

Single-cell RNA sequencing reveals the impaired epidermal differentiation and pathological microenvironment in diabetic foot ulcer

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Abstract

Background: Diabetic foot ulcer (DFU) is one of the most common and complex complications of diabetes, but the underlying pathophysiology remains unclear. Single-cell RNA sequencing (scRNA-seq) has been conducted to explore novel cell types or molecular profiles of DFU from various perspectives. This study aimed to comprehensively analyze the potential mechanisms underlying impaired re-epithelization of DFU in a single-cell perspective.

Methods: We conducted scRNA-seq on tissues from human normal skin, acute wound, and DFU to investigate the potential mechanisms underlying impaired epidermal differentiation and the pathological microenvironment. Pseudo-time and lineage inference analyses revealed the distinct states and transition trajectories of epidermal cells under different conditions. Transcription factor analysis revealed the potential regulatory mechanism of key subtypes of keratinocytes. Cell-cell interaction analysis revealed the regulatory network between the proinflammatory microenvironment and epidermal cells. Laser-capture microscopy coupled with RNA sequencing (LCM-seq) and multiplex immunohistochemistry were used to validate the expression and location of key subtypes of keratinocytes.

Results: Our research provided a comprehensive map of the phenotypic and dynamic changes that occur during epidermal differentiation, alongside the corresponding regulatory networks in DFU. Importantly, we identified two subtypes of keratinocytes: basal cells (BC-2) and diabetes-associated keratinocytes (DAK) that might play crucial roles in the impairment of epidermal homeostasis. BC-2 and DAK showed a marked increase in DFU, with an inactive state and insufficient motivation for epidermal differentiation. BC-2 was involved in the cellular response and apoptosis processes, with high expression of TXNIP, IFITM1, and IL1R2. Additionally, the pro-differentiation transcription factors were downregulated in BC-2 in DFU, indicating that the differentiation process might be inhibited in BC-2 in DFU. DAK was associated with cellular glucose homeostasis. Furthermore, increased CCL2 + CXCL2+ fibroblasts, VWA1+ vascular endothelial cells, and GZMA+CD8+ T cells were detected in DFU. These changes in the wound microenvironment could regulate the fate of epidermal cells through the TNFSF12-TNFRSF12A, IFNG-IFNGR1/2, and IL1B-IL1R2 pathways, which might result in persistent inflammation and impaired epidermal differentiation in DFU.

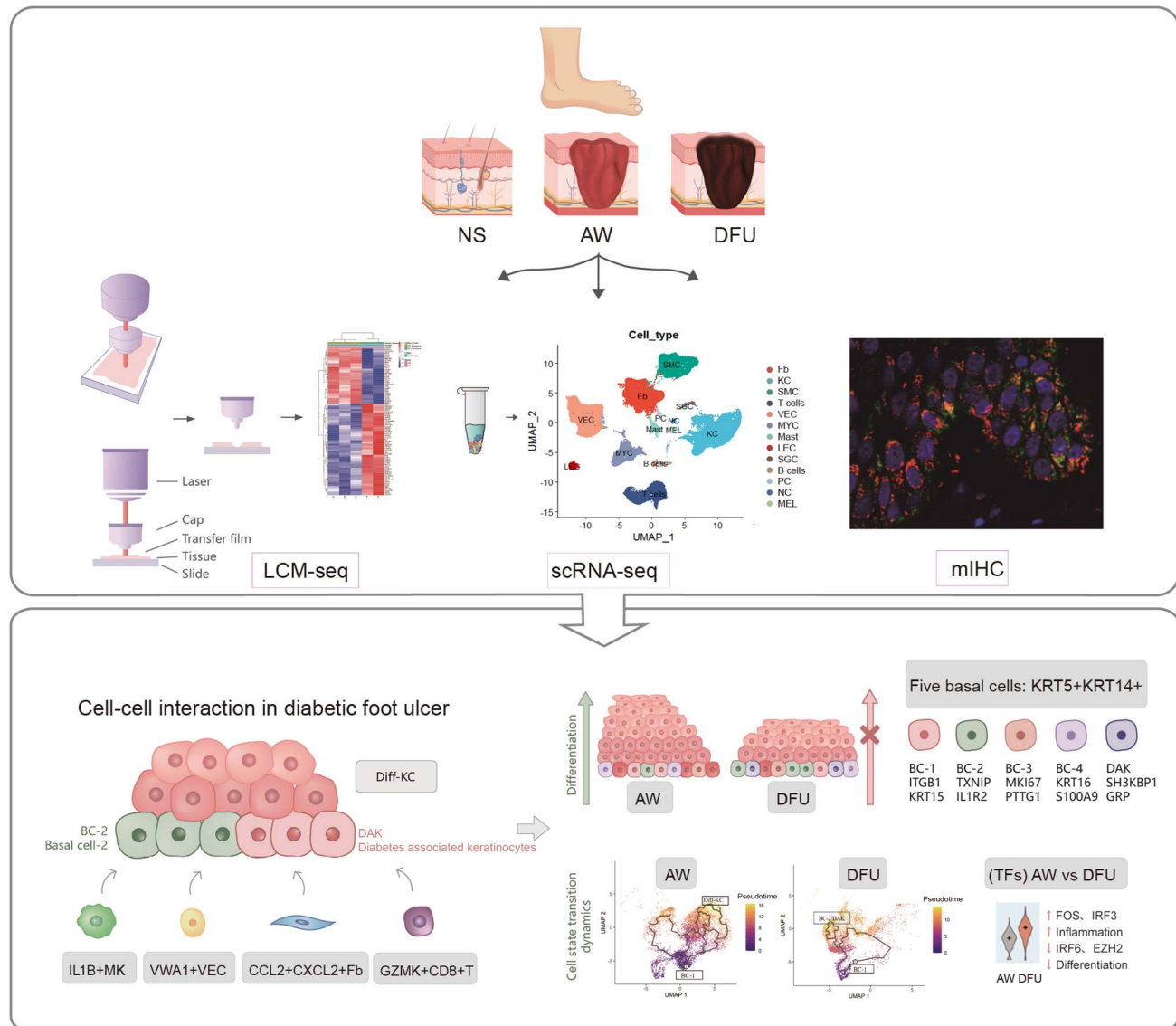
Conclusions: Our findings offer novel insights into the pathophysiology of DFU and present potential therapeutic targets that could improve wound care and treatment outcomes for DFU patients.

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Graphical Abstract



Keywords: Diabetic foot ulcer; Re-epithelization; Wound microenvironment; Epidermal homeostasis; Single-cell RNA sequencing

Highlights

- We identified key keratinocytes associated with impaired epidermal differentiation in DFU, including basal layer cells (BC-2) and diabetes-associated keratinocytes (DAK).
- We revealed the phenotypic changes and abnormal epidermal differentiation trajectory in DFU, characterized by an inactive state and insufficient differentiation motivation.
- We revealed the cellular changes in the microenvironment of DFU, which may regulate the behavior and fate of epidermal cells through cell-cell interactions.
- Critical cells (BC-2 and DAK), marker genes (TXNIP, IL1R2 and IFITM1) and TFs (IRF6 and EZH2) could serve as promising targets for the treatment of DFU.

Background

Diabetic foot ulcer (DFU) is one of the most common and complex complications of diabetes and is characterized by significant morbidity, amputation, mortality, and healthcare expenditure [1, 2]. Current standard management methods for DFU include glycemic control, revascularization, debridement, reconstructive surgery, and various advanced interventions [3–5]. However, satisfactory outcomes have not yet been achieved, probably because the potential and complex pathophysiology of DFU remains incompletely understood [6].

Previous studies have reported that impaired wound healing in DFU involves various factors, including elevated blood glucose, excessive reactive oxygen species, peripheral neural or vascular diseases, and infection [7, 8]. These factors induce alterations in the wound microenvironment, including sustained inflammation, poor granulation tissue, impaired re-epithelialization and inhibited angiogenesis [9]. However, the specific cellular and molecular mechanism of DFU still needs to be further investigated for additional therapeutic options.

Single-cell RNA sequencing (scRNA-seq) is an advanced and high-throughput technology that allows researchers to discover detailed molecular profile changes at the single-cell level and uncover potentially unexpected biological discoveries related to diseases [10]. scRNA-seq has been conducted to explore novel cell types or molecular profiles of DFU from various perspectives, including the increased expression of JMJD3 in diabetic foot ulcer macrophages, the differentiation profile of mononuclear macrophages, a unique population of fibroblasts associated with wound healing and the altered molecular signatures of mast cells after the topical application of mast cell stabilizers in DFU [11–15]. Nonetheless, the mechanisms underlying the impaired re-epithelialization of DFU in a single-cell perspective have not been reported.

It was well-established that the comparison of the cellular and molecular features among homeostasis, acute wound healing, and diabetic wound healing can help us to understand the underlying mechanisms of skin maintenance, repair and aberrations that occur in diseases [16, 17]. Previous studies have identified four distinct basal cell states and mapped the molecular and metabolic heterogeneity between normal skin (NS) and acute wound (AW) in a comprehensive single-cell perspective, which revealed enhanced cell fate and state fluidity during wound healing [16, 18, 19]. Also, fewer differentiated keratinocytes and upregulated inflammatory molecules were observed in the DFU tissues than in NS [11, 12]. However, the differences between AW and DFU at the single-cell level have not been comprehensively investigated.

Here, we performed scRNA-seq analysis on NS, AW, and DFU tissues. Our study provided a comprehensive landscape of these tissues and highlighted distinct cell subsets that contribute to their unique characteristics. Initially, we identified key keratinocytes that were associated with impaired epidermal differentiation in DFU, including basal layer cells (BC-2) and diabetes-associated keratinocytes (DAK). Pro-differentiation transcription factors (TFs) were repressed, whereas proinflammatory TFs were upregulated in BC-2 and DAK. Secondly, we examined the phenotypic changes and abnormal epidermal differentiation trajectory in DFU, which were characterized by an inactive state and insufficient differentiation motivation. Thirdly, we revealed the cellular changes in the DFU microenvironment, which

may lead to poor granulation tissue, impaired angiogenesis, and sustained inflammation. These alterations may regulate the behavior and fate of epidermal cells through cell–cell interactions (CCI). Furthermore, the expression and location of BC-2 and DAK were validated via laser-capture microscopy coupled with RNA sequencing (LCM-seq) and multiplex immunohistochemistry (mIHC). Taken together, our findings identified potential therapeutic targets, including critical cells (BC-2 and DAK), marker genes (TXNIP, IL1R2, and IFITM1), and identified TFs (IRF6 and EZH2), that may promote re-epithelialization and improve the wound microenvironment, thereby facilitating the healing of DFU (Graphic Abstract).

Methods

Subjects

Our research included three NS samples, three AW samples, and three DFU samples. NS tissues were collected from nondiabetes mellitus (non-DM) patients who underwent foot surgery for various reasons, such as foot deformity correction. Discarded skin samples from the feet were collected for analysis. AW tissues were obtained from non-DM patients with acute wounds that required surgery due to incisions or avulsions. The tissues from the peri-wound were collected within 3–5 days postinjury. We collected DFU tissues from DM patients who had foot ulcers for more than one month and who required debridement. The samples were collected from the wound edge during debridement surgery. The size of each sample ranged from 1–2 cm². Subjects who had any conditions or medications that could affect wound healing were excluded from the study. For detailed information about the patients see Table 1.

Preparation of single-cell suspensions from skin samples

Human skin samples were stored in MACS Tissue Storage Solution (Miltenyi, 130–100–008) on ice and washed with 1 × PBS three times. The blood stains and fatty layers were removed from the tissues. These tissues were then cut into 0.5 mm² pieces and incubated in dissociation solution [0.3% collagenase IV (Thermo Fisher, 17 104 019) + 0.2% dispase II (Thermo Fisher, 17 105 041) + 0.2% hyaluronidase (Biofrox, 1141) + 0.02% DNase I (Thermo Fisher, 18 047 019)] in DMEM in a 37°C water bath with shaking for 2–3 h at 100 rpm. The digestion was terminated with DMEM (+10% FB + 1% Pen/Strep) and then filtered with a 100-μm cell strainer. The remaining tissues were digested with 0.25% trypsin–EDTA (Thermo Fisher, 25 200–072) at 37°C for 3–5 min, terminated with DMEM (+10% FB, +1% Pen/Strep), and centrifuged at 300 × g for 5 min at 4°C. The total cell pellet was filtered with a 70-μm cell strainer and resuspended in 1 × PBS (+0.04% BSA, Sigma, B2064). Red blood cell lysis buffer (Sigma, R7757) was then added, and the mixture was incubated for 3–5 min and then centrifuged at 300 × g for 5 min at 4°C. The mixture was resuspended in 100 μl of dead cell removal microbeads (MACS 130–090–101), and the dead cells were removed via a Miltenyi Dead Cell Removal Kit (MACS 130–090–101). The suspension was resuspended in 1 × PBS (+0.04% BSA), centrifuged at 300 g for 3 min at 4°C (repeated twice), and finally resuspended in 50 μl of 1 × PBS (+0.04% BSA). The overall cell viability was confirmed by

Table 1. Clinical characteristics of the patients

No.	Sample ID	Sex	Age/years old	HbA1c/%	Wound area/cm ²	DM duration/years
1	DFU-1	Male	65	11.25	11.36	16
2	DFU -2	Female	68	13.87	12.52	13
3	DFU -3	Male	60	10.99	10.83	20
4	AW-1	Female	59	5.68	9.39	–
5	AW-2	Male	63	5.72	9.21	–
6	AW-3	Female	69	5.39	10.33	–
7	NS-1	Female	55	5.11	–	–
8	NS-2	Male	57	5.89	–	–
9	NS-3	Male	63	5.23	–	–

DFU diabetic foot ulcer, AW acute wound, NS normal skin, DM diabetes mellitus

trypan blue exclusion, and the results were above 85%. Single-cell suspensions were counted via a Countess II Automated Cell Counter, and the concentration was adjusted to 700–1200 cells/ μ l.

Single-cell cDNA and library preparation

Single-cell cDNA, library preparation, and 3'-end single-cell RNA sequencing were performed by GENE DENOVO (Guangzhou, China). The single-cell suspension was washed twice and loaded into 10X Chromium to capture single cells according to the manufacturer's instructions for the 10X Genomics Chromium Single-Cell 3' Kit (V3). The following steps for cDNA amplification and library construction were performed according to the standard protocol. The libraries were sequenced via the Illumina NovaSeq 6000 sequencing system. GENE DENOVO performed postprocessing and quality control using the 10X Cell Ranger package (v2.1.0, 10X Genomics). The reads were aligned to the GRCh38 reference assembly (v2.2.0, 10X Genomics).

Single-cell RNA sequence data processing

The sequencing results were demultiplexed and converted to FASTQ format via Illumina bcl2fastq software. Sample demultiplexing, barcode processing and single-cell 3' gene counting were performed via Cell Ranger version 3.1.0. The data were aligned to the Ensembl genome GRCh38/GRCm38 reference genome. A total of 100 000 single cells were collected from nine patients and processed via 10X Genomics Chromium Single-cell 3' solution. The cell Ranger output was loaded into Seurat (version 3.1.1) for use in dimensionality reduction, clustering, and analysis of the scRNA-seq data. Overall, the quality control criteria were as follows: >500 and <5000 genes/cell and <15% mitochondrial gene expression. Genes that were expressed in fewer than three cells and identified as doublets were excluded. The data were normalized with a scale factor of 10 000.

Analysis and visualization of processed sequencing data

We performed Seurat in R (v 4.3.0) and analyzed all the datasets. We identified the top 2000 most variable genes based on their average expression and dispersion as highly variable genes (HVGs). Furthermore, we reduced the dimensionality of the data by performing principal component analysis (PCA) on HVG. The expression values of genes can be calculated via the 'Normalization' function of Seurat software. PCA was performed using a normalized expression value, and the top 10 PCs were used for clustering analysis. The weighted shared

nearest neighbor (SNN) graph-based clustering method was selected. The Wilcoxon rank-sum test was employed to identify marker genes for each cluster via default parameters, and the FindAllMarkers function in Seurat was used. This selects marker genes expressed in more than 10% of the cells in a cluster and an average log2 (fold change) is greater than 0.25.

Gene set enrichment analysis

The functional enrichment of DEGs at $P < .05$ was analyzed via the R package ClusterProfiler v3.12.0. Gene set enrichment analysis (GSEA) was also conducted via the GSVA package (v1.42.0), and gene sets and molecular signatures were obtained from the Molecular Signatures Database. Normalized enrichment scores were obtained via 1000 gene set permutations, and a cutoff P -value of .05 was used to filter the significant enrichment results.

Pseudo-time and lineage inference

The R package Monocle 3 (version 0.2.3.0) was used to determine the differentiation trajectories of keratinocytes, which have also been shown to be heterogeneous. The results were visualized via a UMAP plot.

Cell cycle analysis

The cyclone tool of the R package scrn (version 1.18.1) was applied to calculate the cell cycle scores, including the G1, S, and G2M scores. The results were visualized to show the proportion of the cell cycle in distinct clusters or subgroups.

Gene set score analysis

Gene sets were acquired from the MSigDB database (<https://www.gsea-msigdb.org/gsea/msigdb/>). The AddModuleScore function in the Seurat R package was used to calculate the signature score of each gene set in each cell type.

Transcription factor analysis

The R package SCENIC (version 1.2.2) was used to analyze the changes in the transcriptional regulons of DAK and BC-2 between AW and DFU. The adj. P -value < .05 was considered to indicate statistical significance. The activity of the top 10 regulons of each subgroup is displayed as a heatmap.

CCI analysis

CellChat (v1.1.3) was used to perform cell–cell communication between CCL2 + CXCL2 + Fb, VWA1 + VEC, IL-1B + MKs, GZMK + CD8 + T cells and keratinocytes (BC-2 and

DAK). The results revealed potential ligand–receptor interactions and a statistical framework for inferring lineage-specific interactions.

LCM-seq

We conducted LCM-seq on two AW samples and three DFU samples. One AW sample was lost due to technical limitations. The workflow of LCM-seq is described below. We quickly removed unnecessary adipose tissue and bloodstains from the tissues. The tissues were subsequently stored in RNA, embedded in OCT medium, and sliced into 20- μ m thick glass slides. The slices were stained with hematoxylin and eosin, and the target keratinocytes were collected via a laser capture microdissection system. We subsequently conducted RNA extraction, quality control and quantification, library preparation and sequencing, and analysis of the extracted RNA. Complete-linkage hierarchical clustering was performed on normalized counts and subsequently portrayed as a heatmap via the R function heatmap. An unpaired *t*-test with the Benjamini–Hochberg procedure for adjusted *P*-values was used to calculate differentially expressed genes with a threshold of $P < .05$. Significantly expressed genes were entered into Metascape (version 3.5, <http://Metascape.org>) for enrichment analysis via Gene Ontology (GO) biological process terms (version 2020-09-16). All genes in the human genome were used. *P*-values were calculated based on the cumulative hypergeometric distribution, and *Q* values were calculated via the Benjamini–Hochberg procedure for multiple testing. A term was deemed overrepresented when $P < .01$, with a minimum count of 3, and an enrichment factor > 1 .

Hematoxylin and eosin staining

Skin samples were collected and fixed in a solution containing 4.0% paraformaldehyde. After the samples were embedded in paraffin and sectioned into 5- μ m-thick slices, they were stained with hematoxylin and eosin (H&E) for general observation under an Olympus BX61 microscope.

IHC

We inactivated endogenous peroxidase by incubating the sections with 3% hydrogen peroxide at room temperature for 15 min after deparaffinization and rehydration. The nonspecific binding sites were subsequently blocked by the addition of 5% BSA at 37°C for 30 min. The sections were incubated at 4°C overnight with a rabbit monoclonal antibody against MKI67 (1:1000; ab16667, Abcam) diluted in PBS containing 1% BSA. After being washed with PBS, the sections were incubated for 60 min at 37°C with a goat anti-rabbit (H + L) HRP secondary antibody (1:1000, BS13278, Bioworld) diluted in PBS. The sections were subsequently counterstained with hematoxylin and mounted with a neutral resin. The images captured with a Nikon microscope (Nikon, Tokyo, Japan) were analyzed with Image-Pro Plus software.

miHC

Dewaxed paraffin skin sections were subjected to heat-mediated antigen retrieval (citrate buffer; pH = 6) and labeled with a rabbit polyclonal antibody against IL1R2 (1: 1000; DF6682, Affinity Biosciences), a mouse monoclonal antibody against TXNIP (1: 1000; ab210826, Abcam), a rabbit monoclonal antibody against KRT15 (1: 1000; ab52816, Abcam), a rabbit monoclonal antibody against SH3KBP1 Ab (1: 1000; Ab151574, Abcam), a rabbit monoclonal antibody against

GRP (1: 1000; ab236050, Abcam), a rabbit monoclonal antibody against KRT10 (1: 1000; ab76318, Abcam), a rabbit polyclonal antibody against IFITM1 (1: 100; DF2513, Affinity Biosciences), and a rabbit polyclonal antibody against IFITM3 (1: 100; DF8270, Affinity Biosciences). The slides were incubated with a secondary antibody followed by avidin-biotin complexes and peroxidase activity was detected with a diaminobutyric acid (DAB) substrate. The captured images were viewed under an inverted fluorescence microscope (IX83, OLYMPUS, Japan) and arranged with Image-Pro Plus software.

Statistical analysis

GraphPad Prism (v.9.0) was used for the experimental data, and R (v.4.3.0), RStudio (v.4.3.0), and Python (v.3.7.4) were used for the sequencing data and matching clinical variables in the statistical analysis. Descriptive statistics, including means, standard deviations, and proportions, were used to summarize the study variables. The Shapiro–Wilk test was employed to assess the normality of the data distribution for each continuous variable. For comparisons of continuous variables, Student's *t*-test (two groups) or analysis of variance (ANOVA, three or more groups, Bonferroni posthoc test) was utilized to determine significant differences among groups when the data were normally distributed (P -value $> .05$, Shapiro–Wilk test). In cases where data were not normally distributed, the Mann–Whitney U test (two groups) or Kruskal–Wallis test (three or more groups, Dunn's posthoc test) were applied. For paired samples, the paired *t*-test was used for normally distributed difference scores, and the Wilcoxon signed-rank test was used for scores that did not follow a normal distribution. Categorical data were analyzed using the Chi-square test or Fisher's exact test, depending on the expected counts in contingency tables. The sample size of the scRNA-seq libraries was not predetermined using any statistical technique. Unless specified otherwise, every experiment was carried out three or more times using biologically distinct samples. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$, (ns) $P > .05$.

Results

Cell heterogeneity in NS, AW, and DFU tissues at the single-cell level

We performed scRNA-seq analysis on nine samples, including three NS samples, three AW samples, three DFU samples (Figure 1a, Table 1). After ensuring adequate quality control, we obtained transcriptomes from 98 038 cells, which were subsequently annotated into 13 cell lineages (Figure 1b–e, Fig. S1a and b). Compared with NS, DFU showed fewer fibroblasts (Fb) and more keratinocytes (KC) and vascular endothelial cells (VEC). In contrast to those in AW, increased KC, smooth muscle cells (SMC), and VEC numbers and decreased immune cell numbers were observed in DFU (Figure 1d), which was consistent with previous findings from the scRNA-seq analysis of DFU [11, 12]. Furthermore, HE analysis demonstrated that DFU had a thicker epidermis and poorer granulation tissue formation, with more immature nucleated cells in the uppermost layer of the epidermis, indicating abnormal keratinocyte maturation in DFU (Figure 1f and g) [20]. In summary, these data provided a representative cellular atlas and proportions for each group, and we can observe the increased quantity and abnormal

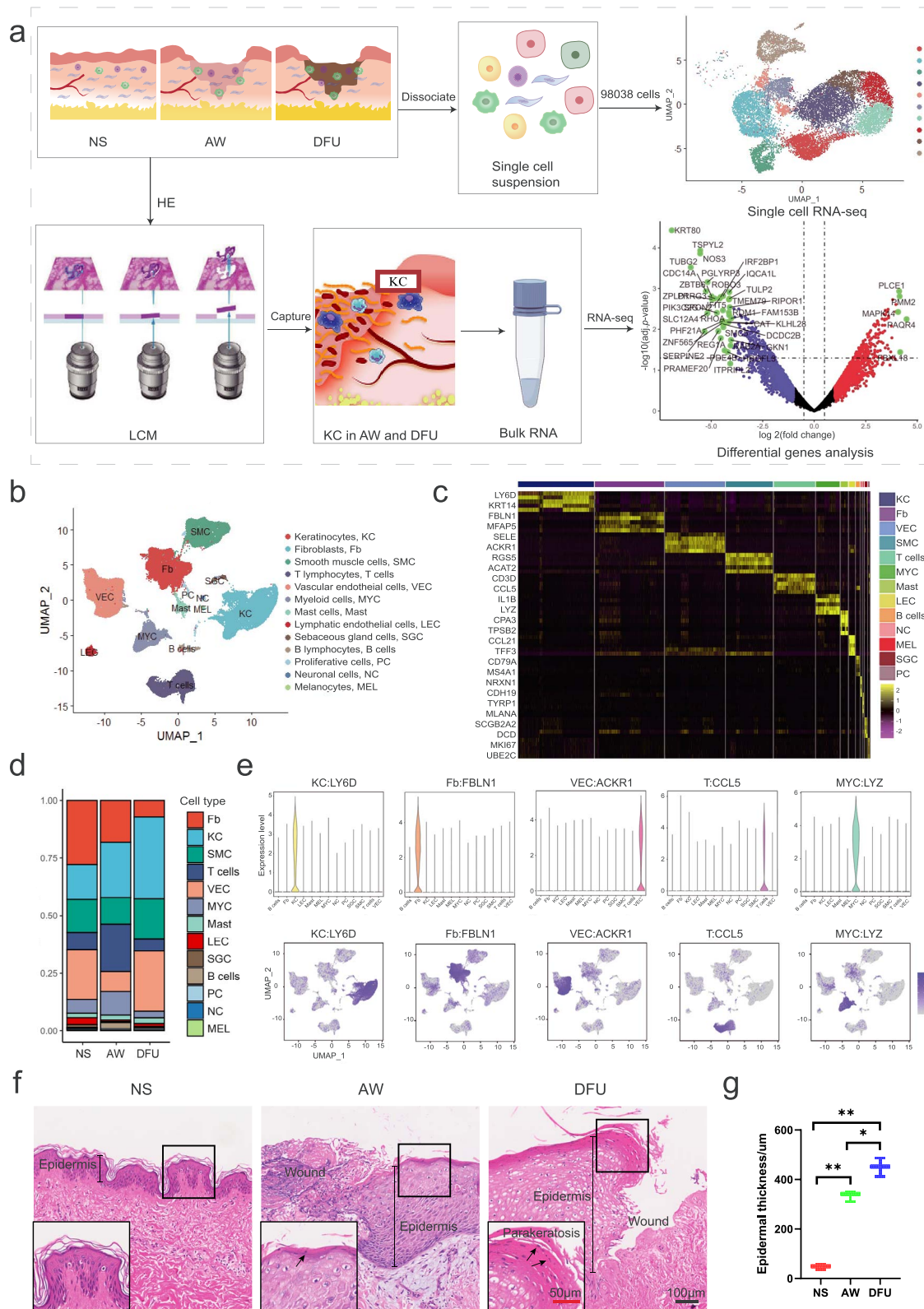


Figure 1. Atlas of various cell types in NS, AW, and DFU tissues at the single-cell level. **(a)** Workflow for scRNA-seq and LCM-seq. **(b)** Clusters and manual annotations of 98 038 cells obtained from NS, AW, and DFU tissues. **(c)** Heatmap of the marker genes in each cluster. Two representative genes are displayed on the left. The color key from purple to yellow indicates low to high levels of gene expression. **(d)** Stacked bar plot of the proportions in each cluster. **(e)** Violin plots and feature plots of marker genes (LY6D, FBLN1, ACKR1, CCL5, and LYZ) that are significantly expressed in KC, Fb, VEC, T cells, and MYC, respectively. **(f)** HE staining of NS, AW, and DFU tissues. The brackets indicate the thickness of the epidermis and the wound edges are visible. The arrows highlight the parakeratosis in the stratum corneum at a higher magnification. The black scale bar represents 100 μ m, and the magnification is 200X. The red scale bar represents 50 μ m, and the magnification is 400X. **(g)** Statistical analysis of epidermal thickness; $n = 3$ for each group. The data represent the mean $\pm$ SD. * p < 0.05, ** p < 0.01. Normal skin (NS), acute wound (AW), diabetic foot ulcer (DFU), laser-capture microscopy (LCM), fibroblasts (Fb), keratinocytes (KC), smooth muscle cells (SMC), T lymphocytes (T cells), vascular endothelial cells (VEC), myeloid cells (MYC), mast cells (mast), lymphatic endothelial cells (LEC), sebaceous gland cells (SGC), B lymphocytes (B cells), proliferative cells (PC), neuronal cells (NC), and melanocytes (MEL)

morphology of KC in DFU. Further mechanisms regarding phenotypic changes and abnormal epidermal homeostasis need to be detected.

Identification of DAK and increased BC-2 in DFU

Keratinocytes (KC) act as the main cell type in the re-epithelialization process, increasing significantly in DFU compared with AW and NS. However, the specific heterogeneity and changes in the biological function of KC under different conditions remain unknown. We further divided KC into ten distinct subpopulations (Figure 2a and Table S1), including five subtypes of basal cells (BC-1, BC-2, BC-3, BC-4, and DAK) that highly expressed KRT5 and KRT14, spinous cells (SC) that expressed the early differentiation genes KRT1 and KRT10, and granular cells (GC) that expressed the late differentiation genes IVL, SPINK5 and SPRR1B (Figure 2b and Fig. S2a) [12, 19]. Compared with those of AW and NS, the KC of DFU highly expressed genes associated with inflammation and stress response, including FOS, JUN, IFITM3, and CXCL14, which were enriched in the IL-17 and TNF signaling pathways (Fig. S2b and c).

Among these basal cells (Figure 2c and d), BC-1 was considered basement membrane cells, with the biological functions of hemidesmosome assembly and extracellular structure organization [21]. The decreased BC-1 in DFU was confirmed by KRT15 staining, indicating poor continuity of the basement membrane in DFU (Fig. S2f and g). BC-2 highly expressed TXNIP, IL1R2, and interferon-stimulated genes (IFITM1 and IFITM3), which were associated with the cellular response and apoptotic processes. mIHC analysis showed a greater number of TXNIP+IL1R2+ cells and IFITM1 + IFITM3+ cells in the DFU than those in the other two groups, which validated the increased BC-2 in the DFU (Figure 2e, f, Fig. S3a and b). BC-3 was distinguished by elevated expression of mitotic markers (PTTG1 and MKI67), which were considered proliferative basal cells [18]. Furthermore, IHC analysis revealed higher expression of MKI67 in the epidermis of DFU, which may result in a thicker epidermis (Fig. S2d and e). BC-4 displayed high expression of KRT16, KRT6C, and S100 family genes (S100A8 and S100A9), which were associated with early epidermis barrier damage [22]. In addition, a distinct subcluster of basal cells in DFU, which highly expressed SH3KBP1 and GRP, was identified as diabetes-associated keratinocytes (DAK). DAK was enriched in pathways associated with the regulation of apoptotic processes and cellular glucose homeostasis. The colocalization of SH3KBP1 and GRP was observed only in the basal layer of the DFU (Figure 2g and h). Furthermore, differentiated keratinocytes (Diff-KC), including SC, GC, and intermediate keratinocytes (inter-KC), were significantly lower in DFU than in NS and AW. This finding was verified by the lower number of KRT10+ cells in DFU through immunostaining analysis (Fig. S2f and h). In summary, these results revealed the heterogeneity of KC and identified crucial subtypes of KC (BC-2 and DAK). We highlighted the imbalanced proliferation and differentiation of epidermal cells in DFU, which was characterized by increased BC and decreased Diff-KC, suggesting an impairment in epidermal homeostasis in DFU [23].

Potential regulatory mechanisms of BC-2 and DAK

Further analysis revealed that BC-2 highly and characteristically expressed the TXNIP, IFITM1, IL1R2, CCL2, FOS, and CXCL14 genes, which were enriched in the

interferon α/γ response signaling pathways and associated with the apoptotic process (Figure 3a, c, d, and e). DAK highly expressed the SH3KBP1, GRP, RHOV, GPX2, and CTSV genes, which were enriched in metabolism-associated pathways (Figure 3b and f). These data suggest that BC-2 was associated with pro-inflammatory and pro-apoptotic processes, whereas DAK was related to metabolic disorders [24, 25]. In addition, we conducted a transcription factor (TF) analysis to explore the potential regulatory mechanism of BC-2 and DAK. The dynamic alterations and potential interactions among TFs define a regulatory network that underlies cellular identity and function [26, 27]. Interestingly, we found that the TFs features of AW were more likely with NS compared to DFU in the heatmap (Figure 3g and h). Moreover, the downregulated TFs in the BC-2 in DFU were enriched in the pathways associated with skin development and keratinocyte differentiation, including IRF6, SIN3A, FOXC1, and EZH2, indicating that the differentiation process might be inhibited in the BC-2 in DFU (Fig. S3c) [28–32]. Similarly, the upregulated TFs in the DAK in DFU were enriched in the pathways associated with cytokine signaling in the immune system and cellular response signaling, including FOS and IRF3, indicating that DAK might have a pro-inflammatory response in the DFU microenvironment (Fig. S3d) [33–35]. These TFs illustrated the potential aberrant differentiation of BC-2 and the pro-inflammatory response of DAK in DFU, which might shed light on future studies.

To explore the specific spatial information of distinct KC subsets and in situ spatial gene expression levels, laser capture microscopy coupled with mRNA-Seq (LCM-Seq) was conducted on the epidermis of the wound edge from two AW and three DFU samples (Fig. S4a). One AW sample was lost because of technological limitations. The heatmap and volcano map revealed distinct differences in gene expression in KC between AW and DFU (Fig. S4b and c). The expression levels of SH3KBP1 and IL1R2 were higher in DFU than those in AW, which was in line with the results of scRNA-seq (Fig. S4d). However, more detailed spatial information needs further exploration with more advanced technology, such as the spatial transcriptome [36].

Impaired epidermal differentiation trajectory in DFU

The increased overall KC and decreased Diff-KC in DFU may result in an imbalance between the proliferation and differentiation of epidermal cells [23]. We performed pseudo-time trajectory analysis to explore the distinct states and transition trajectories of epidermal cells under different conditions. The results revealed a pseudo-temporal trajectory of keratinocyte differentiation from BC-1 to Diff-KC (Figure 4a–c). Along the pseudo-time trajectory, the expression of the stemness gene COL17A1 and cell junction assembly-related gene KRT14 were downregulated, whereas the expression of inflammatory response genes (FOS and IFITM1), the keratin-encoding gene KRT6C, the S100 protein family gene S100A7 and keratinocyte differentiation-specific genes (KRT1 and KRT10) were upregulated [16]. These data may indicate that our putative trajectory was following epidermal stratification and differentiation (Figure 4d) [18]. In addition, cell cycle analysis revealed the distributions and signatures of BC and Diff-KC in different cycling phases (G1, G2M, and S) (Figure 4e). BC-3 showed the highest S score, indicating its proliferative profile (Figure 4f). BC-2 and DAK showed lower G2/M scores than other BCs, suggesting their inactivity and insufficient

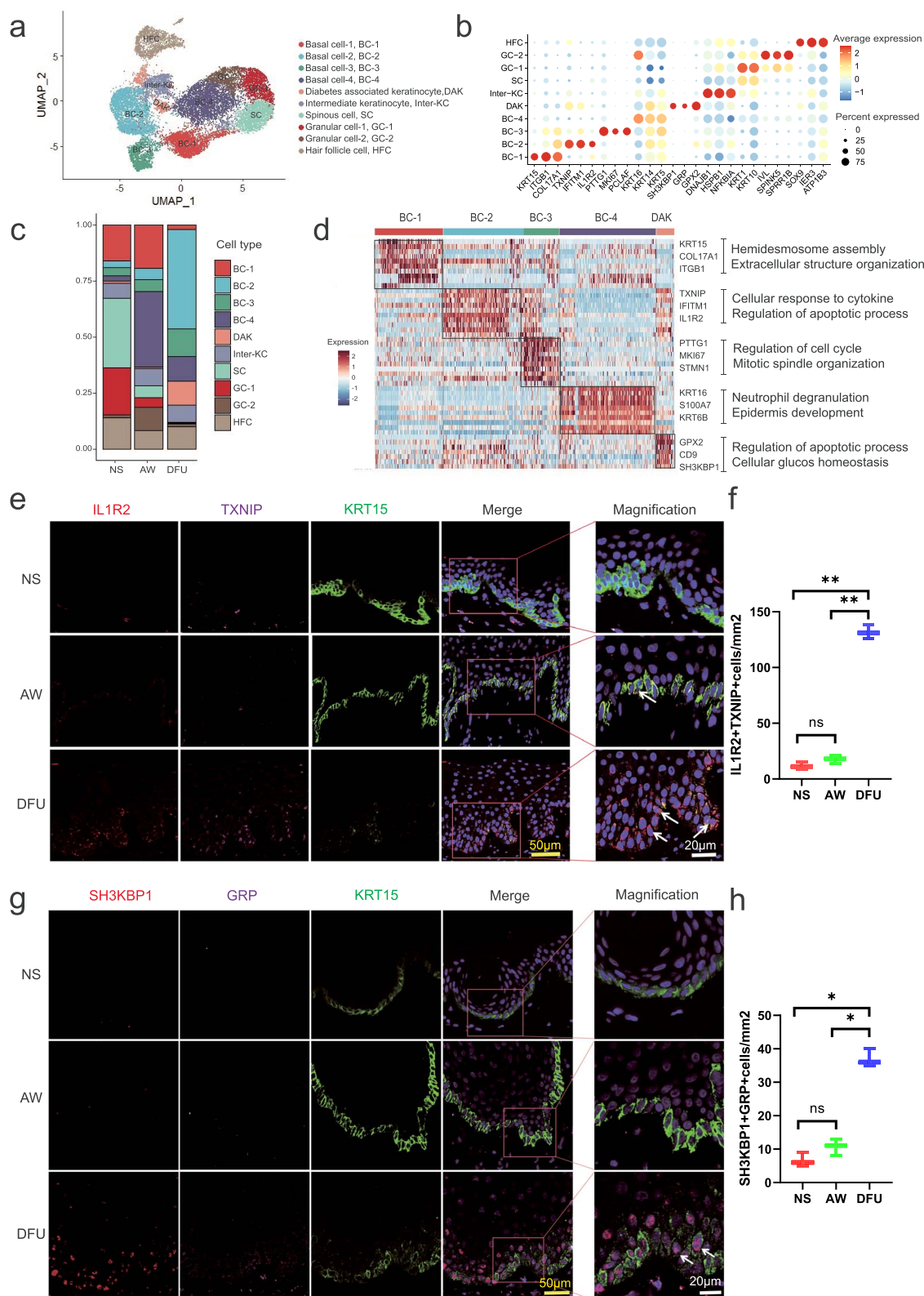


Figure 2. Subtypes of keratinocytes in NS, AW, and DFU tissues. **(a)** UMAP plot of keratinocytes. Keratinocytes are categorized by their respective types and annotated to the right. **(b)** Dot plot of marker genes highly expressed in each KC cluster. **(c)** Stacked bar plot of the proportions in each cluster. **(d)** Heatmap of the marker genes (red) in each basal cell cluster (BC-1, BC-2, BC-3, BC-4, and DAK). The selected marker genes and related biological functions for each cluster are displayed on the right. **(e)** mIHC analysis of IL1R2 (red), TXNIP (purple), KRT15 (green), and DAPI (blue) in each group of samples. Positive cells are labeled with arrows. The yellow scale bar represents 50 μ m, and the magnification was 200X. The white scale bar represents 20 μ m, and the magnification was 400X. **(f)** Quantitative analysis of IL1R2+TXNIP+ cells. $n=3$ for each group. The data represent the mean $\pm$ SD. $**p < 0.01$, (ns) $p > 0.05$. **(g)** mIHC analysis of SH3KBP1 (red), GRP (purple), KRT15 (green), and DAPI (blue) in each group of samples. Positive cells are labeled with arrows. The yellow scale bar represents 50 μ m, and the magnification was 200X. The white scale bar represents 20 μ m and the magnification was 400X. **(h)** Quantitative analysis of SH3KBP1+GRP+ cells. $n=3$ for each group. The data represent the mean $\pm$ SD. $*p < 0.05$, (ns) $p > 0.05$. Basal cells (BC), diabetes-associated keratinocytes (DAK), spinous cells (SC), granular cells (GC), hair follicle cells (HFC), and intermediate keratinocytes (inter-KC)

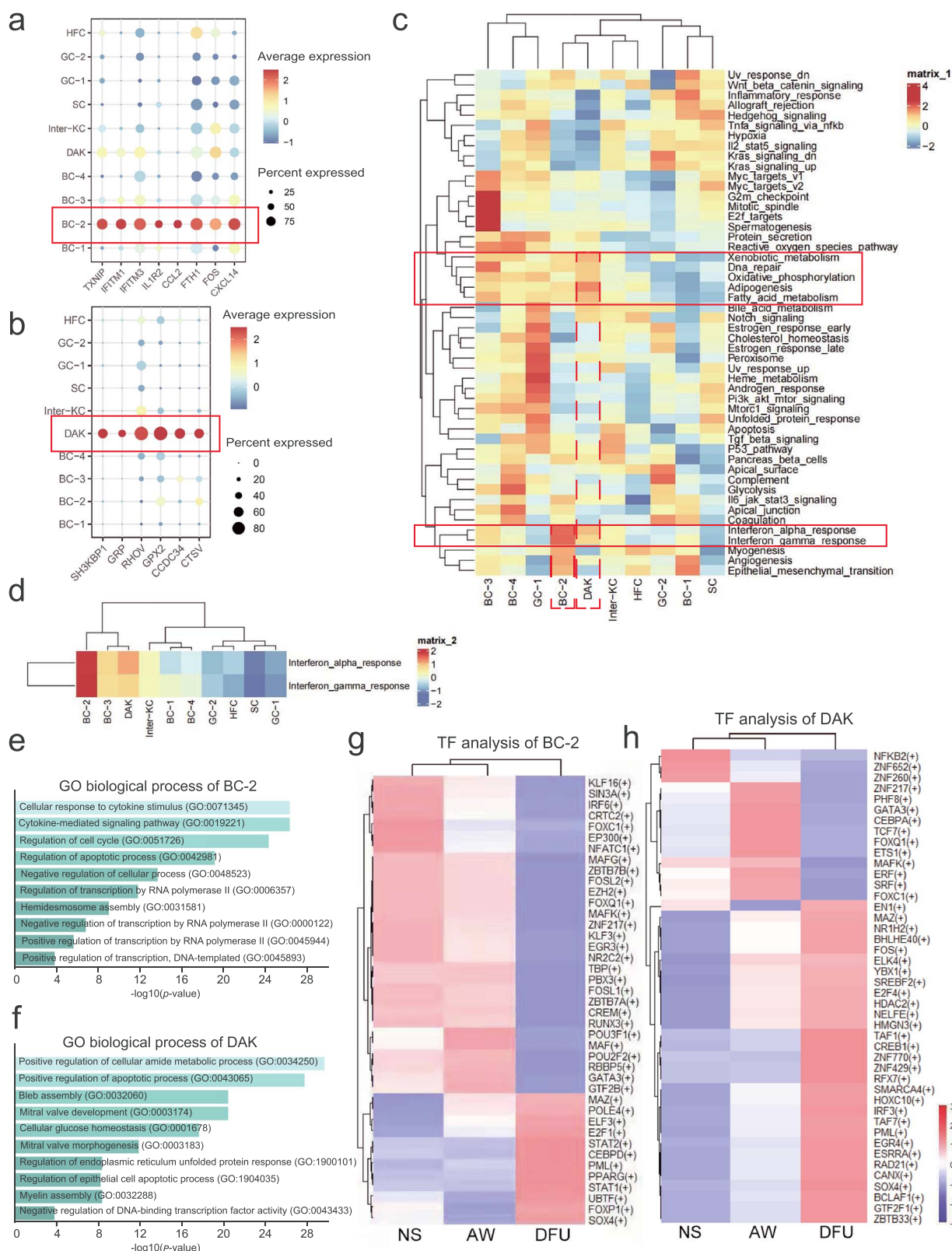


Figure 3. The biological function and potential regulatory mechanisms of BC-2 and DAK. **(a)** Dot plot of the marker genes of BC-2. **(b)** Dot plot of the marker genes of DAK. **(c)** GSEA analysis of the subtypes of KC. The related pathways of BC-2 and DAK are highlighted in the red box. **(d)** GSEA analysis of the subtypes of KC in the IFN- α and IFN- γ pathways. **(e)** GO analysis for biological processes enriched in BC-2. **(f)** GO analysis for biological processes enriched in DAK. **(g)** Heatmap of TFs in BC-2 in each group. **(h)** Heatmap of TFs in DAK in each group. The color key from blue to red indicates low to high levels of TF expression. Interferon-alpha (IFN- α), interferon-gamma (IFN- γ), gene ontology (GO), transcription factors (TFs), basal cells (BC), and diabetes-associated keratinocytes (DAK)

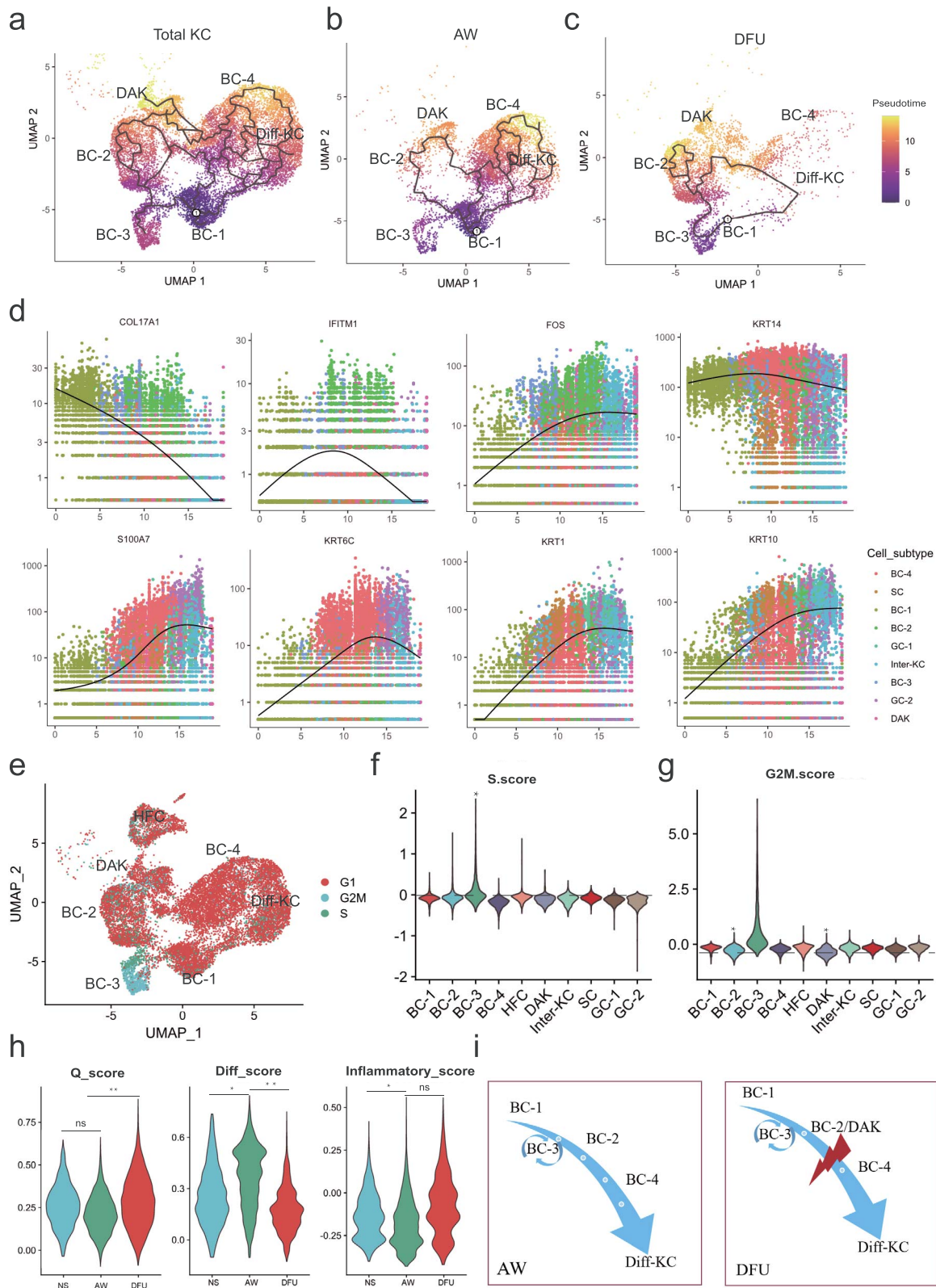


Figure 4. Pseudo-time analysis of KC and impaired epidermal differentiation in DFU. **(a)** Pseudo-temporal cells ordering of the total KC along the differentiation trajectory. **(b)** Pseudo-temporal cell ordering of KC in AW along the differentiation trajectory. **(c)** Pseudo-temporal cell ordering of the KC in the DFU along the differentiation trajectory. The pseudo-time data are depicted in dark purple to light yellow **(d)** The expression levels of the marker genes COL17A1, KRT14, FOS, IFITM1, S100A7, KRT6C, KRT1, and KRT10 along the pseudo-time trajectory. **(e)** UMAP analysis of the cell cycle distribution of KC at different stages. The cells are colored by type and annotated in the graph. **(f)** S score of each KC cluster. **(g)** G2M. Score of each KC cluster. * p -value < 0.05 . **(h)** Gene scoring analysis of the molecular signatures, including the Q score, diff score and inflammatory score. * $p < 0.05$, ** $p < 0.01$, (ns) $p > 0.05$. **(i)** Schematic of epidermal cell differentiation trajectories in AW and DFU. Quiescent score (Q score), differentiated score (diff score), basal cells (BC), diabetes-associated keratinocytes (DAK), and differentiated keratinocytes (diff-KC)

motivation for epidermal differentiation (Figure 4g). Moreover, the basal cells in DFU presented the highest quiescence and inflammation scores and the lowest differentiation score, suggesting that the majority of basal cells in DFU remain in quiescent, proinflammatory and inhibited differentiation states (Figure 4h) [37]. The different trajectories between AW and DFU indicated that basal cells differentiated into Diff-KC successfully in AW but failed in DFU, probably because the epidermal differentiation process ceased in the BC-2 and DAK phases in DFU (Figure 4i). Our results explicated the phenotypic changes and abnormal epidermal differentiation of DFU.

Dermal cell heterogeneity and its pro-inflammatory role in DFU

Dermal stromal cells and immune cells play significant roles in tissue remodeling, angiogenesis, and immunoregulation [7]. Here, fibroblasts (Fb) were divided into nine subpopulations (Figure 5a and d). Among these subclusters, CCL2 + CXCL2 + Fb was significantly increased in DFU and enriched in the MAPK, TNF, and IL17 signaling pathways (Figure 5b and c). According to previous research, CCL2 and CXCL2 could induce continuous infiltration of immune cells, resulting in the release of proinflammatory cytokines such as IL-1 β and TNF- α , thereby causing chronic sustained inflammation in DFU [38].

Vascular endothelial cells (VEC) were regrouped into six distinct subpopulations (Figure 5e and i). The main differences between DFU and the other two groups were the increased VWA1 + VEC and decreased CXCL8 + VEC (Figure 5f). VWA1 + VEC was enriched in the PI3K-Akt and AGE-RAGE pathways and associated with the regulation of epithelial cells, which was closely related to glucose metabolism and epithelial cell proliferation (Figure 5g and h). In addition, CXCL8 + VEC was associated with the response to hypoxia and the regulation of vasculature development and enriched in the IL-17, HIF-1, TNF, and NF- κ B signaling pathways, suggesting that CXCL8 + VEC may regulate angiogenesis during wound healing via hypoxia and inflammation-associated pathways (Figure 5j and k). However, the number of CXCL8 + VEC was lower in DFU compared to AW, indicating poor angiogenesis in DFU.

Four macrophage (MK) subclusters were identified (Fig. S5a, e, and f). IL1B + MK and C1QA + MK represented M1 and M2 macrophages, respectively, and no significant differences were detected between the M1/M2 ratio of AW and DFU (Fig. S5c and d). This may indicate that even though the general polarization appears similar, differences arise in the gene expression of particular markers [39]. MT1G was considered a negative regulator of NF- κ B and significantly decreased in DFU compared to AW, which may result in sustained inflammation and impaired transition to the proliferative stage of the wound healing process (Fig. S5b) [40].

Seven subclusters of T cells were identified in this study (Fig. S5g). GZMA + CD8 + T cells were increased whereas Treg cells were decreased in DFU compared with those in AW (Fig. S5h). GZMA + CD8 + T cells were associated with interferon-gamma, antigen presentation, and phagocytosis signaling pathways and may contribute to an 'immune-aging' phenotype as well as enhance aging-related inflammatory processes (Fig. S5i and j) [41].

To summarize, these data elucidated the main cellular and molecular changes in the dermal microenvironment of DFU,

characterized by inadequate tissue remodeling, abnormal angiogenesis, and persistent inflammation, ultimately leading to refractory DFU.

CCI between the proinflammatory microenvironment and epidermal cells

Epidermal stem cells exhibit excellent heterogeneity during homeostasis and skin diseases, with changes in cell properties, gene expressions, and biological functions that may be caused by the alternative microenvironment [42]. We performed CCI analysis to explore the regulatory network between the main changed cellular subsets (CXCL2 + CCL2 + Fb, IL1B + MK, GZMA + CD8 + T cells, and VWA1 + VEC) and the key subpopulations of KC (BC-2 and DAK) in DFU. Our results revealed the interaction relationships between these cell subpopulations (Figure 6a). CCL2 + CXCL2 + Fb may regulate KC through the TNFSF12-TNFRSF12A pathway, which may be related to cell apoptosis and the pro-inflammatory response. In addition, IL1B + MK may regulate epidermal inflammation through the IL1B-IL1R2 and TNF-TNFRSF1A pathways. Similarly, GZMA + CD8 + T cells may secrete inflammatory factors such as GZMA and IFNG to regulate the biological functions of BC-2 and DAK. These factors may induce the activation of epidermal inflammation (Figure 6b–d). The analysis of communication patterns revealed four distinct patterns for both outgoing and incoming signals in both secreting cells and target cells (Figure 6e). The incoming signaling pathways of BC-2 and DAK were dominated by pattern 1, including EGF, IL1, IFN-II, PERIOSTIN, IGF, and TWEAK signaling pathways (Figure 6f). Notably, GZMA + CD8 + T cells act as senders and influencers of the IFN-II signaling pathway, whereas IL1B + MK cells act as senders and influencers of the IL-1 signaling pathway (Figure 6g and h). Taken together, BC-2 and DAK may be regulated by proinflammatory cell subsets through paracrine mechanisms, which may be associated with proinflammatory and pro-apoptotic pathways.

Discussion

DFU is one of the most common chronic wounds, with high morbidity and amputation rates [43]. Several studies have performed scRNA-seq to explore the molecular and cellular mechanisms of DFU [12, 13]. Nevertheless, the potentially impaired epidermal differentiation mechanisms in DFU compared to AW have not been reported. Our results revealed the cellular landscape of NS, AW, and DFU tissues at the single-cell level and exhibited the profiles of the alternative microenvironment in DFU. We identified two critical subtypes of KC (BC-2 and DAK), that were related to impaired epidermal homeostasis and may be regulated by proinflammatory genes, TFs and proinflammatory cells in DFU. The comprehensive analysis of epidermal heterogeneity and alterations in differentiation dynamics in DFU provided potential therapeutic targets for DFU.

The heterogeneity of keratinocytes has been extensively investigated in various skin tissues derived from healthy skin and skin diseases [44]. It was well established that KC was divided into basal cells and differentiated KC. Keratinocytes progressively lose expression of basal markers, while they concomitantly express differentiation-related markers during differentiation. Multiple basal cell populations were identified

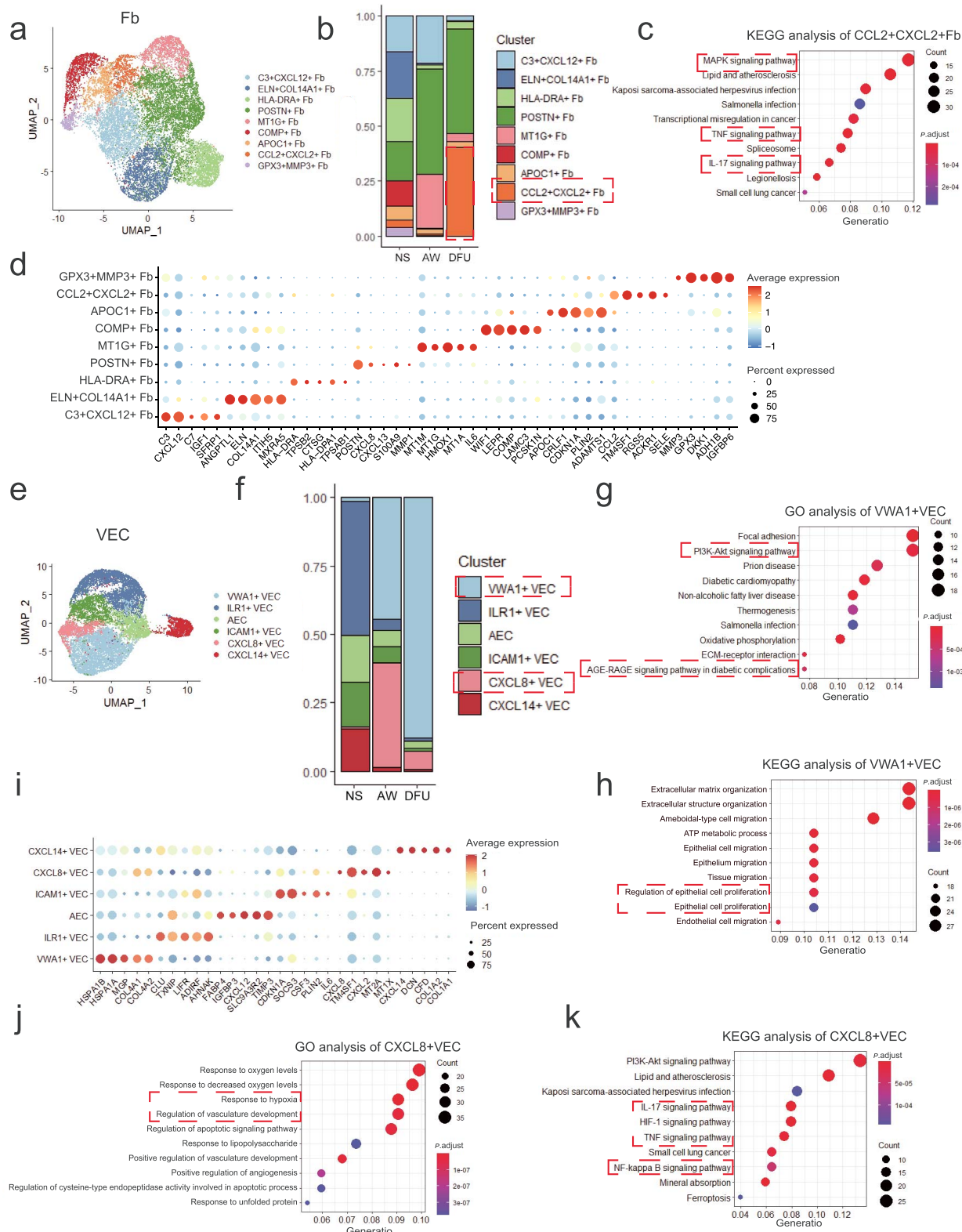


Figure 5. The heterogeneity of fb and vec in NS, AW, and DFU. **(a)** UMAP plot of Fb. The cells were colored according to orthogonally generated clusters and labeled by manual cell type annotation. **(b)** Stacked bar plot of the proportions of subclusters of Fb in each group. CCL2+CXCL2+ Fb is highlighted with a red box. **(c)** KEGG analysis of CCL2+CXCL2+ Fb. The count indicates the number of genes and the color key from blue to red indicates the range of P-values. **(d)** Dot plot of the marker genes in each Fb cluster. The color key from blue to yellow indicates the average expression of genes. The node size is positively associated with the proportion of cells expressing specific markers. **(e)** UMAP plot of VEC. **(f)** Stacked bar plot of the proportions of VEC subclusters in each group. VWA1+ VEC and CXCL8+ VEC are highlighted with red boxes. **(g)** GO analysis of VWA1+ VEC. **(h)** KEGG analysis of VWA1+ VEC. **(i)** Dot plot of marker genes highly expressed in each VEC cluster. **(j)** GO analysis of CXCL8+ VEC. **(k)** KEGG analysis of CXCL8+ VEC. Fibroblasts (fb), vascular endothelial cells (VEC), gene ontology (GO), and Kyoto Encyclopedia of genes and genomes (KEGG)

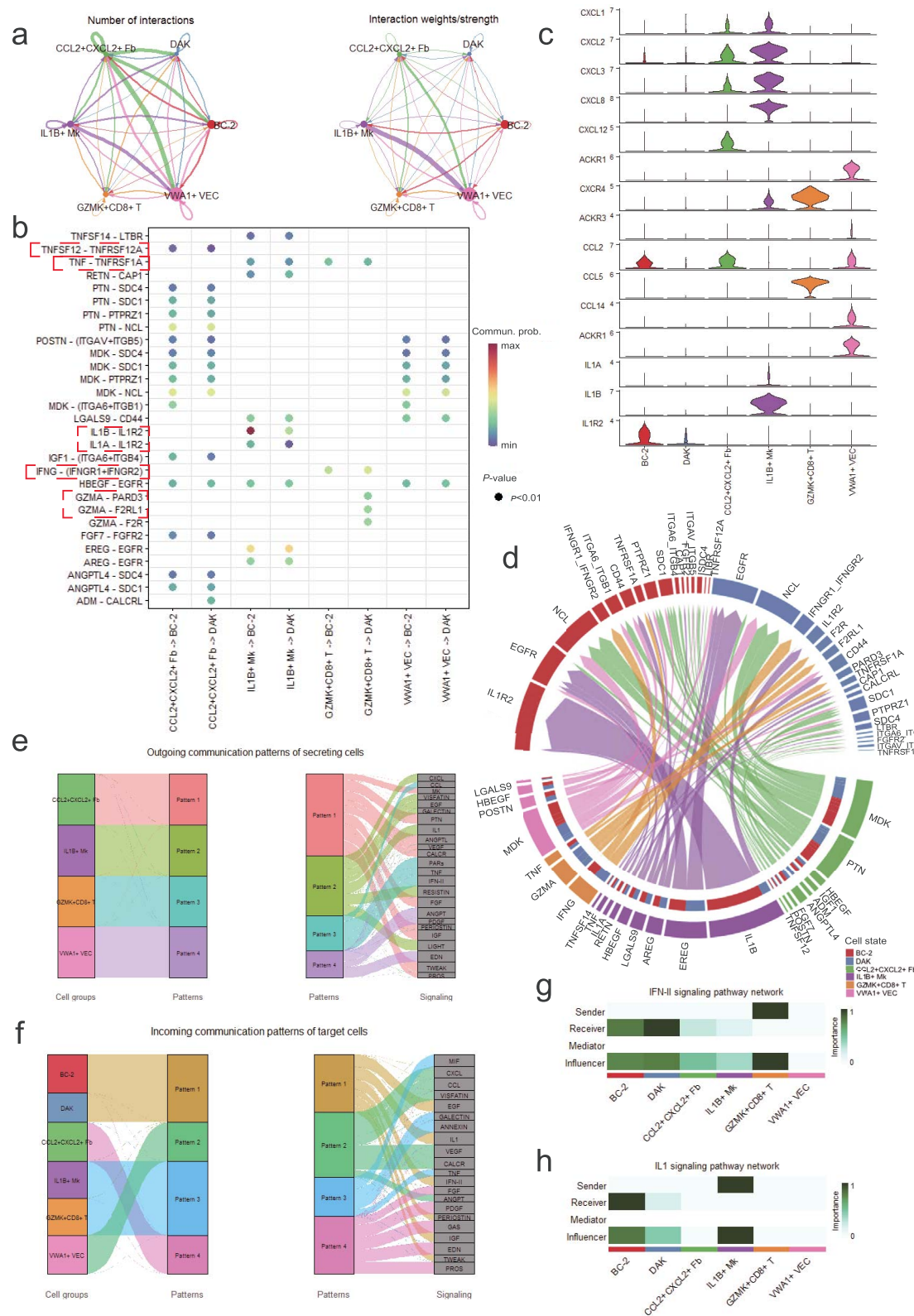


Figure 6. CCI analysis between the proinflammatory microenvironment and key KC subpopulations (BC-2 and DAK) in DFU, **(a)** the number and strength of potential ligand–receptor pairs between different clusters. **(b)** Dot plot of the potential ligand–receptor interactions between ligand-expressing cells and receptor-expressing cells. The color key from green to red indicates the probability of communication. **(c)** Violin plots of selected genes in ligand cells and receptor cells. **(d)** Circle graph of potential ligand–receptor interactions between ligand-expressing cells and receptor-expressing cells. **(e)** Outgoing communication patterns of secreting cells. **(f)** Incoming communication patterns of target cells. **(g)** IFN-II signaling pathway network in selected cells. **(h)** IL1 signaling pathway network in selected cells

based on their transcriptional identities by hierarchical differentiation in the normal skin and skin diseases, usually including the quiescent stem cell, the committed progenitor and proliferative basal cells that maintain epidermal homeostasis [45]. However, the cellular and molecular features of keratinocytes in DFU at the single-cell level have not been fully investigated. In our study, we identified four classic basal cells and a specific DAK subgroup. Among these BCs, BC-2 was considered as an early-response subcluster enriched for immediate early genes and genes associated with cellular stress and apoptosis, such as TXNIP and IFITM [46–48]. Besides, BC-2 exhibited similar molecular features with the specific subtype of KC in pressure ulcers, which was associated with delayed wound healing [49]. Previous studies reported the enhanced inflammatory response induced by TXNIP and IFITM1 in diabetes or wound healing, but their functions in the KC of DFU have not been reported. Similarly, DAK was identified as a distinct subpopulation in DFU, with high expression levels of the SH3KBP1 and GRP genes, and associated with glucose metabolism. Previous research has reported that glucose uptake was required for proliferating keratinocytes to ensure cell proliferation and differentiation [50, 51]. However, hyperglycemia and impaired insulin signaling might impair glucose utilization of skin keratinocytes as well as skin proliferation and differentiation [52]. In addition, keratinocytes were more susceptible to apoptosis in the presence of high glucose, which was consistent with our findings of DAK [51]. As far as we know, a specific DAK subgroup in DFU has not been reported yet. Exploring the function of these critical cells in epidermal pathophysiology is promising for the treatment of DFU.

Epidermal homeostasis is thought to be achieved by the proliferation of basal cells in the basement membrane followed by transit amplification and terminal differentiation of Diff-KC. The potential mechanisms of epidermal homeostasis have been compiled into various models, including a single population of committed progenitor cells that directly self-renew or differentiate, a hierarchical lineage and two independent stem cells that regenerate at different lineage trajectories [16, 18]. Our findings supported a hierarchical lineage of relatively inactive stem cells that resulted in faster cycling and committed progenitor cells that subsequently exited the cell cycle and exhibited terminal differentiation to maintain epidermal homeostasis. Under conditions of skin homeostasis and acute wounds, epidermal differentiation commences at the quiescent basal cells BC-1, progresses to a stress response associated with BC-2 and BC-4, and ultimately differentiates into Diff-KC. However, the trajectory may be arrested in the BC-2 and DAK phases in the DFU, indicating the KC differentiation process in DFU was inhibited, failing to rebuild the barrier of the epidermis. Our results revealed the potential molecular mechanism and dynamic changes in DFU. Lineage differentiation predictions and multiple possibilities need further verification.

Alterations in the microenvironment in DFU have been reported, resulting in an elevated inflammatory state and cell apoptosis. We provided similar findings and more detailed and comprehensive information about the changes in biological processes and interactions with the epidermis. The TNFSF12-TNFRSF12A and POSTN-ITGB5 signaling pathways may regulate the proliferation, adhesion, stress response, apoptosis, and differentiation of BC-2 and DAK through interactions with CCL2+CXCL2+ Fb. TNFSF12 (TWEAK) has been reported to be a key factor in regulating the fate of KC and

could be a therapeutic target for dermatitis and psoriasis [53, 54]. The expression of POSTN is upregulated in skin injury, resulting in delayed wound healing [55, 56]. Additionally, immune cells may secrete proinflammatory factors such as IL1B, GZMA, and IFNG to influence the behavior of epidermal cells. These results may corroborate the notion of an ineffective inflammatory response in DFU, characterized by dysfunctional immune cells and a high concentration of proinflammatory cytokines, thereby resulting in persistent inflammation and impaired epidermal differentiation in DFU [57].

The shortcomings and limitations of this study are listed as follows. Firstly, the sample size in scRNA-seq ($n = 9$) was relatively small. A larger sample size could provide more convincing results. Secondly, wound healing is a dynamic and complex biological process. However, the samples included in our study were obtained at the inflammatory stage during the wound-healing processes. The detailed and comprehensive information on transcriptome dynamics at the different stages needs to be further detected. Finally, we identified some promising targets for treating DFU by scRNA-seq, but the function of these targets needs further verification. In the future, we may design more accurate and effective methods for treating DFU according to the key therapeutic targets.

Conclusion

In summary, our study revealed a comprehensive single-cell landscape of DFU, AW, and NS tissues and highlighted distinct cell subsets that contribute to their unique characteristics, especially in KC. The marker genes (TXNIP, IL1R2, and IFITM1), TFs (IRF6 and EZH2), ligands, and receptors involved in the regulation of BC-2 and DAK could serve as promising targets for the treatment of DFU.

Supplementary data

Supplementary data is available at *Burns & Trauma Journal* online.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Yiling Liu, (Formal Analysis [equal], Investigation [equal], Methodology [equal], Validation [equal]), Peng Wang (Formal Analysis [equal], Investigation [equal], Methodology [equal], Validation [equal]), Jingting Li (Software [equal], Supervision [equal]), Lei Chen (Software [equal], Supervision [equal]), Bin Shu (Software [equal], Supervision [equal]), Hanwen Wang (Visualization [equal]), Hengdeng Liu (Visualization [equal]), Shixin Zhao (Visualization [equal]), and Junli Zhou

(Julin Xie (Conceptualization [equal], Funding Acquisition [equal], Project Administration [equal], Resources [equal])).

Data availability

Data supporting the results of this study can be obtained from the corresponding author upon reasonable request.

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