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ORIGINAL RESEARCH

Diabetes Mellitus Inhibits Hair Follicle Regeneration by Inducing Macrophage Reprogramming-Mediated Pyroptosis

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Background: Diabetes mellitus (DM) is known to inhibit skin self-renewal and hair follicle stem cell (HFSC) activation, which may be key in the formation of chronic diabetic wounds. This study aimed to investigate the reasons behind the suppression of HFSC activation in DM mice.

Methods: Type 1 DM (T1DM) was induced in 6-week-old mice via streptozotocin, and hair follicle growth was subsequently monitored. RNA sequencing, bioinformatics analyses, qRT–PCR, immunostaining, and cellular experiments were carried out to investigate the underlying mechanisms involved.

Results: T1DM inhibited HFSC activation, which correlated with an increase in caspase-dependent programmed cell death. Additionally, T1DM triggered apoptosis and pyroptosis, predominantly in HFSCs and epidermal regions, with pyroptosis being more pronounced in the inner root sheath of hair follicles. Notably, significant cutaneous immune imbalances were observed, particularly in macrophages. Cellular experiments demonstrated that M1 macrophages inhibited HaCaT cell proliferation and induced cell death, whereas high-glucose environments alone did not have the same effect.

Conclusion: T1DM inhibits HFSC activation via macrophage reprogramming-mediated caspase-dependent pyroptosis, and there is a significant regional characterization of cell death. Moreover, T1DM-induced programmed cell death in the skin may be more closely related to immune homeostasis imbalance than to hyperglycemia itself. These findings shed light on the pathogenesis of diabetic ulcers and provide a theoretical basis for the use of hair follicle grafts in wound repair.

Keywords: diabetes mellitus, caspases, hair follicle stem cells, chronic wounds, cutaneous immune disorder

Introduction

Diabetic chronic wounds (DCWs), also known as diabetic ulcers, are common skin complications of diabetes. Approximately 340 million people worldwide have diabetes, approximately one-third have diabetes-related skin diseases, and about one-fifth have DCWs.[1–3] DCWs are difficult to heal, and there are no effective medications or methods to prevent and treat DCWs other than nursing management.[4,5] Notably, 0.03%-1.5% of patients suffer from lifelong disability due to amputation of their limbs due to long-term ulcers.[6] The formation of DCWs is associated with peripheral neuropathy, difficulty in vascular regeneration, inhibition of skin cell growth, persistent inflammation, and a high glycemic environment that is conducive to microbial growth.[7–11] Despite many targeted solutions, effective prevention and treatment strategies remain elusive, highlighting unknown pathological mechanisms and underscoring the urgency for novel solutions.

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Hair follicles (HFs) are important accessory organs that maintain normal physiological function and homeostasis of the skin and can promote wound healing.[12,13] HFSCs are important constituent cells of HFs, and the activation of HFSCs is the key to HF regeneration.[14,15] Following skin injury, HFSCs migrate to trauma areas, where they differentiate into keratinocytes to promote re-epithelialization. Recent studies have shown that HFSC activation is vital in the formation and development of DCWs. Diabetes mellitus (DM) inhibits the activation and proliferation of Lgr5 hFSCs in the dorsal skin of mice through the inhibition of WNT-dependent pathways, which in turn reduces skin renewal.[10] This means that fragile skin is more prone to injury and difficult to heal.[16] Additionally, HFSCs exhibit regional variations in characteristics and environmental sensitivities. Those from the occipital region have better environmental adaptability and can rapidly adapt to the new environment, proliferate, and differentiate even after being grafted. Therefore, the occipital region is also known as the dominant donor site for HF grafts.[17] In contrast, the scarcity and fragility of foot HFs might contribute significantly to diabetic foot. Numerous clinical studies have confirmed that HF grafting is an effective way to accelerate the healing of DCWs.[18–20] Therefore, we suggest that maintaining the health of HFs is crucial for maintaining skin homeostasis and repair ability. Additionally, we hypothesized that DM may also involve other mechanisms affecting HFSCs in addition to inhibiting their activation and proliferation.

Programmed cell death (PCD) is a genetically regulated, active form of cell death encompassing 12 recognized types. Several forms of PCD have been shown to be associated with DM. For example, a high-glucose environment inhibits autophagy by disrupting YTHDC1/SQSTM1 signaling pathway expression, thereby reducing the healing of DCWs.[21] A previous study reported that apoptosis-related mRNAs are significantly upregulated in human diabetic foot ulcers.[22] A cellular study confirmed that high-glucose medium inhibits the migration and proliferation of human immortalized epidermal cells (HaCaTs) while promoting apoptosis.[23] Another mouse-based study suggested that DCWs combined with *Candida albicans* infection may be associated with the caspase-1/NLRP3 inflammasome pyroptosis pathway.[24] These studies all revealed that DCWs are associated with PCD, albeit through diverse mechanisms. However, these studies were mainly based on pre-existing DCWs, and no one has yet studied diabetic skin before it broke.

DM is known to cause systemic immune dysfunction and a persistent cutaneous low-grade inflammatory state.[25,26] DCWs are also associated with a persistent inflammatory state.[26] Moreover, inflammation can induce PCD pathways, such as pyroptosis and apoptosis. Pyroptosis is associated primarily with the host immune response to microbial infection or cellular stress. This leads to the release of proinflammatory molecules, which may contribute to the clearance of pathogens but may also result in tissue damage and inflammation-related diseases.[27] Pyroptosis is driven primarily by the activation of inflammatory caspases, including caspase-1, −4, −5, and −11, and is typically characterized morphologically by rapid cell swelling, membrane rupture, and the release of inflammatory intracellular contents. Apoptosis plays a crucial role in various physiological and pathological processes, including embryonic development, tissue homeostasis, and the elimination of damaged or unwanted cells. Apoptosis is driven primarily by the activation of initiator caspases (eg, caspase-8 and caspase-9) and executioner caspases (eg, caspase-3, caspase-6, and caspase-7) and is typically characterized morphologically by cell shrinkage, chromatin condensation, nuclear fragmentation, and the formation of apoptotic bodies. The classical pathway for pyroptosis involves NLRP3/caspase-1/GSDMD/IL-1βandIL-18, whereas the classical pathway for apoptosis involves caspase-3/GSDME. Currently, there are no studies investigating the roles of pyroptosis in the context of HFs.

We hypothesize that DM may precipitate HFSC death through the activation of PCD-related signaling pathways. This may be the key to explaining the reduced skin renewal ability of DM through the inhibition of HF regeneration. This study aimed to investigate the mechanisms through which DM inhibits HFSC activation. We examined the effects of type I DM (T1DM) on HFs in animal experiments and screened the mechanism and key factors involved via RNA sequencing. We subsequently assessed the influence of inflammatory and high-glucose environments on HaCaTs in cellular experiments.

Methods

Ethics

This study was conducted in strict accordance with all relevant national regulations and institutional policies for the care and use of laboratory animals. The study protocol was approved by the Animal Care and Use Committee of Peking University Shenzhen Hospital (2024–263).

Male C57BL/6 mice, sourced from Charles River (Beijing, China), were housed at our animal center. The mice were kept under constant temperature conditions (22–26°C) and subjected to a 12-hour light/dark cycle, with ad libitum access to food and water. Prior to any procedures, the mice were anesthetized with isoflurane (970–00026–00; RWD, Shenzhen, China). Euthanasia was performed by injecting an excessive amount of pentobarbital sodium.

Animals

The experiment commenced when the mice reached six weeks of age. After a 16-hour fast, 20 mice were injected with 120 mg/kg streptozotocin (STZ). The STZ was dissolved in 0.028 mol/L citric acid and 0.022 mol/L sodium citrate buffer at pH 4.4. The method is derived from Ref.[28] The recommended STZ dose is 200 mg/kg. However, at this dose, the mortality rate of the mice we purchased was extremely high. Therefore, we reduced the STZ dose to improve animal survival. Blood glucose levels were monitored on the 2nd and 7th days post-injection, and mice whose blood glucose levels were ≥ 16.7 mm were included in the experiment. The general standard for diabetes is usually a blood glucose concentration of ≥ 11.1 mm. However, in this study, when the blood glucose concentration reached this level, it gradually returned to normal a few days after the model was established. Hence, this standard was not adopted in this study. On the 7th day of the experiment, the hair on the backs of the mice were removed with Veet® hair removal cream. The regrowth of hair and the transition of the HF cycle were subsequently observed.

Laser Speckle Imaging and Analysis

Laser speckle imaging is a technique utilized to detect microcirculation and is highly sensitive to interference from extraneous information such as hair and skin composition. For imaging, the mice were positioned beneath the laser speckle device (RFLSI-ZW, RWD, China), ensuring uniform photographic height and device settings throughout the experiment.

Previous observations revealed that skin with HFs in the telogen phase can be observed to have clear microcirculation imaging (in warm colors) through laser speckle after hair removal. However, activated HFs or growing hairs have a significant impact on imaging. The cold color areas increase significantly and may even show no color. The activation of HFs and hair growth can be evaluated on the basis of this principle. The "cold-toned area/hair-removed area" was subsequently used to evaluate hair growth. Similarly, under naked-eye observation, areas of HF activation and hair growth can be observed. "(hair growth area + HF activation area)/hair-removed area" was used to evaluate hair growth.

Histology

Dorsal skin tissues were carefully collected and fixed in a 4% paraformaldehyde (PFA) solution for 2 days. Subsequently, 5 μm thick paraffin-embedded sections were prepared on slides. Following dewaxing, the slides were subjected to staining using the Sirius Red/Fast Green kit (9046, Chondrex, USA) in accordance with the manufacturer's recommended protocol. Microscopy images of the stained sections were captured for documentation.[29]

Tunel

Following dewaxing, the paraffin sections were subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining to identify dead cells. The staining reagents were applied as per the provided instructions (G1501, Servicebio, China), with specific details outlined in previous descriptions.[30] Images were captured using a Revolve FL microscope (Discover Echo, USA).

Immunohistochemistry

Slides were deparaffinized and immersed in either 0.01M citrate buffer (pH 6.0) or 0.01M Tris/EDTA buffer (pH 9.0), then microwaved for 10 minutes. Then, slides were incubated with various primary antibodies: Caspase1 (A21296, ABclonal, China), Caspase4 (A19305, ABclonal, China), GSDMD (M02842, BOSTER, USA), GSDME (A7432, ABclonal, China), and IL18 (A23076, ABclonal, China). After washing, the slides were treated with enzyme-labeled goat anti-rat IgG polymer (PV-6000, ZSGB-BIO, China) and developed with DAB (ZLI-9017, ZSGB-BIO, China) as per

the manufacturer's instructions. The slides were counterstained with hematoxylin, dehydrated, and sealed. Images were captured using an orthogonal fluorescence microscope (Olympus BX53, Tokyo, Japan).

Immunofluorescence

The sections were baked in an oven at 60°C for 1 hour. Next, they were dewaxed with alcohol and placed in citrate for antigen retrieval (microwaved to 96°C and left for 30 minutes). The endogenous enzyme was removed by incubation with 3% H_2O_2 for 10 minutes at room temperature. The samples were then incubated with 3% BSA for 30 minutes. Next, the primary antibody (NOS2, A3774, ABclonal, China) was added, and the mixture was incubated at 4°C overnight. The next day, after washing with PBS, the secondary antibody (ARG1, A4923, ABclonal, China) was added, and the samples were incubated for 50 minutes at room temperature. The nuclei were subsequently stained with DAPI. The slides were sealed with resin and photographed for observation under a fluorescence microscope. DAPI emits blue fluorescence, FITC emits green fluorescence, and CY5 emits red fluorescence.

RNA Sequencing and Analysis

The samples were preserved in RNAwait (BL621A, Biosharp, China) at 4°C overnight and subsequently stored on dry ice before shipment to the RNA-sequencing company Suzhou Panomix. The company conducted the relevant data analysis. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) of the National Genomics Data Center (Nucleic Acids Res 2022),[31] China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA017036), which are publicly accessible at https://ngdc.cncb.ac.cn/gsa. Here, the data were subjected to secondary analysis and processing. The CIBERSORT analysis is described in the online description (https://cibersortx.stanford.edu).[32]

qRT–PCR

Following sequencing, the RNA samples were transcribed into complementary DNA (cDNA) using a synthesis kit (K1622, Thermo, USA). A quantitative real-time PCR (qRT–PCR) kit was obtained from Bimake (B21203, Bimake, Shanghai, China). All the experimental steps adhered strictly to the manufacturer's instructions.[33] The qRT–PCR analysis was carried out using an ABI-Q3 apparatus (ABI, Foster, USA), with *Gapdh* serving as the reference gene for normalization.

Cell Culture

HaCaT (SCSP-5091, CAS Cell Bank, China) and RAW264.7 (CL0266, Fenghui, China) cells were obtained commercially and cultured in DMEM (10–014-CVR, Corning, New York, USA) supplemented with 10% FBS (AUS-01S-02, Cell-box, China) and penicillin/streptomycin, with passaging performed every three days. Monoclonal cultures of cells were also established using this method. The reason for the use of HaCaT cells in this study is that epidermal stem cells are an important constituent of HFs and a classic cell type for skin research. However, HFSCs are generally difficult to culture after isolation and rapidly differentiate. Moreover, there are numerous types of HFSCs, and the isolation of subtypes is extremely challenging.

Transwell Assay

The Transwell assay was performed according to previous experimental protocols.[34] Briefly, RAW264.7 cells were placed in the upper chamber for 24 hours of induction culture, with M1 induction conditions of 100 ng/mL LPS (BS904, Biosharp, China) + 10 ng/mL IFN-γ (C746, novoprotein, China). Next, the medium was changed to normal medium, and the cells were cocultured with HaCaTs in the lower chamber. The experiments were performed after 48 hours of coculture.

Cell Death Assessment

Cell death was assessed using Hoechst33342/PI (BL116A, Biosharp, China). Hoechst 33342, which is capable of penetrating the cell membrane, emits blue fluorescence when bound to DNA. Upon cell membrane rupture, PI enters the cell and binds to DNA, resulting in red fluorescence and indicating cell death.

Cell Viability

A total of 5000 cells were incubated in a 96-well plate for 24 hours. The cells were subsequently treated with different glucose concentrations for 48 hours and subjected to CCK-8 (T1210, Tsinglight, China) testing for an additional two hours. The optical density (OD 450 nm) values were then recorded using an Epoch plate reader (BioTek, Winooski, USA).

Statistics

The data are presented as the mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism software (Version 6, San Diego, USA). A p value of ≤ 0.05 was considered statistically significant. *$p \leq 0.05$. **$p \leq 0.01$. ***$p \leq 0.001$. ****$p \leq 0.0001$.

Results

T1DM Inhibits HF Regeneration

To understand the effects of T1DM on HFs, we performed continuous observations following modeling. Comparisons were made using littermates. In general, the HFs of mice aged 6–8 weeks are in the resting (telogen) phase, during which the skin appears white. The HFs subsequently transition into the anagen (growth) phase, and the skin gradually changes to a blue–black color. The results revealed statistically significant differences in hair regrowth on the twelfth day following shaving. Under normal lighting, the dorsal skin of the control mice mostly turned blue–black, and remarkable hair growth was observed. In contrast, large areas of pinkish-white color remained on the backs of the T1DM mice (Figure 1A). Laser speckle quantification demonstrated that, in comparison with those in control mice, both hair regrowth and HF regeneration were significantly delayed in T1DM mice (Figure 1A and B). Similarly, direct assessment of hair regrowth areas further confirmed the reduced regrowth speed in T1DM mice (Figure 1B). Furthermore, the growth of hair is usually associated with the activation of HFs. The histological results revealed that T1DM significantly inhibited the activation of HFSCs (Figure 1C and D).

T1DM Provokes Pyroptosis Pathways in the Skin

To investigate the mechanism by which T1DM inhibits HFSC activation, RNA sequencing was employed to screen the mechanism and key factors involved. The results revealed that T1DM significantly upregulated pyroptosis-related pathways while inhibiting apoptosis-related pathways (Figure 2A and B). These findings were confirmed by qRT–PCR (Figure 2C). To further determine the area where pyroptosis takes place, TUNEL was used for localization. This method is capable of simultaneously detecting pyroptosis and apoptosis. The TUNEL results markedly increased positive fluorescence in the T1DM group compared with the control group, which was predominantly localized to HFSCs and the epidermal layer (Figure 2D). Taken together, the RNA sequencing results suggested that these TUNEL results may be dominated by pyroptosis or that PCD may occur in different regions.

T1DM Provokes Caspase-Dependent Pyroptosis in the Inner Root Sheath

Skin and HFs contain multiple stem cell populations,[35,36] and it remains unclear which regions of stem cells undergo pyroptosis. Therefore, immunohistochemistry is selected to locate the specific area where pyroptosis occurs. The immunohistochemistry results revealed that the expression of caspase-1, caspase-4, GSDMD, GSDME, and IL-18 was mainly observed in the inner root sheath of HFs (Figure 3A), suggesting that T1DM-induced inhibition of HFSC activation may be linked to the onset of focal pyroptosis in the inner root sheath. The structure of the HF is shown in Figure 3B.[37] This finding also implies that other HFSCs and keratinocytes may undergo apoptosis.

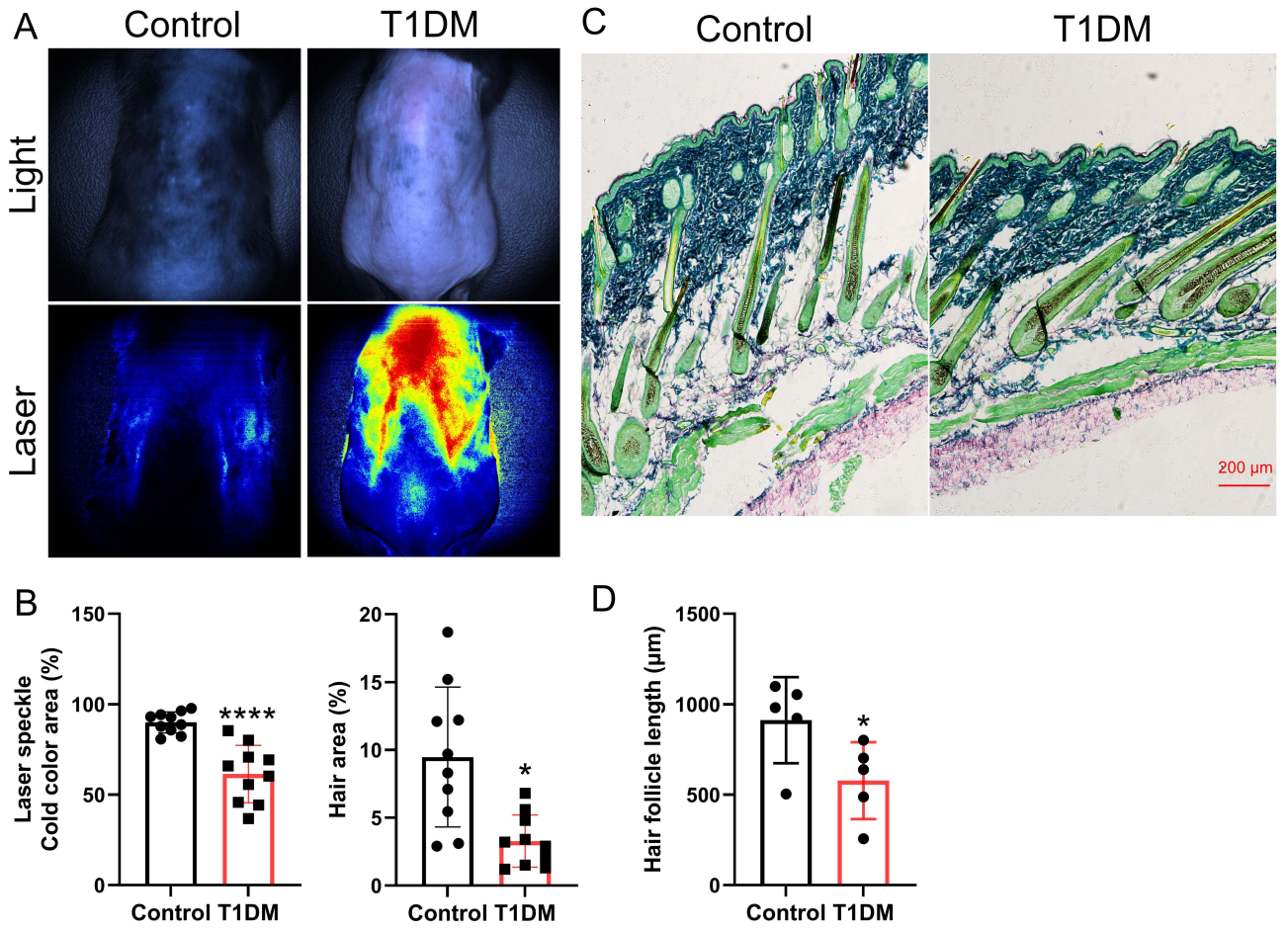

Figure 1 T1DM inhibits HF regeneration. (**A**) Representative pictures of laser speckle. "Light" refers to ordinary illumination, and "laser" is the image taken by laser speckle photography. Images under normal illumination are directly used for the statistics of hair regrowth. Warm light regions represent blood flow dynamics, and cold color regions represent areas of high signal interference. (**B**) Statistics of the cold color region (control vs T1DM, 89.94±5.804 vs 61.42±15.88%, n=10) and hair regrowth region (control vs T1DM, 9.472±5.151 vs 3.28±1.196%, n=10). (**C**) Representative images of Sirius Red/Fast Green staining of the skin. (**D**) HF length (control vs T1DM, 911.8 ±237.7 vs 577.4±212.1%, n=5). *$p \leq 0.05$. ****$p \leq 0.0001$.

T1DM Disrupts Cutaneous Immune Homeostasis

Pyroptosis, also known as cellular inflammatory necrosis, is intricately linked to the immune response. To explore the impact of T1DM on the skin immune system, we conducted an in-depth analysis of RNA-seq data via CIBERSORT, a bioinformatics tool capable of scrutinizing 22 immune cell types. The findings revealed variations in immune cell types associated with macrophages, including monocytes, macrophage subtypes, and mast cells (Figure 4A and B). Furthermore, CD4+ T cells, NK cells, and resting dendritic cells also exhibited differences. The absence of statistical significance for some observations might stem from an insufficient sample size. These results imply that T1DM may disrupt immune homeostasis in the skin, potentially leading to cellular pyroptosis. Nevertheless, the precise location of immune cell aggregation and the underlying mechanism of this influence remain elusive.

T1DM Regulates Phenotypic Changes in Macrophages

The regulation of immune homeostasis and regeneration of HFSCs heavily relies on macrophage phenotypic changes.[14,38] Consequently, this study focused on examining the distribution and phenotypic alterations of macrophages. The findings indicated that macrophages (F4/80 staining) were predominantly concentrated in the lower part of the HFs, specifically in the inner root sheath region (Figure 5A), suggesting their potential direct impact on HFSCs. In other words, the area where pyroptosis occurs coincides with the area of positive staining of macrophages.

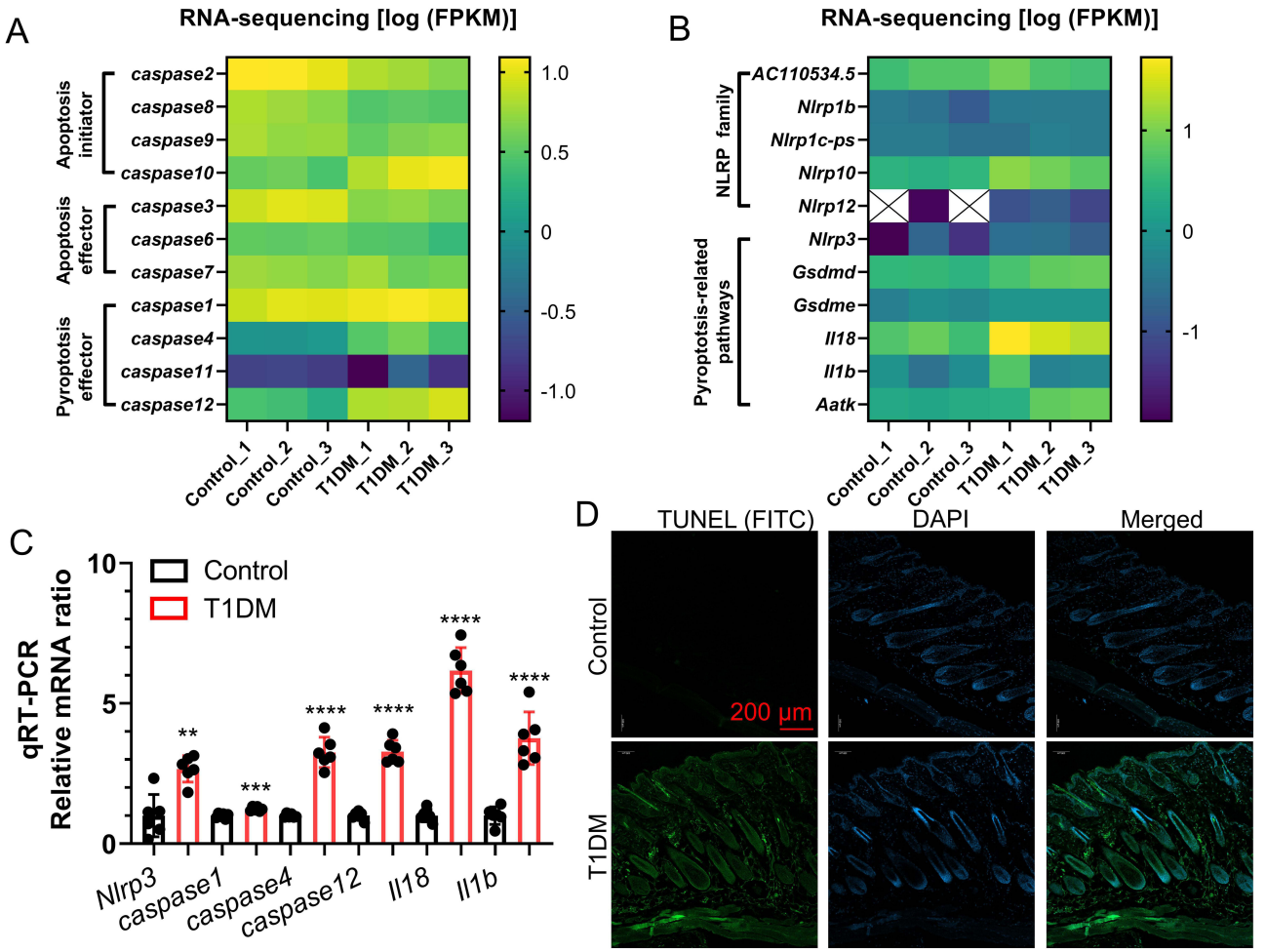

Figure 2 T1DM promotes pyroptosis pathways in the skin. (**A**) Expression of the caspase family, the main effector factor of apoptosis and pyroptosis. N=3. (**B**) The expression of pyroptosis-related pathways. (**C**) qRT–PCR showed consistent trends. T1DM increases the RNA expression levels of *Nlrp3*, *Caspase1*, *Caspase4*, *Caspase12*, *Il18* and *Il1b* in the skin. N=6. (**D**) Representative images of TUNEL staining. The green fluorescence indicates that T1DM activates the pyroptosis or apoptosis pathway in the skin. Positive staining was distributed in the epidermis, hair follicles and dermis layers. $^{**}p \leq 0.01$. $^{***}p \leq 0.001$. $^{****}p \leq 0.0001$.

Further analysis targeted the well-characterized M1 and M2 macrophage subtypes, which are known for their roles in the injury response and repair processes, respectively. For each phenotype, six marker genes were selected for testing. The outcomes demonstrated that T1DM influenced the macrophage phenotype, leading to increased expression of more M1 genes (Figure 5B). Consistently, immunofluorescence staining revealed that T1DM increased NOS2 expression in the inner root sheath and epidermis (Figure 5C). These findings imply that T1DM may modulate HFSC regeneration by altering the macrophage phenotype within HFs.

M1 Macrophages Directly Cause the Death of HaCaTs

The relationship between HFSC pyroptosis and the hyperglycemic microenvironment remains unclear. To elucidate the underlying mechanisms, two potential pathways through which T1DM may induce pyroptosis were investigated. First, T1DM may trigger M1 macrophage polarization, leading to cell death. Alternatively, T1DM may directly induce cell death due to the high-glucose environment. The initial investigations focused on the impact of M1 macrophages on HaCaTs, which are important members of HFSCs and the epidermis. Due to their rapid differentiation following in vitro culture, HFSCs are not ideal for research purposes in this context. Transwell experiments revealed that M1 macrophages led to a reduction in cell density, morphological alterations, inhibition of colony formation, and cell death (Figure 6A–D). These findings suggest that M1 polarization directly triggers the death of HFSCs. However, the specific effects of hyperglycemia on skin cells warrant further investigation.

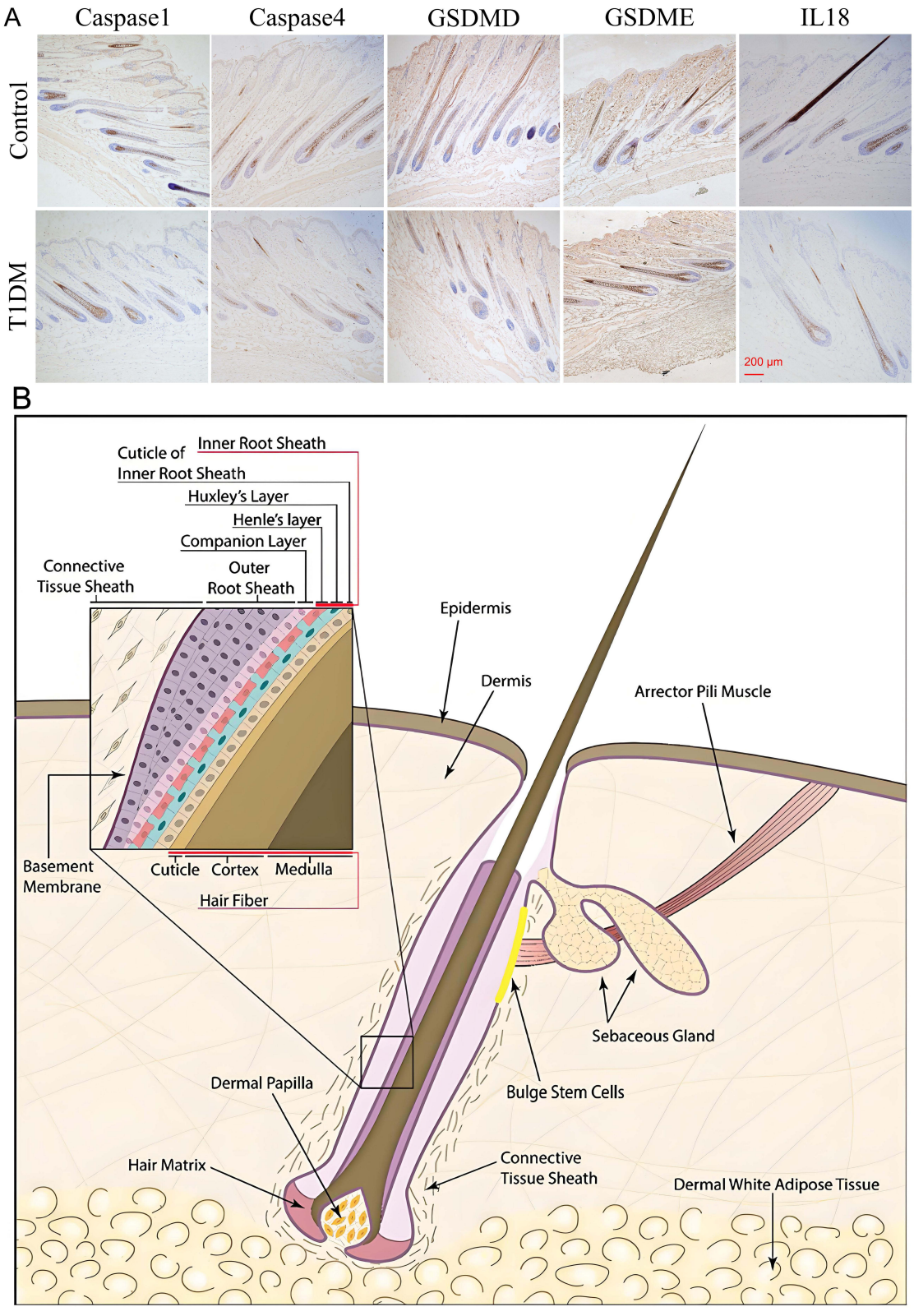

Figure 3 T1DM induces caspase-dependent pyroptosis in the inner root sheath. (**A**) The expression levels of Caspase-1, Caspase-4, GSDMD, GSDME, and IL-18 in the skin. (**B**) The structure of the HF. Reprinted with permission from Kiani MT, Higgins CA, Almquist BD. The Hair Follicle: An Underutilized Source of Cells and Materials for Regenerative Medicine. *ACS Biomaterials Science & Engineering*. 2018;4:1193–1207. Copyright 2018 American Chemical Society.[37]

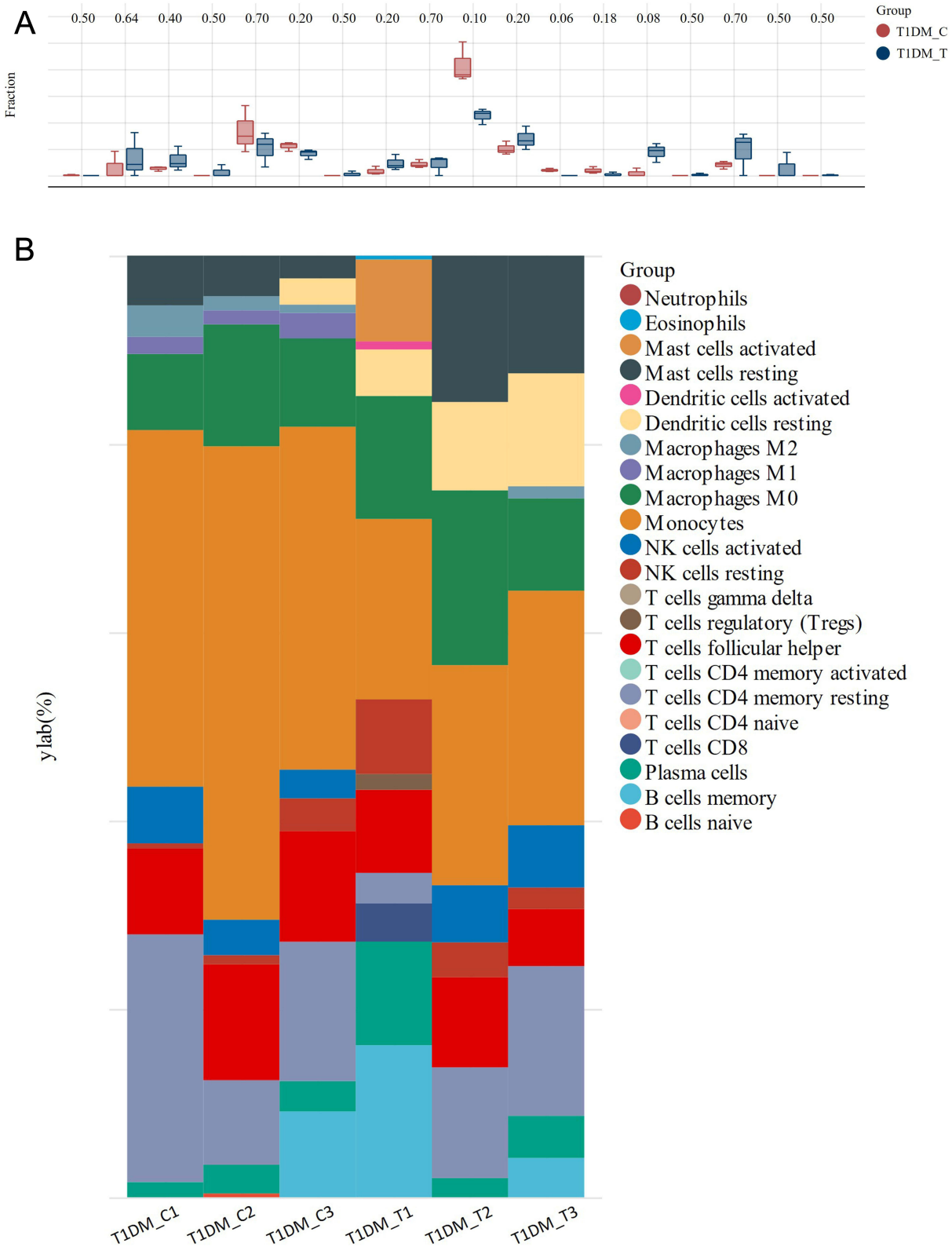

Figure 4 T1DM induces an imbalance in cutaneous immune homeostasis. (**A**) Comparison of the relative expression of 22 immune cell types between the two groups. (**B**) Results of the relative percentages of 22 immune cell types. T1DM_C represents the T1DM project control group. T1DM_T represents the T1DM project T1DM group. N=3.

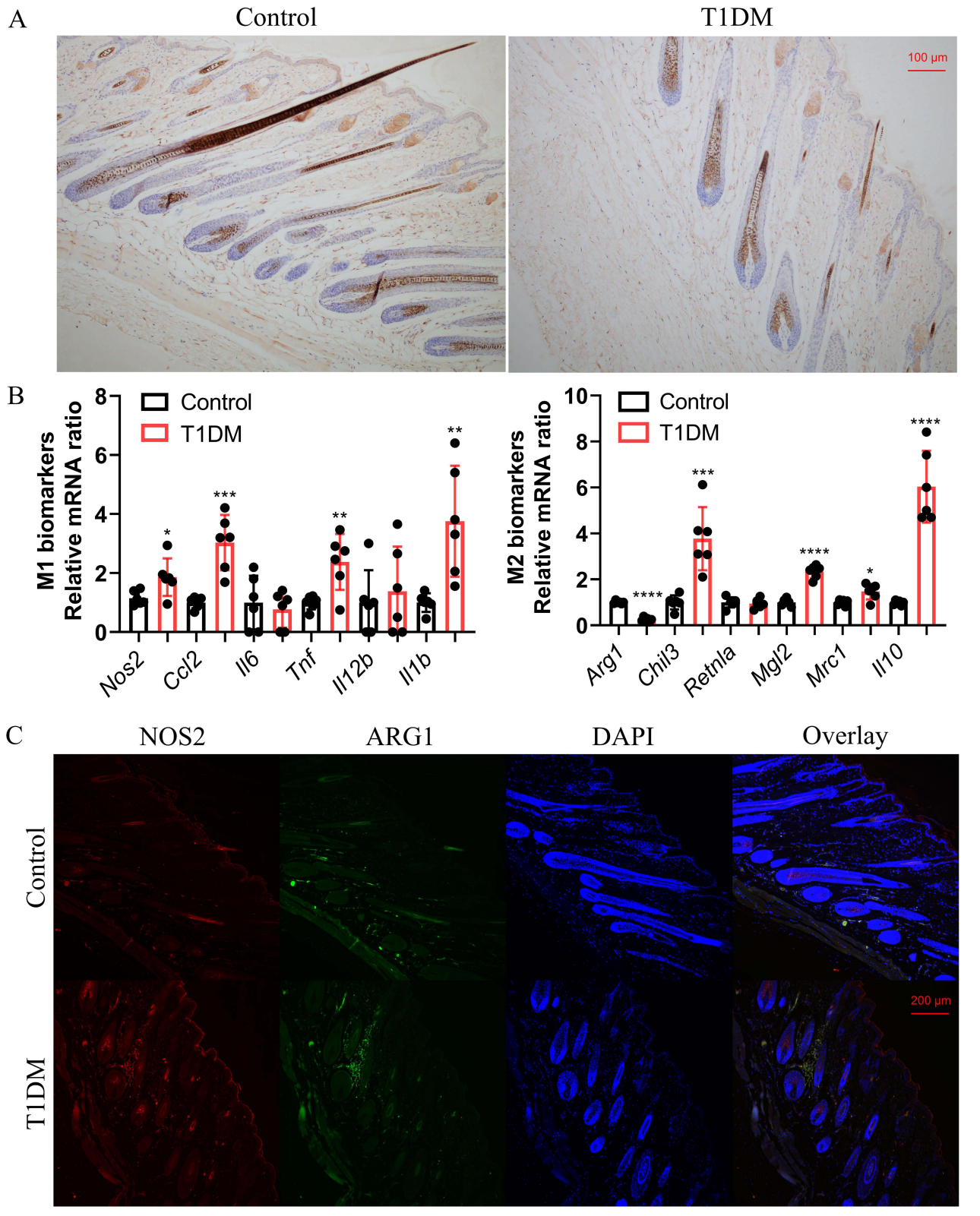

Figure 5 T1DM affects macrophage polarization. (**A**) Representative images of staining for F4/80, a macrophage biomarker. The positive staining in the inner root sheath area of the HFs was the most significant. (**B**) Polarization of M1 and M2 macrophages. M1- and M2-related factors were significantly upregulated in the T1DM group, but the expression of M1-related factors was increased. N=6. (**C**) Representative images of immunofluorescence staining for NOS2 (red) and ARG1 (green). *$p \leq 0.05$. **$p \leq 0.01$. ***$p \leq 0.001$. ****$p \leq 0.0001$.

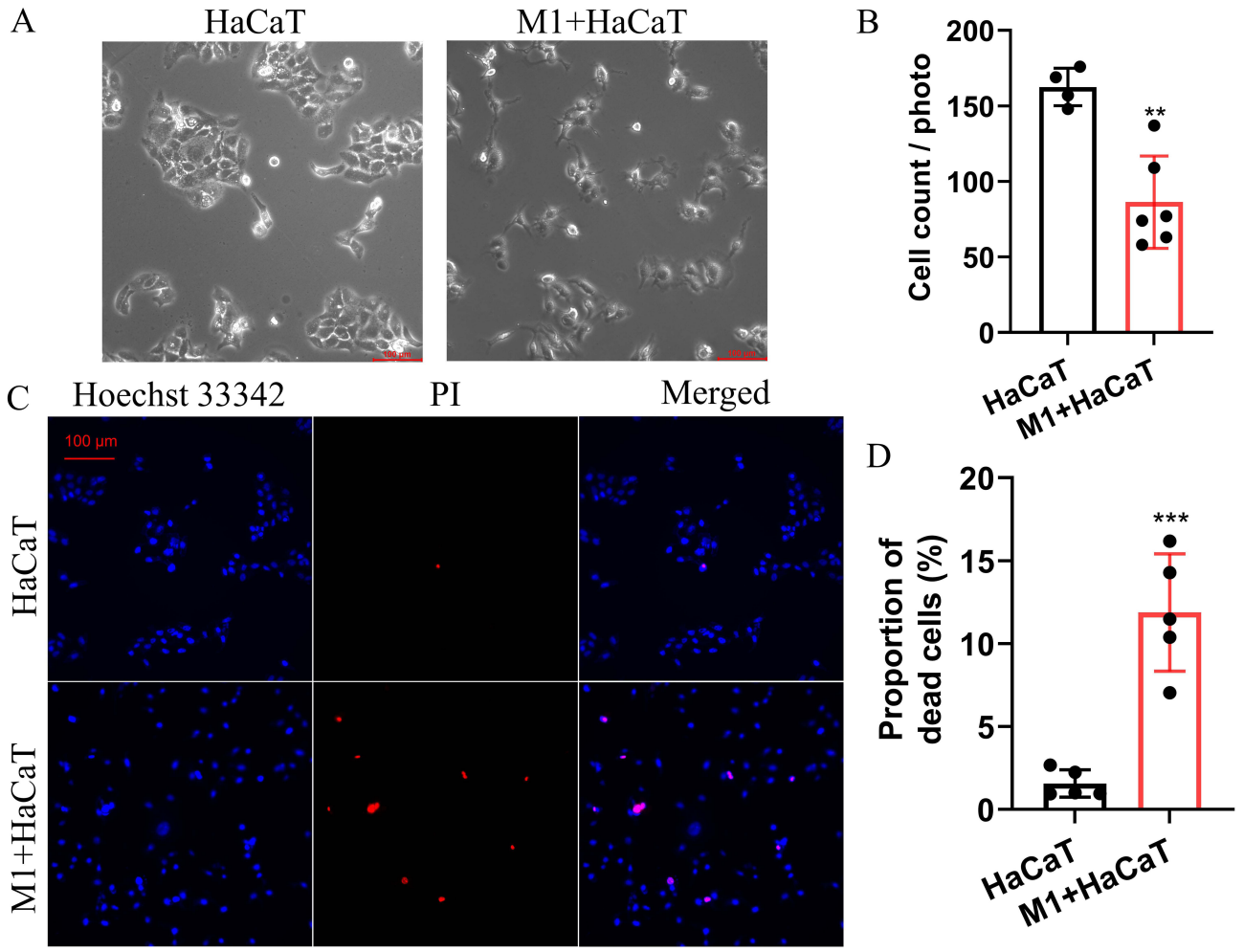

Figure 6 M1 macrophages directly cause the death of HaCaTs. (**A**) Representative images of the growth of HaCaTs in normal and Transwell cultures. M1 represents polarized macrophages. (**B**) The number of HaCaTs was significantly lower in the inflammatory culture environment (HaCaT cells vs M1+HaCaT cells, 162.5±12.45 vs 86.33 ±30.55, respectively; n=5). (**C**) Representative images of cell survival. (**D**) Inflammatory culture environments accelerated cell death (HaCaT vs M1+HaCaT, 1.564±0.829 vs 11.87±3.537%, n=5). **$p \leq 0.01$. ***$p \leq 0.001$.

A Moderately High-Glucose Environment Does Not Directly Cause the Death of HaCaTs

To investigate the direct impact of a high-glucose environment on cell survival, we conducted in vitro experiments to simulate various glucose concentrations. Initially, we established a glucose concentration range for investigation. In humans, the normal blood glucose concentration falls within the range of 4.4–6.1 mm, with a maximum blood glucose concentration of 11.1 mm one hour post-meal, which is considered indicative of potential diabetes mellitus. Some patients with severe diabetes usually have a blood glucose concentration of 30–40 mm, but patients at this level may develop ketoacidosis or even fall into a coma. In this study, the diagnostic criterion for T1DM in mice was set at 16.7 mm, which already represents a considerably elevated concentration. In a few cases, mice can reach a blood glucose concentration of 30 mm. Notably, the standard high glucose concentration in DMEM is 25 mm. Consequently, 5 mm, 25 mm, 30 mm, and 50 mm were selected as the standards for cell culture in our experiments.

To mimic the normal blood glucose concentration in the human body, we initially acclimated HaCaTs in medium containing 5 mm glucose for one month. The cell cultures were subsequently subjected to experiments involving varying glucose concentrations. First, a monoclonal cell culture was utilized to investigate cell proliferation (Figure 7A and B). The findings revealed a slight upward trend in the cell proliferation rate when the glucose concentration was less than 30 mm, although this increase was not statistically significant. This may also be because the sample size of each group

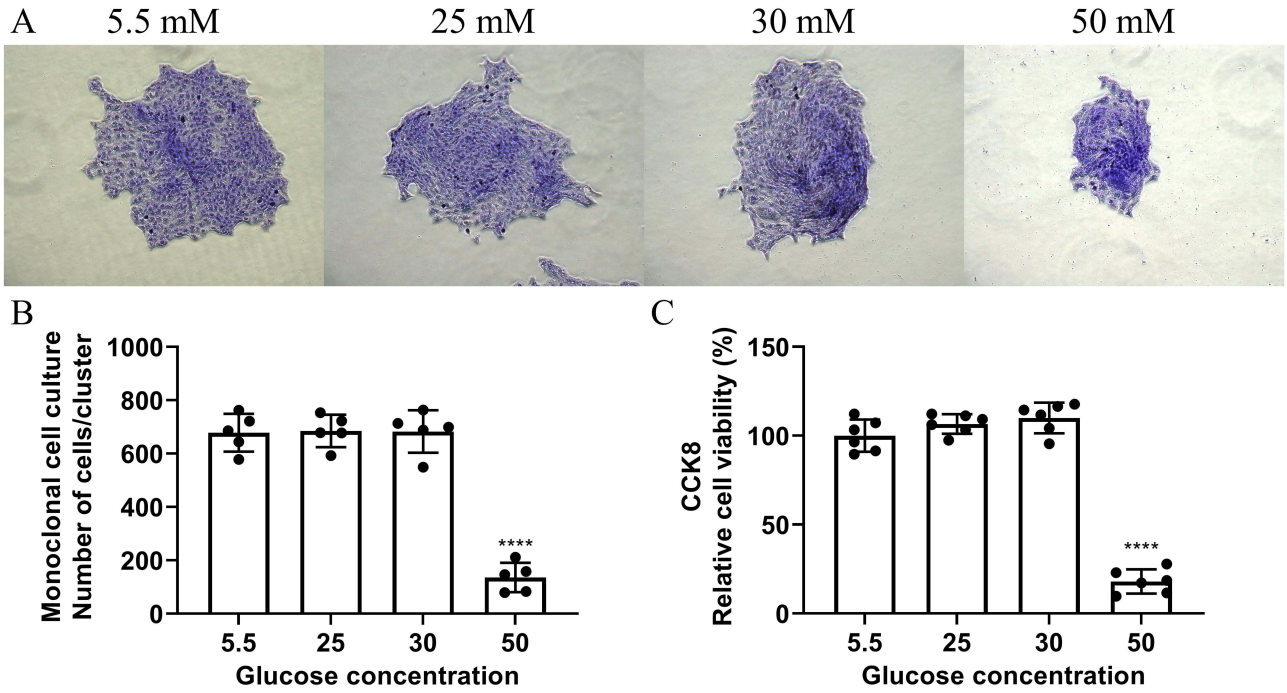

Figure 7 Moderately high-glucose environments did not affect HaCaTs. (**A**) Growth of monoclonal HaCaTs at different glucose concentrations. A glucose concentration of 50 mm significantly inhibited the growth of monoclonal cells. (**B**) A concentration of 50 mm glucose inhibited cell growth; below 30 mm, it did not. (**C**) A concentration of 50 mm glucose inhibited cell viability; below 30 mm, it did not. ****$p \leq 0.0001$. Compared with 5.5 mm.

was insufficient. However, a glucose concentration of 50 mm significantly inhibited cell proliferation. Consistent with these findings, the results of the CCK8 analysis of cell viability in the conventional culture environment were the same (Figure 7C). These observations suggest that moderate increases in glucose concentration do not adversely affect cell proliferation or viability and may even promote cell survival. However, excessively high glucose concentrations impede cell proliferation.

Discussion

DCWs pose a significant global medical challenge, necessitating a deep understanding of their underlying pathology to develop innovative treatments. This study elucidates the mechanism by which T1DM triggers the activation of caspase-dependent pyroptosis by disrupting immune homeostasis, which ultimately leads to the death of HFSCs and keratinocytes, as well as pyroptosis of HFSCs in the inner root sheath. Interestingly, we observed that moderate increases in glucose concentration did not lead to HFSC death. This novel mechanism may provide valuable insights into the formation of DCWs and potentially serve as a therapeutic target for future treatments of DCWs.

While HFSCs have been implicated in wound healing, few studies have explored the correlation between diabetes-induced follicular growth inhibition and DCWs. Clinical studies have demonstrated that skin grafts incorporating follicles from high-quality donor sites promote better healing of DCWs than do abdominal skin grafts alone.[19] The lower HF density in the feet than in the legs, trunk, and head highlights the critical role of HFSCs in maintaining skin renewal and DCW prevention. Previously, we demonstrated that DM inhibits HFSC activation and skin renewal but did not provide further explanation.[10] The present study provides a more detailed elaboration, revealing that T1DM promotes a proinflammatory environment through immune homeostasis disruption, which triggers PCD in HFSCs. Given the rapid renewal rate of the skin, compromised regeneration can accelerate ulcer formation and delay healing. This effect may be more pronounced in the feet because of lower follicle density. This explains why follicular grafting is particularly effective in healing DCWs.

Importantly, the appropriate increase in glucose concentration did not affect HaCaT growth. This phenomenon might be related to the specific region and condition of the stem cells. For example, while a glucose concentration of 30 mm can

be detrimental to humans, it may be tolerable for HFSCs in the occipital region. Previous reports have indicated that the glucose requirements of HFs vary across different regions, with HFs in the head generally exhibiting a relatively high energy demand.[39,40] Furthermore, stem cells display varying sensitivities to glucose during activation and differentiation.[41,42] For example, muscle stem cells have distinct glucose requirements during proliferation, resting, and differentiation.[43] These findings suggest that the tolerance of stem cells to glucose is dependent on their physiological state, with higher energy demand correlating with increased glucose tolerance. Consequently, densely populated HFs with rapid hair growth exhibit greater glucose tolerance, whereas the opposite is observed for follicles in the foot region. This mechanism elucidates the resilience, proliferation, and differentiation of HFs post-grafting into diabetic wounds.

Furthermore, this study provides a crucial theoretical foundation for the future utilization of engineered HFSCs in treating DCWs. Given that wounds in diabetic patients are difficult to heal, employing non-invasive or minimally invasive techniques for wound repair is a superior option. An increasing number of studies have confirmed that somatic cells can be induced into induced pluripotent stem cells (iPSCs) through reprogramming.[44] Moreover, iPSCs and skin stem cell populations can be cultivated and induced into precursor cells or HF organoids, which can then be used for transplantation.[45,46] A recent study demonstrated that after iPSCs are induced into HF organoids, they can be transplanted into the skin and grow normally.[46] Thus, using any accessible cells to be induced into HFs in vitro and then transplanted into DCWs is clearly an excellent treatment strategy. This is also the direction we are currently exploring.

This study revealed another pivotal finding, indicating that T1DM triggers the death of skin stem cell populations through the induction of cutaneous immune reprogramming, resulting in diverse patterns of cell death in different regions. Importantly, pyroptosis occurs in HFSCs within the inner root sheath, a region that plays a crucial role in HFs because of its proximity to both the hair and HFSCs in the outer root sheath. Studies have demonstrated that HFs contain multiple stem cell populations with distinct functions and transdifferentiation capabilities, which can compensate for each other.[35] Therefore, we suggest that T1DM instigates a pro-inflammatory microenvironment within the inner root sheath, leading to pyroptosis of HFSCs in this region. This may result not only in a deficiency of support for hair growth but also in the inhibition of outer root sheath regeneration through the inhibition of transdifferentiation. This ultimately leads to the suppression of hair growth and skin self-renewal.

This study has several limitations. First, methods for restoring immune homeostasis, a highly complex but vital aspect of both skin health and overall health, have not been investigated. Second, the study did not explore additional diabetes-related immune cell subtypes. Third, the cellular experiments excluded HFSCs from the inner root sheath because of their high differentiation potential in vitro and the absence of an established culture technique. However, given the pivotal role of keratinocytes in HF development, HaCaTs were utilized as proxies to study HFSCs. While HaCaTs are commonly used as substitutes for both keratinocytes and HFSCs in related studies, their responses may not precisely mirror those of actual cells. Therefore, investigating relevant experimental protocols is an important direction for future research. Fourth, the study revealed inconsistent patterns of cell death in HFSCs and keratinocytes in different regions. Given the substantial complexity of coculturing various immune cells and skin cells, further exploration will be time intensive. Future studies are also planned to employ spatial high-throughput sequencing methods. Fifth, HF grafting has been demonstrated to be applicable for treating DCWs. However, in actual situations, this treatment method still causes a small amount of damage. The optimal approach is to culture HFSCs through in vitro expansion and then create organoids. This project involves a significant amount of work and requires further research.

Conclusion

In conclusion, this study revealed for the first time that T1DM triggers PCD in the epidermis and HFSCs, specifically leading to pyroptosis in HFSCs within the inner root sheath through the regulation of macrophage reprogramming in this region. Furthermore, T1DM-induced PCD of the skin may be linked to an imbalance in immune homeostasis rather than hyperglycemia. These findings offer a novel theoretical basis for understanding the pathophysiological process of DCWs and provide potential therapeutic strategies for HF grafting.

Abbreviations

DCWs, chronic diabetic wounds; HaCaT, human immortalized epidermal cell line; HF, hair follicle; HFSC, hair follicle stem cell; iPSC, induced pluripotent stem cell; PCD, programmed cell death; STZ, streptozotocin; T1DM, type I diabetes mellitus.

Data Sharing Statement

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) of the National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA017036), which is publicly accessible at https://ngdc.cncb.ac.cn/gsa.

Ethics

This animal study was conducted in strict accordance with all relevant national regulations and institutional policies for the care and use of animals. The study protocol was approved by the Animal Care and Use Committee of Peking University Shenzhen Hospital (2024-263).

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Author Contributions

All authors made a significant contribution to the work reported, whether 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; agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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