitment [27, 31]. To determine whether the expression of CCL20 was abnormal in diabetic mice, we examined the expression of CCL20 in the dermis around the wounds of diabetic mice. The results showed reduced levels of CCL20 in the dermis around the wounds of diabetic mice compared with wild-type controls (**Figure 4B**). Therefore, our data suggest that impaired CCL20/CCR6 chemokine signaling contributes to the weakened recruitment of dermal Vγ4 γ δ T cells in the wound skin of diabetic mice, which results in only a slight increase of dermal Vγ4 γ δ T cells following wounding compared with wild-type controls.

Reduced levels of IL-23 and IL-1β in the dermis around wounds result in impaired IL-17 production in dermal Vγ4 γ δ T cells in diabetic mice

Because dermal Vγ4 γ δ T cells are the major IL-17-producing cells in the skin, we evaluated whether IL-17 production by these cells was influenced in diabetic mice. We found that dermal Vγ4 γ δ T cells from wild-type controls produced significant amounts of IL-17. In contrast, dermal Vγ4 γ δ T cells from diabetic mice secreted minimal IL-17 (**Figure 5A**). Previous studies have demonstrated that dermal Vγ4 γ δ T cells require a combination of IL-23 and IL-1β stimulation to produce IL-17 [11]. Thus, we investigated the expression of IL-23 and IL-1β in the dermis around the wounds of diabetic mice. The results showed that the levels of IL-23 and IL-1β in the skin were obviously reduced in diabetic mice compared with wild-type controls (**Figure 5B**). Meanwhile, local IL-23 and IL-1β administration significantly increased IL-17 production by dermal Vγ4 γ δ T cells at the wound edge in diabetic mice (**Figure 5A**). The results indicate that the reduced levels of IL-23 and IL-1β result in the reduction of IL-17 production in dermal Vγ4 γ δ T cells in the wound skin of diabetic mice. Thus, we propose that impaired IL-17 production in dermal Vγ4 γ δ T cells, along with reductions in dermal Vγ4 γ δ T cell numbers at the wound edge, are important mecha-

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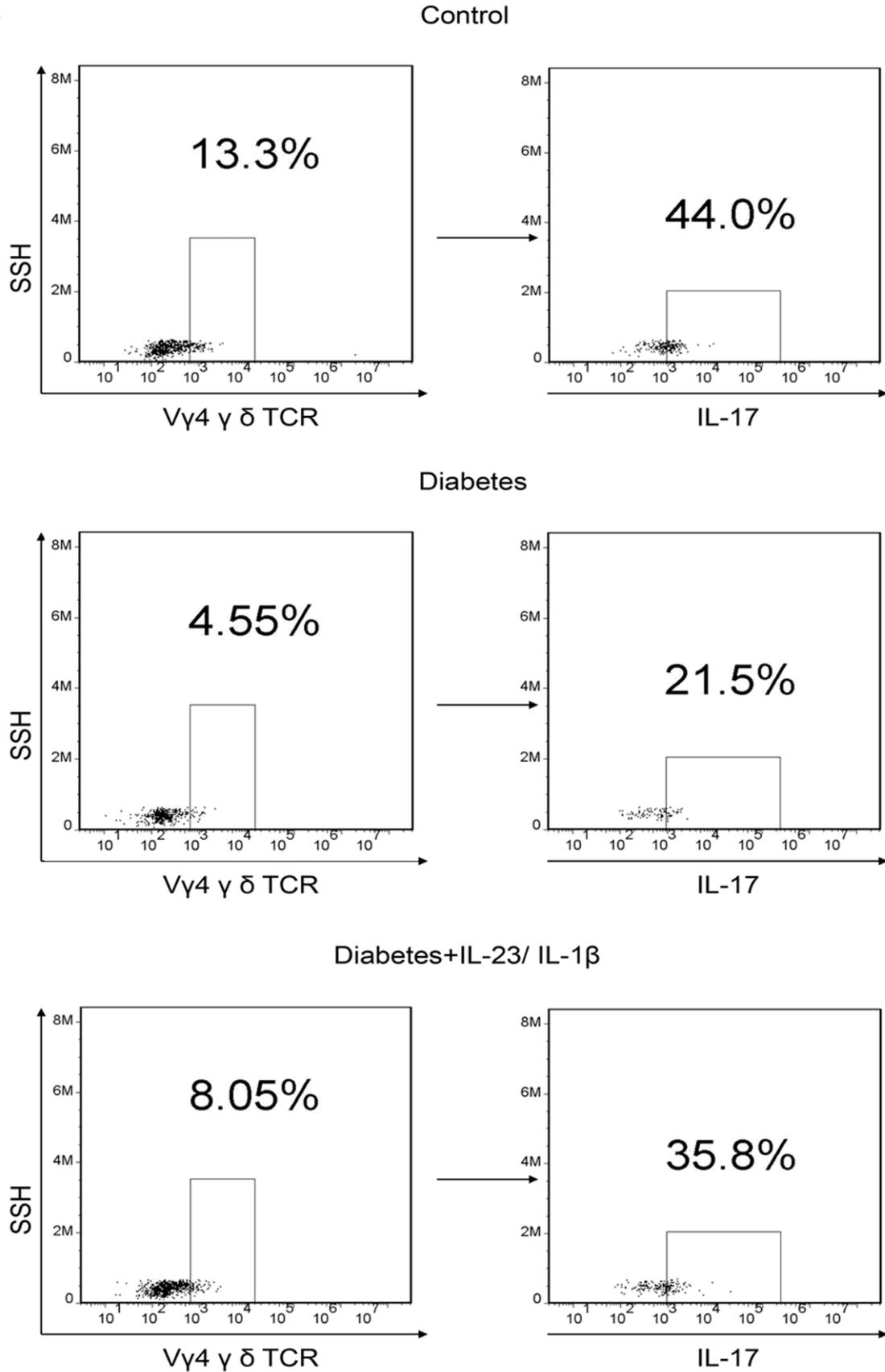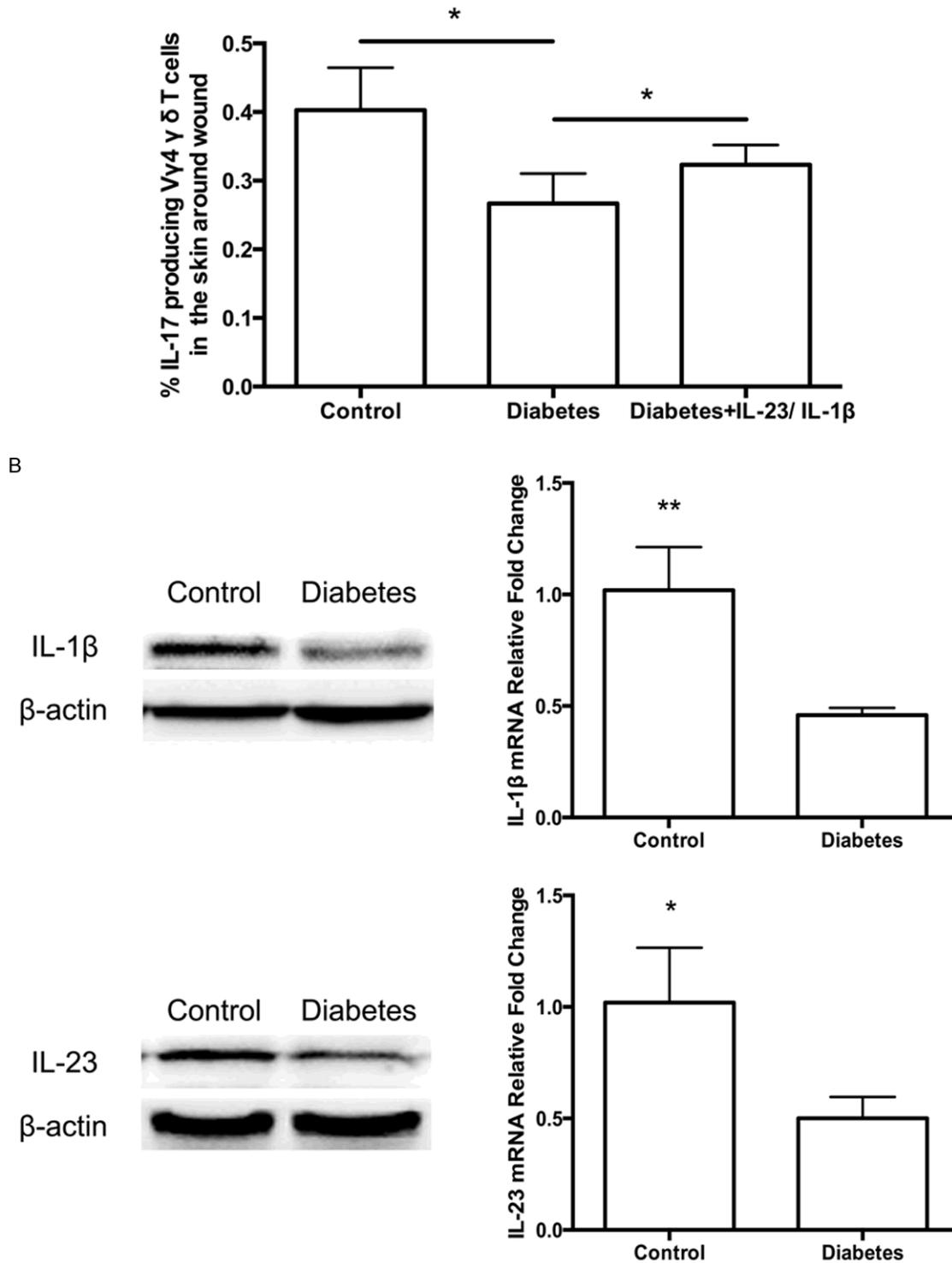
Figure 5. Reduced levels of IL-23 and IL-1β in the dermis around wounds result in impaired IL-17 production by dermal Vγ4 γ δ T cells in diabetic mice. Wild-type C57BL/6J mice were administered daily i.p. injections of STZ or vehicle control for 6 days and received full-thickness wounds in their back skin 4 weeks after STZ treatment. A. Dermal Vγ4 γ δ T cells from wild-type controls produced significant amounts of IL-17, and dermal Vγ4 γ δ T cells from diabetic mice secreted minimal IL-17. Local IL-23 and IL-1β administration significantly increased IL-17 production by dermal Vγ4 γ δ T cells in diabetic mice. On day 1 after wounding, single-cell suspensions of the dermis in STZ-induced diabetic or control mice were obtained and stained for IL-17 and Vγ2 TCR to examine the production of IL-17 in dermal Vγ4 γ δ T cells by using FACS. IL-23 (1 μg) and IL-1β (1 μg) or vehicle control was intradermally injected daily on the

back skin of STZ-induced diabetic mice for 2 days before wounding. B. Reduced expression of IL-1β and IL-23 in the dermis around the wounds of diabetic mice at both the mRNA and protein level. On day 1 after wounding, the dermis around the wounds of STZ-induced diabetic or control mice was obtained to evaluate the expression of IL-1β and IL-23 by western blot and quantitative PCR. *p < 0.05 and **p < 0.005 vs vehicle control (two-tailed, unpaired Student's t-test).

nisms underlying the markedly reduced IL-17 levels in the skin around the wounds of diabetic mice.

Discussion

Diabetes is a clinically common metabolic disorder that is associated with some skin complications, including skin barrier disruption, increased infection and chronic non-healing wounds. Considering that dermal Vγ4 γ δ T cells serve as an important part of the resident cutaneous immunosurveillance program [18], we investigated whether these cells were associated with diabetic skin complications. Strikingly, we observed that the maintenance and recruitment of dermal IL-17-producing Vγ4 γ δ T cells were impaired, and IL-17 production by these cells was weakened in diabetic mice. These factors were a major cause of the markedly diminished levels of IL-17 in the skin of diabetic mice. Because reduced IL-17 production at the wound edge was closely associated with delayed wound closure in diabetic mice, defects in dermal Vγ4 γ δ T cells may be an important mechanism underlying delayed wound healing in diabetic mice. To our knowledge, this is the first description correlating dermal Vγ4 γ δ T cells with diabetic skin complications.

Interleukin 17 (IL-17) is a pleiotropic cytokine that acts on many cells closely associated with inflammation and wound healing. A recent study reveals that IL-17 induces the expression of regenerating islet-derived protein 3-alpha (REG3A) in keratinocytes [4]; this protein is highly expressed in keratinocytes during wound repair and can promote keratinocyte proliferation. IL-17 can induce VEGF expression in keratinocytes and promote angiogenesis [5, 6], and it also induces an atypical M2-like macrophage subpopulation associated with wound healing [8]. IL-17 can increase mesenchymal stem cell migration to promote tissue regeneration [7]. IL-17 is involved in the attraction of neutrophils and induces the expression of multiple host-defense molecules in keratinocytes to promote healing [9, 10]. Meanwhile, IL-17a$^{-/-}$ mice exhibit delayed wound healing [10]. Herein, we

observed reduced IL-17 in intact skin and in the skin around the wounds of diabetic mice, and local IL-17 administration promoted wound healing in diabetic mice. Thus, we propose that decreased IL-17 at the wound edge results in impaired wound repair in diabetic mice.

The skin serves as an important barrier to protect the body against environmental threats and can immediately repair itself when it is harmed. This function is mediated partly by resident dendritic epidermal T cells (DETC), which can sense skin injury and produce cytokines and growth factors to regulate immune responses and promote repair [32]. In contrast to this well-recognized subset, there exists a population of Vγ4 γ δ T cells in the dermis. A recent study revealed that IL-17 production was significantly decreased after Vγ4 T cell depletion, suggesting that these Vγ4 γ δ T cells are the predominant source of IL-17 in the skin [11]. Our results showed that dermal Vγ4 γ δ T cells were reduced in the intact skin of diabetic mice compared with wild-type controls, suggesting the impaired maintenance of Vγ4 γ δ T cells in the dermis of diabetic mice. Considering that IL-17 plays an important role in skin barrier function, these reduced dermal IL-17-producing Vγ4 γ δ T cells may be closely associated with disruptions in the skin barrier in diabetic mice.

Considering that dermal Vγ4 γ δ T cells are IL-7R^{hi}, in contrast to DETCs, which are IL-7R^{lo} [29], IL-7 is a candidate trophic factor for the local maintenance of Vγ4 γ δ T cells in the skin. A recent study has shown that both the frequency and the number of dermal γ δ T cells are significantly reduced in IL-7$^{-/-}$ mice, and there is a significant increase in the number of dermal γ δ cells in mice transgenically overexpressing IL-7 [18]. Our results demonstrated that the levels of IL-7 were obviously reduced in the skin of diabetic mice, and dermal Vγ4 γ δ T cells displayed a significant increase after local IL-7 supplementation. These findings suggest that the impaired maintenance of Vγ4 γ δ T cells results from reduced levels of IL-7 in the skin of diabetic mice. Because IL-7 is produced by epidermal keratinocytes, we further investigated

what may regulate IL-7 production in keratinocytes.

The Akt/mTOR pathway has been shown to be a central metabolic regulator that plays a pivotal role in the pathogenesis of diabetes [23-25]. Our results showed that impairment of the mTOR pathway by rapamycin resulted in obviously reduced IL-7 production by keratinocytes and decreased IL-7 levels in the skin of mice. These findings indicate that activation of the mTOR pathway is critical for IL-7 production in keratinocytes. Because IL-7 is mainly produced by keratinocytes in the skin, we propose that reduced IL-7 production in keratinocytes resulting from impairments in the mTOR pathway subsequently leads to decreased IL-7 levels in the skin. Furthermore, we found that the phosphorylation of mTOR pathway was weakened in the epidermis of diabetic mice. Thus, we propose that the decreased IL-7 production in the skin is closely associated with weakened activation of the mTOR pathway in the epidermis of diabetic mice, which leads to the impaired maintenance of Vγ4 γ δ T cells in the dermis of diabetic mice.

Furthermore, we found that Vγ4 γ δ T cells in the dermis of diabetic mice were only increased slightly following wounding; however, Vγ4 γ δ T cells in the dermis of wild-type controls revealed an outstanding increase following wounding. These results suggest that there may be impaired recruitment of dermal Vγ4 γ δ T cells following wounding in diabetic mice. The chemokine receptor CCR6 is associated with the recruitment of γ δ T cells [27, 28], and dermal Vγ4 γ δ T cells constitutively express CCR6 [29]. Meanwhile, CCL20 serves as ligand for CCR6 [30], which dominantly influences γ δ T cell recruitment [27, 31]. Our results showed decreased levels of CCR6 in dermal Vγ4 γ δ T cells and reduced expression of CCL20 in the dermis of diabetic mice. This indicates that impaired CCL20/CCR6 chemokine signaling leads to the impaired recruitment of dermal Vγ4 γ δ T cells following wounding in diabetic mice.

A recent study reveals that IL-23 together with IL-1β activates splenic γ δ T cells to produce IL-17 [33]. Dermal Vγ4 γ δ T cells secrete large amounts of IL-17 following combined IL-23 and IL-1β stimulation, and IL-23 or IL-1β alone failed to stimulate them to produce IL-17 [11]. The cellular sources of IL-23 in the skin are mainly infiltrating DCs and monocytes in the dermis [34, 35], as well as keratinocytes and langerhans cells in the epidermis [36, 37]. Psoriatic skin keratinocytes and infiltrating inflammatory cells secrete large amounts of IL-1β [38]. Our results showed reduced levels of IL-23 and IL-1β in the dermis of diabetic mice, and the dermal Vγ4 γ δ T cells from diabetic mice secreted minimal IL-17 compared with the wild-type controls. Meanwhile, local IL-23 and IL-1β administration significantly increased IL-17 production by dermal Vγ4 γ δ T cells at the wound edge in diabetic mice. This suggests that the reduced levels of IL-23 and IL-1β in the dermis weaken IL-17 production in dermal Vγ4 γ δ T cells in diabetic mice. Thus, we conclude that impaired IL-17 production by dermal Vγ4 γ δ T cells combined with diminished numbers of these cells is a major cause of the marked reduction in IL-17 levels in the skin around the wounds of diabetic mice.

Taken together, our results emphasize the importance of IL-17-producing Vγ4 γ δ T cells in the dermis in diabetic wound healing. The impaired maintenance of dermal Vγ4 γ δ T cells is caused by the reduced production of IL-7 in the skin of diabetic mice, which is closely associated with weakened activation of the mTOR pathway in the epidermis of diabetic mice. Weakened CCL20/CCR6 chemokine signaling leads to the impaired recruitment of dermal Vγ4 γ δ T cells following wounding in diabetic mice. Meanwhile, the reduced levels of IL-23 and IL-1β in wound skin results in the impaired production of IL-17 by dermal Vγ4 γ δ T cells in diabetic mice. Therefore, diminished dermal Vγ4 γ δ T cells and impaired IL-17 production by these cells are important mechanisms underlying the markedly reduced IL-17 levels in the skin around wounds in diabetic mice. Because reduced IL-17 at the wound edge is closely associated with delayed wound closure, defects in dermal Vγ4 γ δ T cells may be an important mechanism that results in delayed wound healing in diabetic mice.

Disclosure of conflict of interest

The authors declare no competing financial interests. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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