Insights into Keratinocyte and Immunologic Landscape in Cutaneous Graft-Versus-Host Disease through Single-Cell Transcriptomics

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Cutaneous manifestations are the most common presenting sign of chronic graft-versus-host disease (GVHD), and the extent of cutaneous involvement is also highly correlated with prognosis. Very little is understood about the underlying pathogenesis underpinning injury at this location, especially the contribution of keratinocytes and other structural skin cells. We performed single-cell RNA sequencing to compare the transcriptome of epidermal and dermal chronic GVHD samples with that of healthy control samples. Our findings reveal unique nonimmunologic and immunologic changes in epidermal keratinocytes and dermal immune cells. Specifically, we observed upregulation of alarmins and inflammatory cytokines and downregulation of anti-reduction−oxidation and activator protein-1 pathway genes in the keratinocyte compartments. In dermal immune cell subsets, we showed increased $CD8^+$ T, $CD4^+$ T, $CD4^+Foxp3^+$ regulatory T, and NK cells in chronic GVHD, accompanied by increased signals of leukocyte functions, inflammatory responses, cytolysis, and macrophage M1 polarization. Finally, we also delineated the donor versus recipient cellular origin of nonimmune and immune cell populations in sex-mismatched chronic GVHD. Taken together, these data reveal complex keratinocyte and immune responses in cutaneous chronic GVHD, supporting future studies of skin cell contributions to pathogenesis and potential local treatment strategies.

Keywords: Cutaneous chronic GVHD, Graft-versus-host disease, Keratinocytes, M1 and M2 macrophages, T cells

INTRODUCTION

Graft-versus-host disease (GVHD) is a severe immune-related complication of allogeneic hematopoietic stem cell transplantation associated with high treatment resistance and mortality that affects 30−50% of transplanted patients (Ferrara et al, 2009). Traditionally divided into acute GVHD (aGVHD) and chronic GVHD (cGVHD) depending on the time of its clinical onset (Vigorito et al, 2009). The skin remains the most frequently affected organ in both subtypes, termed cutaneous GVHD (Santos et al, 2018). Cutaneous manifestations are the most common presenting sign of cGVHD, and the extent of cutaneous involvement is highly correlated with prognosis (Palmer et al, 2016; Strong Rodrigues et al, 2018). In its chronic form, GVHD can present with a wide spectrum of cutaneous features, including lichenoid and sclerodermatous changes, along with histopathological hallmarks of hyperkeratosis, acanthosis, basal cell necrosis, superficial perivascular, or lichenoid lymphocytic infiltrate with perifollicular fibrosis (Strong Rodrigues et al, 2018; Vargas-Díez et al, 2005).

In this study, we focused on cutaneous cGVHD, which has multifaceted underlying mechanisms, including immune cell dysfunctions and distorted tissue repair (Socié and Ritz, 2014). To date, very few studies have addressed the underlying pathogenesis of injury at this location, especially the contribution of keratinocytes (KCs) and other skin cells. We leveraged single-cell RNA sequencing (scRNA-seq) to study the transcriptome of epidermis and dermis in the cGVHD skin lesions. Studies, such as described in this paper, can provide new insights into the mechanisms of skin cGVHD that may help drive future development of novel and effective therapeutics.

RESULTS

scRNA-seq reveals distinctive epidermal KC and immune cell populations in cGVHD

To examine cell-specific transcriptome of cGVHD skin lesions, we performed scRNA-seq of epidermis isolated from a patient diagnosed with nonfibrotic subtype of cGVHD and

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Abbreviations: aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; DEG, differentially expressed gene; GVHD, graft-versus-host disease; K, keratin; KC, keratinocyte; redox, reduction−oxidation; scRNA-seq, single-cell RNA sequencing

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normal skin from a healthy control (normal). The patient with cGVHD underwent matched unrelated donor hematopoietic stem cell transplantation with onset of biopsy-proven cutaneous cGVHD 160 days status after transplantation (Table 1). Clinical photograph at the time of biopsy is shown in Figure 1a. Histologic analysis showed lichenoid dermatitis with hyperkeratosis, hypergranulosis, and chronic dermal inflammation in the patient with cGVHD (Figure 1b and c). A total of 4699 cells in cGVHD and 8662 cells in normal epidermal skin were sequenced, with an average of 1797 genes per cell detected in cGVHD and 2652 genes per cell detected in normal epidermal cells. We performed unsupervised clustering analysis that grouped cells into 26 clusters (Figure 1d). From these clusters, we identified 12 cell types using signature genes (Figure 1e and f). cGVHD epidermis showed lower percentages of basal and suprabasal KCs, myeloid dendritic cells, and melanocytes than the normal skin epidermis (Figure 1g). On the other hand, cGVHD epidermis showed slightly increased percentages of macrophages, $CD4^+$ T cells, $CD8^+$ T cells, Langerhans cells, and plasmacytoid dendritic cells. The presence of endothelial cells, fibroblasts, and smooth muscle cells likely results from technical contamination from the dermis. We analyzed differentially expressed genes (DEGs) in cGVHD versus normal epidermis and identified several proinflammatory genes, including keratin (K) genes *K6A*, *K6B*, *K6C*, and *K16*; *S100A7*, *S100A8*, and *S100A9*; *HLA-DRA*; and *CD74*, as well as *VIM* that were highly expressed cGVHD epidermis (Figure 1h). Gene ontology analysis on DEGs in cGVHD relative to those in normal KCs revealed significantly enriched biological processes, including cornification, immune, and antimicrobial responses (Figure 1i).

Upregulation of inflammatory genes and downregulation of reduction–oxidation and regenerative genes in cGVHD epidermal KC populations

Unsupervised clustering analysis originally yielded 12 clusters of KCs, including 6 basal KC clusters and 6 suprabasal KC clusters, which were further subdivided on the basis of histologic locations and degree of differentiation (Figure 2a). Basal KCs were characterized by the expression of *K5/K14*, and KC stem cells were marked by *K15*. Among the basal KCs, KC-B4s were transient amplifying cells, as marked by the cell proliferation marker Ki-67. Spinous, granular, and terminal KCs were characterized by the expressions of *K1/K10*, *K2*, and involucrin gene IVL/FLG genes respectively (Figure 2b) (Wang et al, 2020). KC stem cell percentages markedly decreased in cGVHD and showed downregulation of basal markers, including SYT8 and POSTN (Figure 2c and d). We performed DEG analysis comparing cGVHD with normal KCs and found that *K6A*, *K6B*, *K6C*, *K16*, *S100A7*, *S100A8*, *S100A9*, *PI3*, *SPRR1B*, and *IFI27* were highly expressed in cGVHD KCs, whereas homeostatic cytokines such as CXCL14 and CCL27 as well as reduction–oxidation (redox) genes such as *MT1X* and cell proliferation regulators such as *c-Myc* and *CCND1* were downregulated in cGVHD KCs (Figure 3a). We separately analyzed basal and suprabasal KCs in cGVHD versus normal samples given their distinctive transcriptional profiles in resting state (Figure 3b and c). We then performed pathway analysis, which revealed the upregulations of alarmins, such as *K6A/6B/16/17*, *S100A7/8/9*, and *CD74*, and inflammatory cytokines, such as *IL1A/1B*, *IL36G*, *CCL2/5*, and *CXCL8/9/10* in cGVHD KCs compared with those in normal counterparts (Figure 3d and e). There was a concomitant downregulation of anti-redox genes (eg, *MT1E* and *MT1X*), multiple SELENO proteins that regulate lipid metabolism and protein folding, and activator protein-1 pathway transcription factors of both Jun (*Jun*, *JunB*, and *JunD*) and Fos (*Fos* and *FosB*) family members as well as *MYC*, *HES1*, and *EGR1* gene regulators (Figure 3b, c, f, and g), which control skin regeneration and differentiation (Angel et al, 2001). We validated the unsupervised analyses by performing immunofluorescent staining of additional normal samples and samples from patient with cGVHD, which showed upregulation of S100A7 and K16 in aGVHD and cGVHD (Figure 3h).

Dermal structural and immune cell transcriptional changes in cGVHD

Next, we performed scRNA-seq of dermis isolated from the same patient with cGVHD and healthy control (normal). A total of 7014 cells of cGVHD dermis and 18,244 cells of normal dermis were sequenced, with a median of 1988 genes per cell detected in cGVHD and 1688 genes per cell detected in normal dermis. Unsupervised clustering analysis grouped cells into 32 clusters (Figure 4a). From these clusters, we identified 16 cell types using signature genes (Figure 4b and c). cGVHD dermis showed lower percentages of fibroblasts, endothelial cells, smooth muscle cells, macrophages, mitotic cells, basophils, and B cells than respective counterparts of the normal dermis. On the other hand, cGVHD dermis showed markedly increased immune cells, including $Foxp3^+$ regulatory T cells, $CD8^+$ T cells, $CD4^+$ T effector cells, and NK cells (Figure 4d). The presence of KCs and melanocytes is likely technical contamination from the epidermis. DEGs analysis in cGVHD versus normal dermis revealed that *PTPRC*, *HLA-DRB5*, *IL32*, *CORO1A*, *CD52*, *HCST*, *SRGN*, *CD2*, *COTL1*, and *TRAC* were highly expressed in cGVHD dermis (Figure 4e). Gene ontology analysis on DEGs in

Table 1. Patient Characteristics

Characteristics	Patients with cGVHD
Transplant indication	Mycosis fungoides
Type of transplantation	Matched unrelated allogeneic stem cell transplantation
Pretransplant conditioning regimen	25 mg/m^2 fludarabine and 140 mg/m^2 melphalan
Post-transplant regimen	Tacrolimus
Time to onset for cutaneous symptoms after transplantation	160 days
Extracutaneous manifestations	GI, with biopsies showing gut GVHD
Grading/scoring	Mild (skin score of 2)
cGVHD subtype	Nonfibrotic
Previously treated?	No
Biopsy location	Left back
Pathologic diagnosis	Lichenoid dermatitis concerning for GVHD, lichenoid type

Abbreviations: cGVHD, chronic graft-versus-host disease; GVHD, graft-versus-host disease.

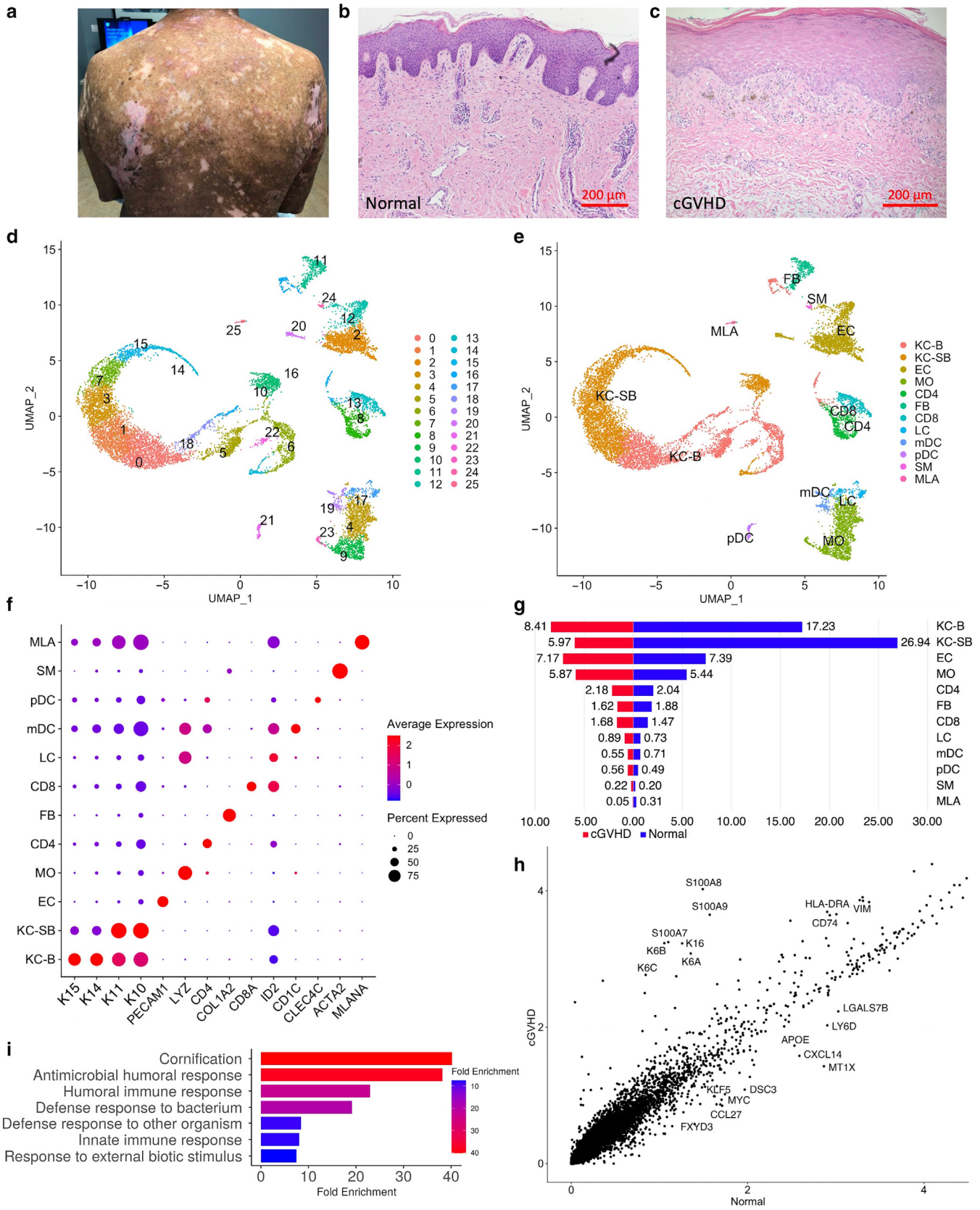

Figure 1. Single-cell RNA sequencing reveals distinctive epidermal keratinocyte and immune cell populations in cGVHD. (**a**) Clinical photograph of patient with cGVHD at the time of biopsy. (**b**) H&E-stained normal skin at ×10 magnification. (**c**) H&E-stained cGVHD sample at ×10 magnification. (**d**) Unsupervised clustering represented in an UMAP plot of the epidermal samples. (**e**) UMAP plot of clusters grouped by cellular identity. (**f**) Dot plot of percentage expressed and average expression of signature genes defining each cellular identity. (**g**) Sample distribution of cell populations by percentage of total cell numbers of normal or cGVHD epidermis skin. (**h**) Top 10 most differentially expressed genes in normal versus cGVHD epidermis skin. (**i**) Gene ontology analysis of most

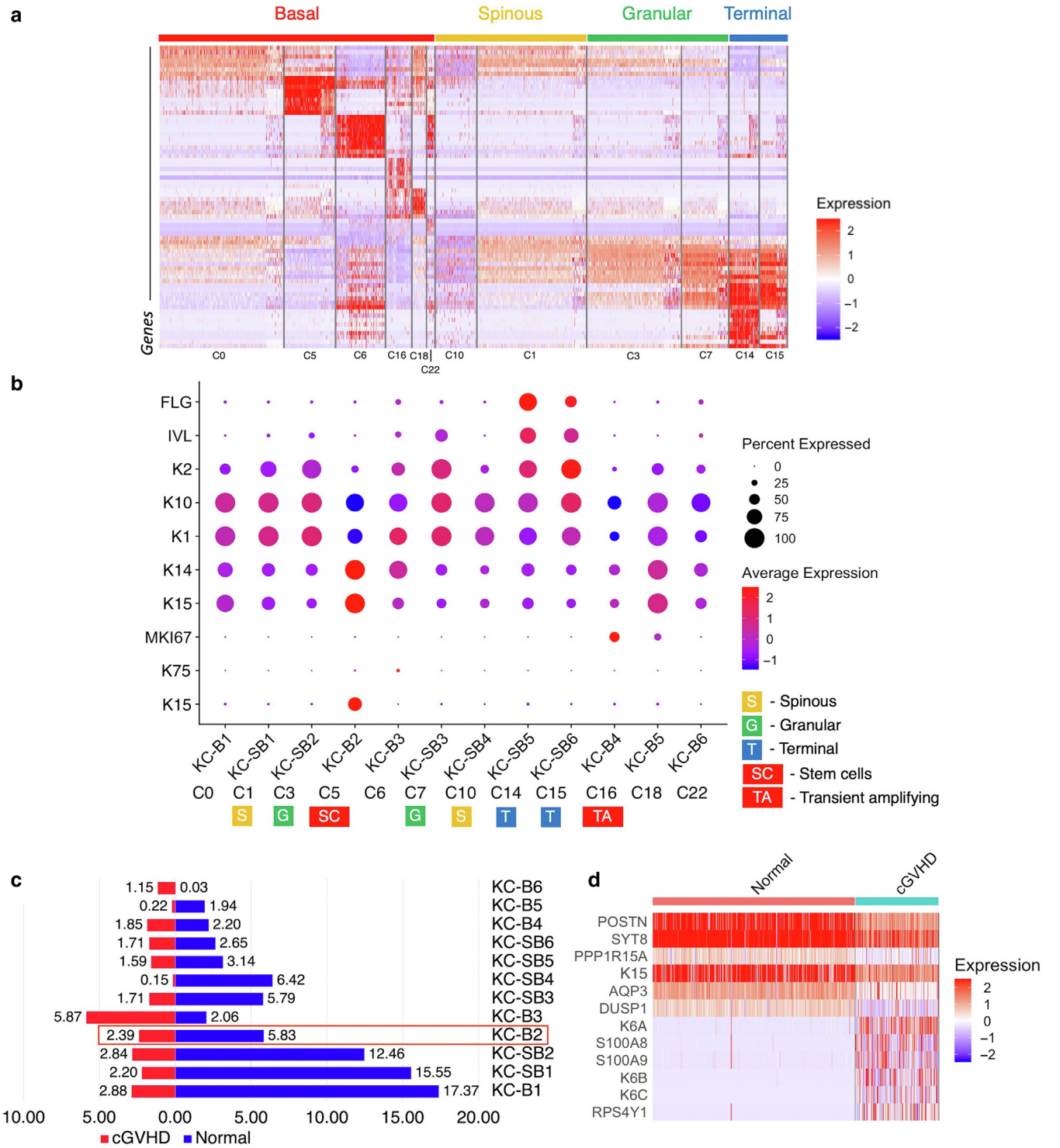

Figure 2. Analysis of keratinocyte subgroups revealed reduction in keratinocyte stem cells in cGVHD. (**a**) Heatmap of 12 clusters of epidermal keratinocytes based on histologic locations. (**b**) Dot plot showing keratinocyte markers. C denotes cluster number. (**c**) Distribution of keratinocyte populations by percentage of total keratinocytes of normal or cGVHD epidermis. Red box highlights K15+ keratinocyte stem cells (cluster 5). (**d**) Heatmap of highly differentially expressed genes in normal versus cGVHD keratinocyte stem cells. cGVHD, chronic graft-versus-host disease; K, keratin.

highly expressed genes in cGVHD epidermal sample. KC-B denotes basal keratinocytes, KC-SB denotes suprabasal keratinocytes, MO denotes macrophages, FB denotes fibroblasts, MITO denotes mitotic cells, BAS denotes basophils, SM denotes smooth muscle, MLA denotes melanocytes, and B denotes B cells. cGVHD, chronic graft-versus-host disease; EC, endothelial cell; IVL, involucrin; K, keratin; LC, Langerhans cell; pDC, plasmacytoid dendritic cell; UMAP, Uniform Manifold Approximation and Projection.

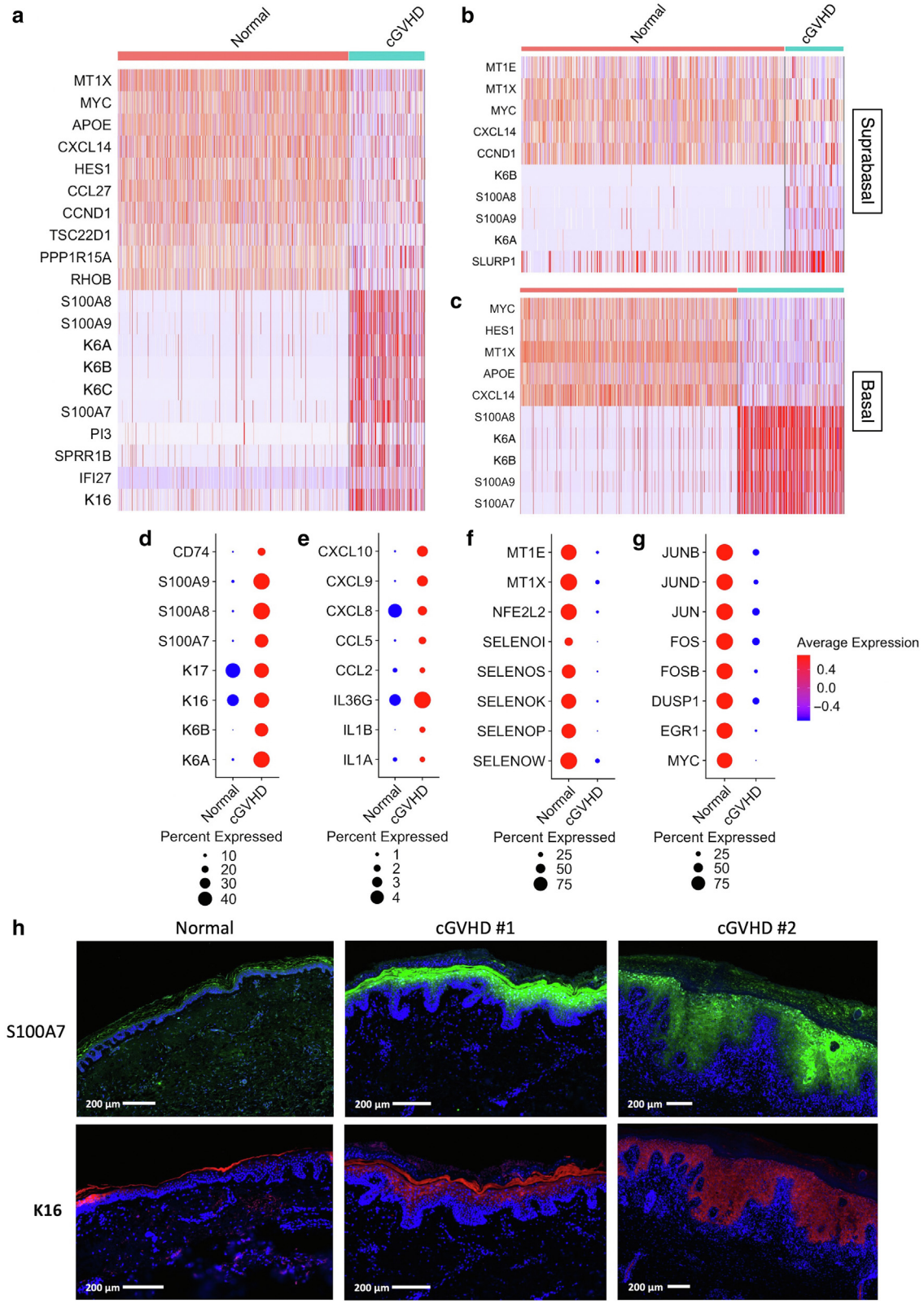

Figure 3. Upregulation of inflammatory genes and downregulation of redox and regenerative genes in cGVHD epidermal keratinocyte populations. (**a**) Heatmap of top 10 most differentially expressed genes in keratinocytes in normal versus cGVHD epidermal samples. (**b**) Heatmap of top 5 most differentially expressed genes in suprabasal keratinocytes in normal versus cGVHD epidermal samples. (**c**) Heatmap of top 5 most differentially expressed genes in basal keratinocytes in normal versus cGVHD epidermal samples. Dot plot showing the percentage expressed and average expression of (**d**) alarmin genes, (**e**) inflammatory cytokines, (**f**) antioxidant genes, and (**g**) AP-1 and other transcription factor genes expressions in normal versus cGVHD samples. (**h**) Immunofluorescent staining of S100A7 and K16 in additional normal and cGVHD samples confirmed increased expressions of S100A7 and K16 in cGVHD. AP-1, activator protein-1; cGVHD, chronic graft-versus-host disease; K, keratin; redox, reduction–oxidation.

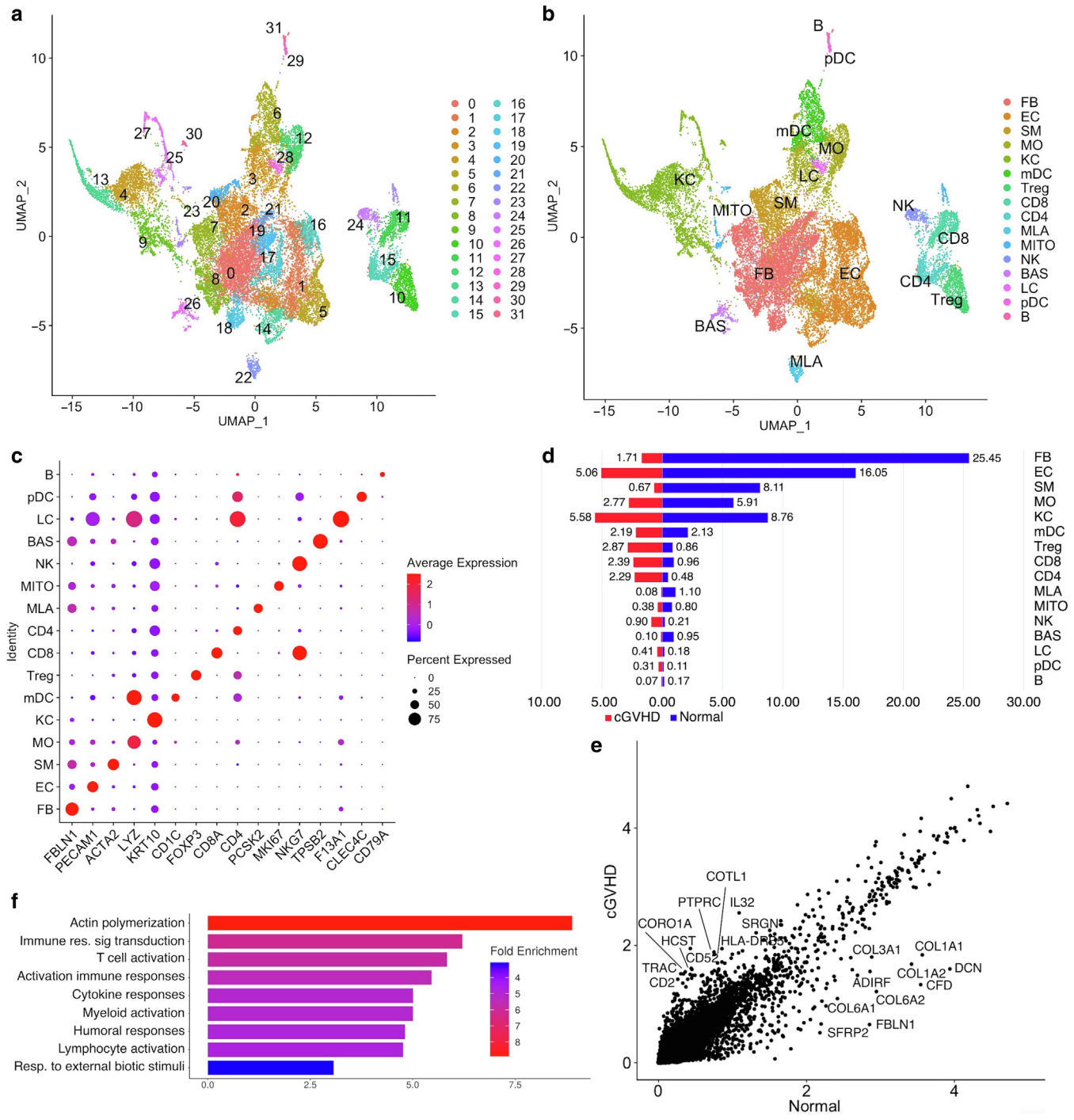

Figure 4. Dermal structural and immune cell transcriptional changes in cGVHD. Unsupervised clustering represented in an (**a**) UMAP plot of the dermal samples, (**b**) UMAP plot of clusters grouped by cellular identity, and (**c**) dot plot of percentage expressed and average expression of signature genes defining each cellular identity. (**d**) Sample distribution of cell populations by percentage of total cell counts of normal or cGVHD dermis. (**e**) Top 10 most differentially expressed genes in normal versus cGVHD dermis skin. (**f**) Gene ontology analysis of most highly expressed genes in cGVHD dermal sample. KC-B denotes basal keratinocytes, KC-SB denotes suprabasal keratinocytes, MO denotes macrophages, FB denotes fibroblasts, MITO denotes mitotic cells, BAS denotes basophils, SM denotes smooth muscle, MLA denotes melanocytes, and B denotes B cells. cGVHD, chronic graft-versus-host disease; EC, endothelial cell; KC, keratinocyte; LC, Langerhans cell; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; Treg, regulatory T cell; UMAP, Uniform Manifold Approximation and Projection.

cGVHD dermis relative to that in normal revealed significantly enriched biological processes, including actin polymerization, immune signal transduction, T-cell activation, myeloid activation, humoral response, and cytokine response (Figure 4f).

Increased inflammatory responses and upregulation of M1 macrophage in cGVHD

We next focused our attention on immune cell populations that showed the most significant changes. We performed DEGs analysis in cGVHD versus normal immune cells and

found that the 4 groups with the most DEGs were $CD8^+$ T cells, $CD4^+$ T cells, NK cells, and macrophages (Figure 5a–d). Collectively, cGVHD groups showed higher markers of inflammation, with macrophages demonstrating higher expression of profibrotic genes such as *CCL18* (Figure 5d).

Classically, macrophages are classified into the proinflammatory M1 macrophages and anti-inflammatory M2 macrophages. Further analysis of the 2 macrophage clusters—3 and 12—revealed higher expression of M1 markers, including signal transducer and activator of transcription genes *STAT1* and *STAT2*, *CXCL9*, and *CXCL10*, in cluster 12 (renamed M1) and M2 markers, including *KLF2*, *KLF4*, *MYC*, and *CEBPB*, in cluster 3 (renamed M2) (Figure 5e) (Lawrence and Natoli, 2011). Whereas the percentages of M2 macrophages in total cells by sample showed a decrease of 1.15% (718 in cell numbers), the percentage of M1 macrophages increased by 2.95% in cGVHD compared with that in normal samples (Figure 5f), yielding an M1/M2 ratio of 0.44 and 1.2 in normal and lesional skin, respectively. In agreement with the proinflammatory phenotype, M1 groups showed elevated expression of inflammatory and activation markers (*C1QA/B/C*, *HLA-DRB5*, *FCER1G*, *TYRO1BP*, *CTSZ/S/B*, and *CXCL9* as well as *FCGR3A*, *FPR3*, *ACP5*, and *CD68*). In contrast, M2 groups expressed increased levels of homeostatic and extracellular matrix remodeling markers (*DCN*, *COL6A2/1A1/1A1/3A1*, *SFRP2*, and *MGP* as well as *VWF*, *PLVAP*, *ID1*, *SELE*, and *SOX18*) (Figure 5g). Finally, lesional M1 macrophages showed higher M1 polarization and inflammatory markers with higher expression of CD68, FPR3, and FCGR3A than normal M1 (Figure 5g).

Gene ontology analysis of highly expressed genes in cGVHD $CD8^+$ T cells, $CD4^+$ T cells, NK cells, and macrophages revealed enrichment in actin polymerization/depolymerization, immune responses and downstream signaling transduction, Fc receptor signaling pathway, cytolysis, and antigen processing and presentation (Figure 6).

Sex-based gene analysis delineates cellular origin of nonimmune and immune cell populations in cGVHD

Finally, we set out to better delineate the cellular origin of structural skin and immune cells in the cGVHD male recipient skin with a female bone marrow donor. We examined the expression of 2 sex-specific genes, X-chromosome *XIST* and Y-chromosome *RPS4Y1*, which are exclusively expressed in females and males, respectively (Staedtler et al, 2013). Female-specific *XIST* showed higher expressions in cGVHD dermal immune cells than in nonimmune cells (Figure 7a). Minimal to no expression of *RPS4Y1* was detected in the normal sample from a female patient (Figure 7b). Male-specific *RPS4Y1* showed higher expressions in cGVHD dermal structural cells, especially endothelial and smooth muscle cells. Similarly, other male genes, such as *USP9Y*, *ZFY*, *TTTY14/15*, *DDX3Y*, *UTY*, *KDM5D*, *EIF1AY*, *TMSB4Y*, and *NLGN4Y*, were absent in majority of immune cells (Figure 8). Few $CD4^+$ and $CD8^+$ T cells of cGVHD sample showed expression of *RPS4Y1* and other male genes (Figures 7b and 8), suggesting male origin.

DISCUSSION

In this study, we sought to characterize the nonimmune and immune cell landscape in cutaneous cGVHD. Through scRNA-seq, we provide preliminary evidence that both KCs and immune cells play an important role in cGVHD skin. We first identified distinctive transcriptional changes in cGVHD epidermal KCs, characterized by significant upregulation of alarmin proteins and inflammatory cytokines and downregulation of anti-redox enzymes and activator protein-1 pathway genes involved in skin regeneration/differentiation. In the dermis, we observed significantly elevated numbers of $CD4^+$, $CD8^+$, and NK cells along with increased expression of genes involved in leukocyte cytotoxic and inflammatory functions in cGVHD. In addition, the number of M2-polarized macrophages significantly decreased along with reduced expression of homeostatic and extracellular matrix–remodeling genes in cGVHD. Finally, we discovered that immune cell infiltrates in the cGVHD skin are primarily of donor origin, whereas structural skin cells are of recipient origin.

Ongoing inflammation and interruption of normal skin repair processes profoundly disrupt normal skin homeostasis in treated cutaneous GVHD. It was first reported by Sale et al (1985) that KCs in the tips of the rete ridges along the edge of the epidermis, a population that represents basal stem cells (Sale et al, 1985; Whitaker-Menezes et al, 2003), preferentially undergo apoptosis in cutaneous GVHD. Loss of epidermal stem cells in cGVHD leads to reduced wound healing and increased risk of microbial infection, both of which further propagate the highly inflammatory pathogenic environment. We observed reduction in KC stem cells along with elevated expressions of KT6A/B, K16, K17, and S100 proteins in cGVHD KCs. The keratin proteins have long been reported to be critical barrier alarmin molecules in other chronic inflammatory skin diseases (Cheng et al, 2018). Loss of K6A/6B in KCs enhances epithelialization potential due to increased KC migration through Src kinase and myosin IIA and is associated with decreased K16 level (Rotty and Coulombe, 2012; Wang et al, 2018; Wong and Coulombe, 2003). Overexpression of K16 can lead to KC hyperproliferation, delay in wound healing, and aberrant innate immunity activation (Wawersik et al, 2001; Zhang et al, 2019). On the other hand, K17 promotes KC hyperproliferation and T helper 1/T helper 17 cytokine production and acts as autoantigen for CD4/$CD8^+$ T cells (Hobbs et al, 2016; Shen et al, 2005; Yang et al, 2018). S100A7/8/9 are cytoplasmic alarmins that enhance inflammation by promoting dendritic cell/microphage activation and leukocyte recruitment (Yang et al, 2017). Interestingly, in the context of psoriasis, S100A7 has been shown to increase inflammatory infiltrates to the skin (Wolf et al, 2010) and simultaneously suppresses the expression of KC differentiation markers, including involucrin, loricrin, K1, and K10, through the NF-κB pathway (Son et al, 2016). Not surprisingly, we also observed upregulation of inflammatory cytokines/chemokines in cGVHD KCs, all of which are known to promote leukocyte infiltration and activation (Orlik et al, 2020). This promotes a feed-forward loop of enhanced KC inflammation evidenced by increased CD74 expression, which is part of

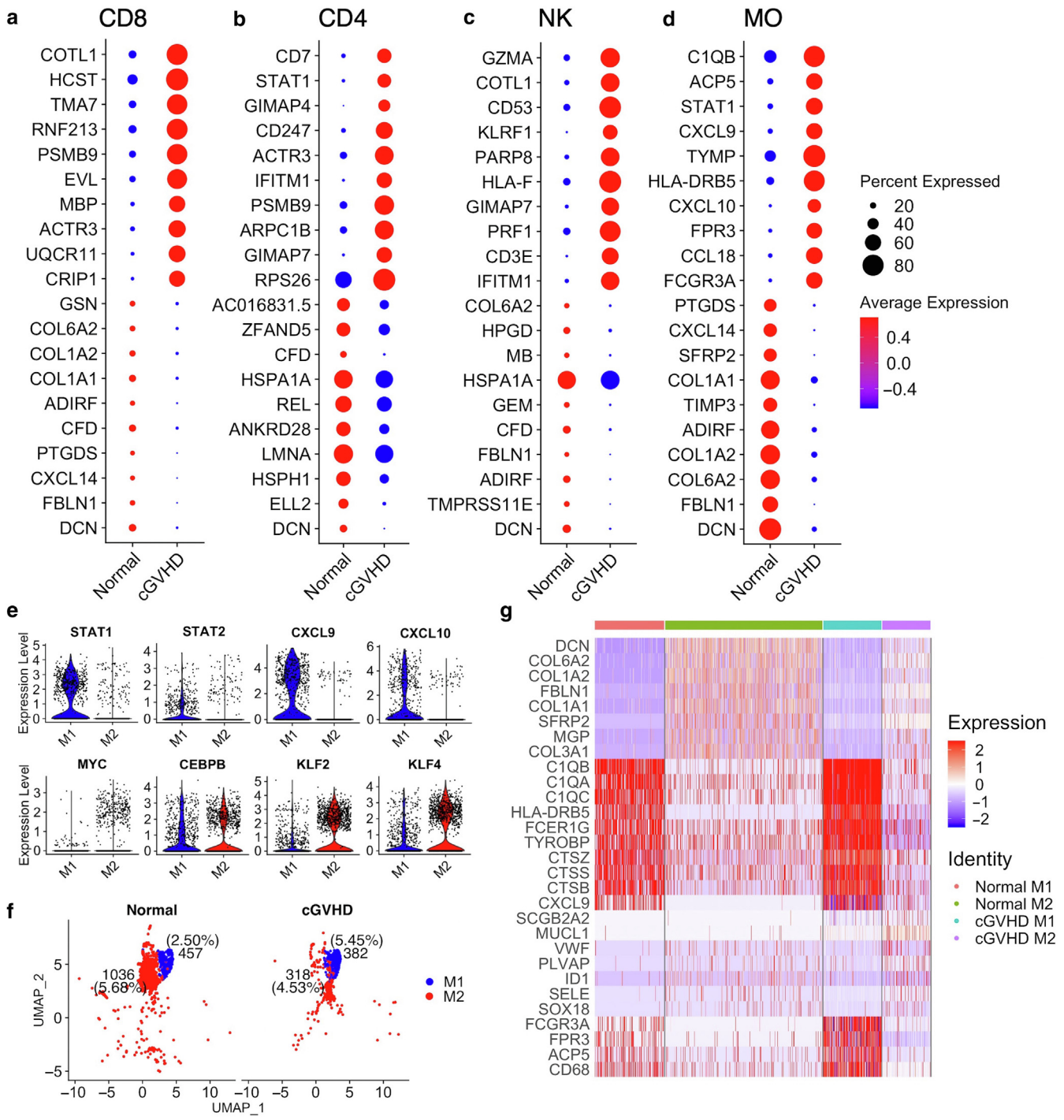

Figure 5. Increased inflammatory responses and upregulation of M1 macrophage in cGVHD. Dot plot showing percentage expressed and average expression of top 10 most differentially expressed genes in (**a**) $CD8^+$ T cells, (**b**) $CD4^+$ T cells, (**c**) NK cells, and (**d**) macrophages. (**e**) Violin plots showing macrophage polarization characterized by signature markers. (**f**) UMAP plots showing total number of M1- and M2-polarized macrophages of normal or cGVHD dermis. (**g**) Heatmap of most differentially expressed genes in macrophage clusters grouped by polarization status and sample. cGVHD, chronic graft-versus-host disease; STAT, signal transducer and activator of transcription; UMAP, Uniform Manifold Approximation and Projection.

the canonical components of major histocompatibility complex class II machinery regulated by IFNg signaling cGVHD KCs (Li et al, 2022). Collectively, these data suggest that epidermal KCs play an intimate role in propagating inflammation in cutaneous cGVHD.

Furthermore, cGVHD epidermal KCs showed decreased ability to protect against oxidative stress and regenerate, as evidenced by the decreased expressions of anti-redox and activator protein-1 pathway genes. Metallothioneins (MT1X and MT1E) as well as NFE2L2/NRF2 are known to upregulate and play a protective role in the epidermis in response to UV irradiation and ROS (Durchdewald et al, 2007; Marionnet et al, 2010; Schäfer et al, 2010). Selenoproteins also significantly contribute to oxidation resistance and tissue

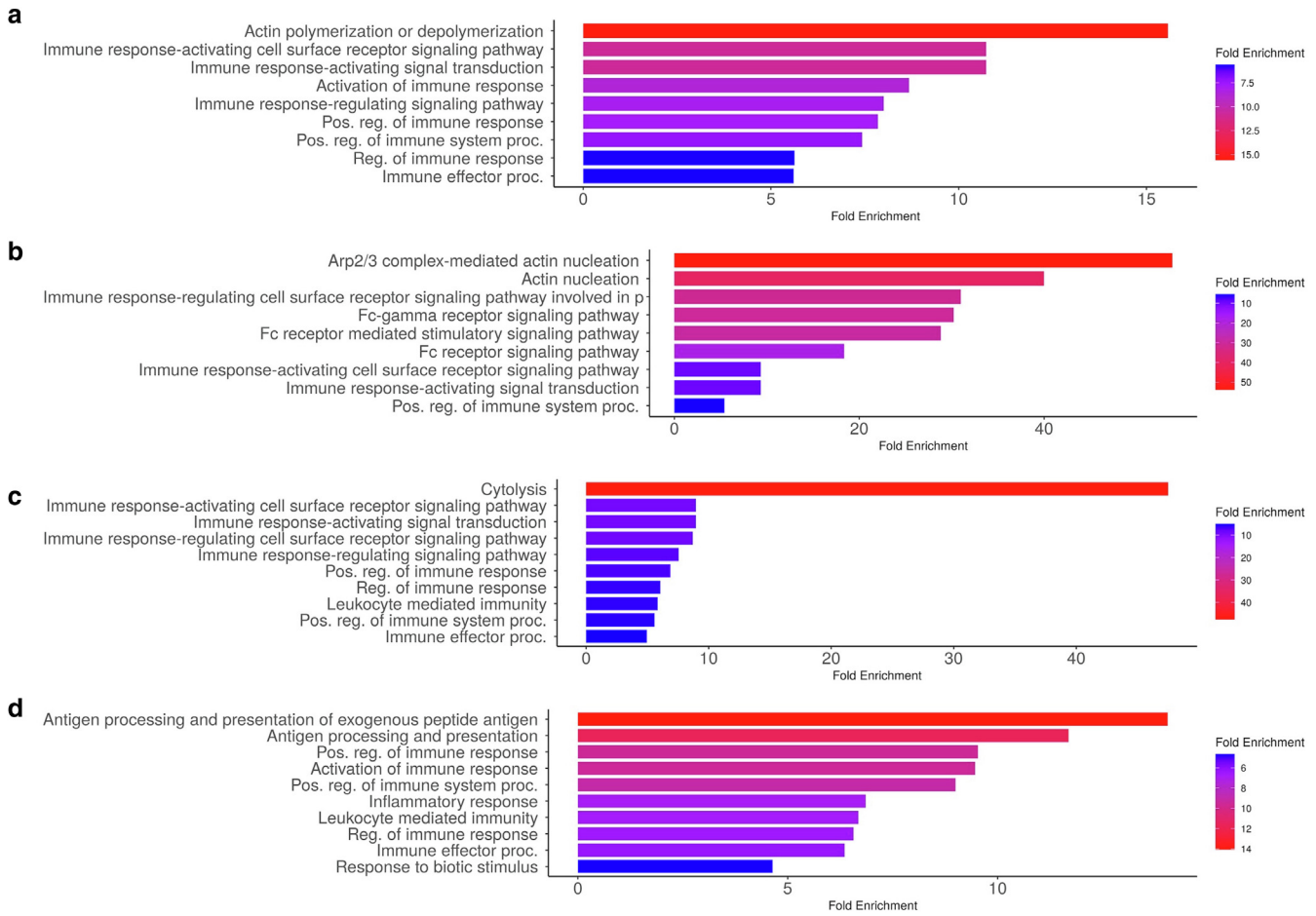

Figure 6. Gene ontology analysis of most highly expressed genes in cGVHD CD8$^+$ T cells, CD4$^+$ T cells, NK cells, and macrophages. (**a**) Gene ontology analysis of most highly expressed genes in cGVHD CD8$^+$ T cells. (**b**) Gene ontology analysis of most highly expressed genes in cGVHD CD4$^+$ T cells. (**c**) Gene ontology analysis of most highly expressed genes in cGVHD NK cells. (**d**) Gene ontology analysis of most highly expressed genes in cGVHD macrophages. cGVHD, chronic graft-versus-host disease.

homeostasis (Zhang et al, 2023). Interestingly, recent redox metabolomics studies showed that increased oxidative stress results in higher TNF-α levels and contributes to GVHD pathogenesis (Suh et al, 2014). In addition, activator protein-1 pathway transcription factors have been implicated in maintaining epidermal KC survival and differentiation and suppression of skin inflammation (Eckert et al, 2013; Zenz et al, 2005; Zhang et al, 2015). Loss of these transcription factors interferes with re-epithelialization in wounded skin, reduces FLG level, and produces an ichthyosis-related phenotype that is associated with increased levels of chemokines, including IFNg, CCL2, CCL5, CXCL9, and CXCL10 (Gangnuss et al, 2004; Young et al, 2017). Finally, there is a significant reduction in the expression of CXCL14, which has been implicated in skin homeostasis, antimicrobial functions, and antitumor immunity against human papillomavirus–positive head and neck cancer (Schaerli et al, 2005; Westrich et al, 2019) in both basal and suprabasal KCs. CXCL14 downregulation is observed in pathogenic states such as psoriasis, atopic dermatitis, and skin allergen sensitization (Lee et al, 2020; Maerki et al, 2009). Taken together, this in part explains the observed clinical phenotype of hyperkeratosis, delayed differentiation, reduced barrier integrity, increased sun sensitivity, and skin cancer risk in patients with cGVHD (Strong Rodrigues et al, 2018; Zampaolo et al, 2017).

Extensive research has highlighted the distinctive roles of immune cells, including lymphocytes, NK cells, and macrophages, in cGVHD (MacDonald et al, 2017). We observed increased percentages of CD8$^+$, CD4$^+$, and NK cells and increased percentage of M1-polarized macrophages in cGVHD. Not surprisingly, many of the most significantly upregulated genes in CD8$^+$ and CD4$^+$ clusters in cGVHD are highly involved in actin rearrangement, antigen recognition, and T-cell activation. Although most targets have not been previously studied in cGVHD, IFN-responsive genes, including *PSMB9,* signal transducer and activator of transcription 1 gene *STAT1*, and *IFITM1*, have been implicated in T-cell subsets in cutaneous GVHD (Hakim et al, 2016; Piper et al, 2022; Zouali et al, 2022). Although less well-studied, NK cells are thought to play dichotomous roles in the development of GVHD (Simonetta et al, 2017). On one hand, NK cell alloreactivity against T cells and antigen-presenting cells plays a protective role against aGVHD. On the other hand, IL-2–activated NK cells produce IFNg and TNFα and promoted aGVHD in a xenogeneic model (Xun et al, 1995).

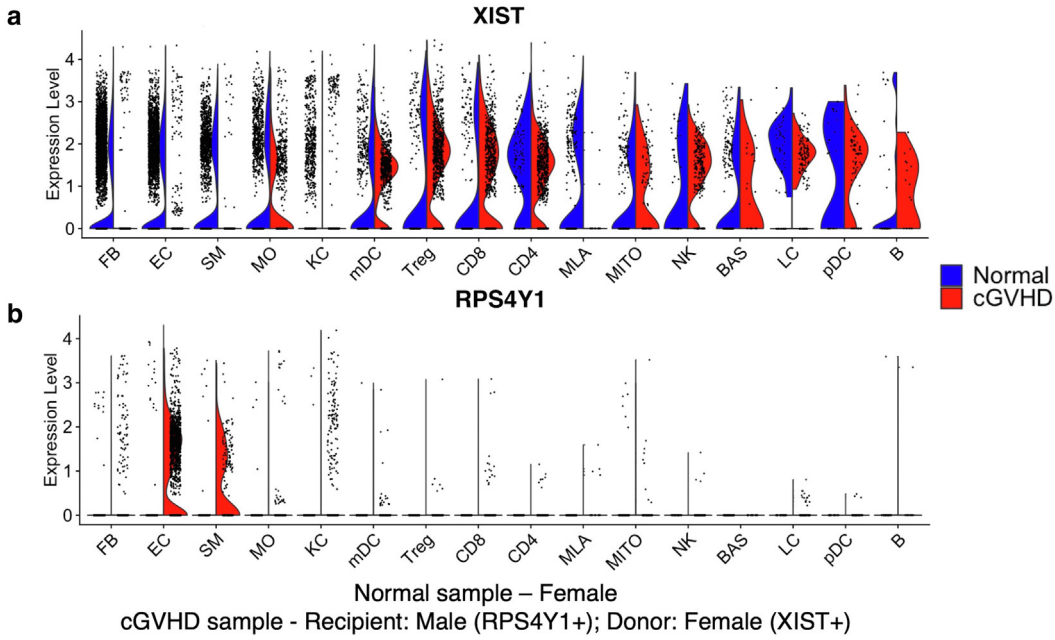

Figure 7. Sex-based gene analysis delineates cellular origin of nonimmune and immune cell populations in cGVHD. Expression of (**a**) XIST and (**b**) RPS4Y1 across all cell types in dermal normal versus cGVHD skin. Normal sample is from a female patient. GVHD sample is from a male recipient of female bone marrow donor. MO denotes macrophages, FB denotes fibroblasts, MITO denotes mitotic cells, BAS denotes basophils, SM denotes smooth muscle, MLA denotes melanocytes, and B denotes B cells. cGVHD, chronic graft-versus-host disease; EC, endothelial cell; KC, keratinocyte; LC, Langerhans cell; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; Treg, regulatory T cell.

Our data showed that cutaneous cGVHD NK cells are highly activated and cytolytic, as evidenced by the upregulation of granzyme A, CD53, activating receptor KLRF1, PRF1, and IFITM1 (Del Zotto et al, 2017; Hanna et al, 2004). Further mechanistic studies are needed to elucidate the specific functions of this cluster of NK cells.

Macrophages have been found to play a crucial role in promoting fibrosis and amplifying inflammatory responses of other immune cells in GVHD (Brüggen et al, 2014; MacDonald et al, 2017). Although previous studies have linked M1 macrophages with aGVHD and M2 macrophages with cGVHD, this can greatly vary in different phases, tissues, and conditions of GVHD (Hong et al, 2020). In addition, most reports linking M2 macrophages with cGVHD did so by measuring M2 markers in the peripheral blood or by altering the polarization status of macrophages prior to transplantation (Alexander et al, 2014; Inamoto et al, 2017). In contrast, our data demonstrated a reduction in M2 compared with that in M1 macrophages in cGVHD and that cGVHD macrophages appear more inflammatory as evidenced by the higher expressions of signal transducer and activator of transcription 1, CXCL9, CXCL10, and CD16A (FCGR4A). Interestingly, we also found that this group of inflammatory cGVHD macrophages secrete CCL18, a profibrotic chemokine (Ossorio et al, 2018; Pochetuhen et al, 2007). Cocho et al (2015) found that CCL18 upregulation is a prominent marker for the dry eyes associated with GVHD. Overall, this suggests that the dichotomous paradigm of associating M1 macrophages with aGVHD and M2 macrophages with cGVHD could be overly simplistic, and further research is needed to better delineate how macrophage polarization affects cGVHD in the skin.

Finally, our data appear to be consistent with previous reports that immune cells in cGVHD skin are mostly donor derived (Santos E Sousaet al, 2018). Nevertheless, a small percentage of host-derived T cells may be present and contributory to pathogenesis (Strobl et al, 2020). Although it is challenging to clearly differentiate between infiltrating and resident immune cells with our current data, the presence of few dots measuring the expression of RPS4Y1 in the cGVHD sample suggests that they may be resident immune cells of recipient origin remaining in the skin. We also found that skin structural cells, especially endothelial cells and smooth muscle cells, have high RPS4Y1 expressions, indicating male recipient origin. However, the lower expressions of RPS4Y1 in fibroblasts and KCs raised the provocative question of whether some structural cell clusters are partially from the female donor or intrinsically lost expression of Y-chromosome genes. Several reports have highlighted that structural cell, such as KCs, endothelial cells, and fibroblasts, can be donor in origin, and the degree of chimerism correlates with GVHD disease severity (Murata et al, 2007; Niino et al, 2005; Ogawa et al, 2005; Willemze et al, 2009). This could also support the notion that donor-derived hematopoietic stem cells may contain pluripotent stem cells.

In conclusion, our dataset revealed a complex nonimmune and immune landscape of nonfibrotic cutaneous cGVHD. To our knowledge, this study utilized scRNA-seq to investigate the complex transcriptomic network in the skin lesion of cGVHD. Although our study was limited by small sample size and the lack of matched nonlesional skin, it nonetheless provided mechanistic insights for downstream analysis. In addition, although the sex-mismatched donor recipient provided opportunity for donor cell identification, it may also contribute to some of the differential gene expressions. Future studies are needed to further elucidate the disease-promoting mechanisms of genes of interests and areas of therapeutic exploration.

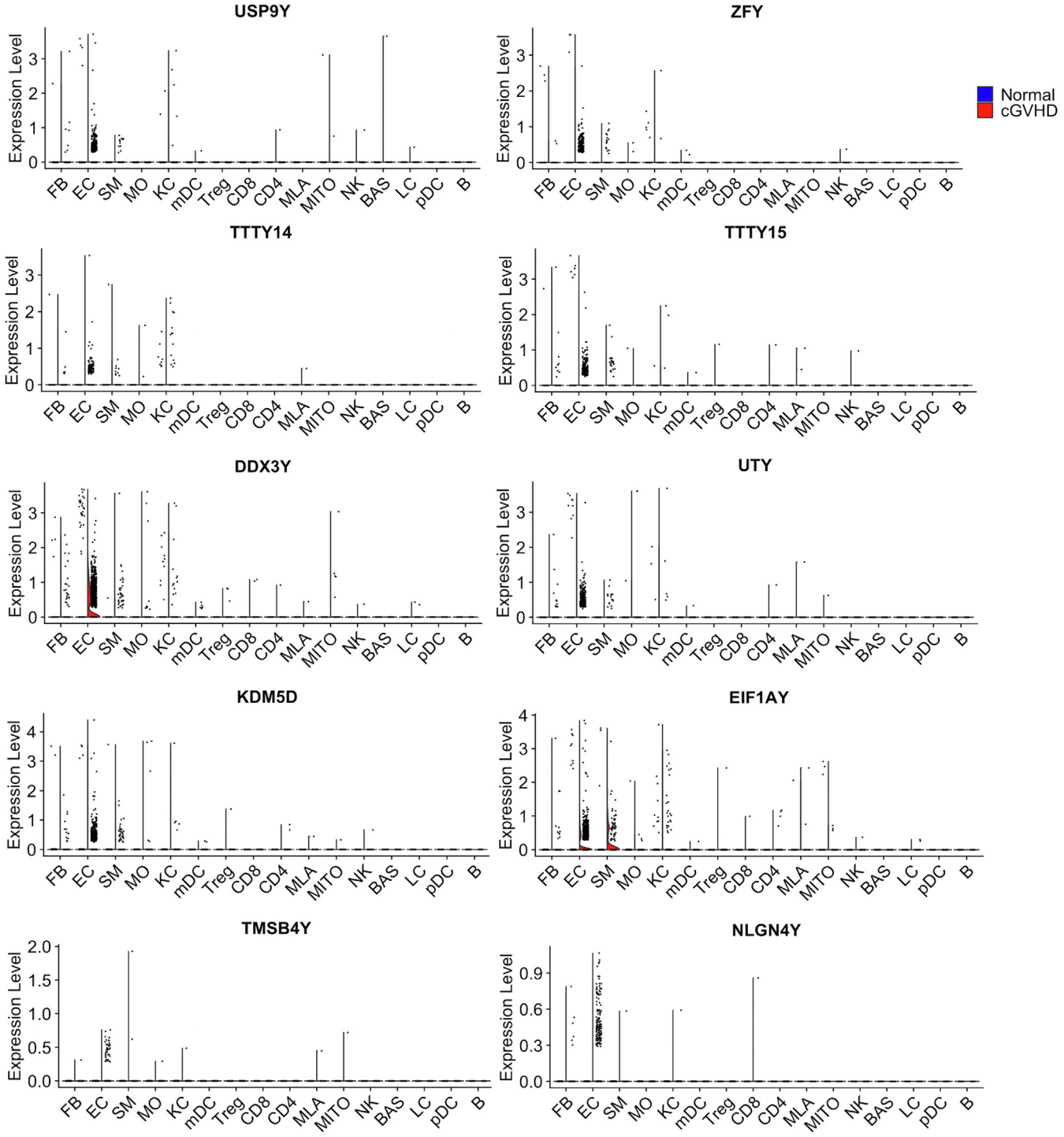

Figure 8. Expressions of Y-chromosome–specific genes in dermal cells. Expressions of *USP9Y*, *ZFY*, *TTTY14*, *TTTY15*, *DDX3Y*, *UTY*, *KDM5D*, *EIF1AY*, *TMSB4Y*, and *NLGN4Y* across all cell types in dermal normal versus cGVHD skin. MO denotes macrophages, FB denotes fibroblasts, MITO denotes mitotic cells, BAS denotes basophils, SM denotes smooth muscle, MLA denotes melanocytes, and B denotes B cells. cGVHD, chronic graft-versus-host disease; EC, endothelial cell; KC, keratinocyte; LC, Langerhans cell; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; Treg, regulatory T cell.

MATERIALS AND METHODS

Tissue preparation from fresh human skin biopsies

Lesional punch biopsy was obtained from a patient with untreated lichen planus–like nonfibrotic cGVHD of the skin in accordance with an institutionally approved institutional review board protocol with patient consent. Surgically discarded abdominal plastic skin was obtained through an exempt institutional review board protocol. After washing twice briefly in cold PBS, the freshly obtained skin biopsies were incubated in 4 ml Dispase (1 unit/ml, Thermo Fisher Scientific) at 4 degrees overnight. Next day, the epidermis and dermis were separated from each other and chopped into small pieces with a pair of scissors. The epidermal pieces were digested with 0.25% trypsin for 15 minutes, and the dermal pieces were digested with collagenase B (1 mg/ml, Sigma-Aldrich) for 1 hour

followed by addition of 0.25% trypsin and incubation for another 30 minutes in 37 °C water bath with agitation at 240 r.p.m. The digestion mixtures were neutralized in 10% fetal bovine serum in DMEM and filtered through 70-μm and then 40-μm strainers. The cell suspension was centrifuged at 300*g* for 5 minutes, washed 3 times, and then resuspended in 0.04% BSA at 1000 cells/μl.

scRNA-seq workflow

We used 10X Genomics Chromium assay for single-cell gene expression analysis (Kanayama et al, 2017). After quality check, the cell suspensions prepared as described earlier were sent to Duke Molecular Genomics core for bar coding, cDNA library construction, and subsequent sequencing on the NovaSeq6000 Illumina sequencing platform at 50 K reads/cell. The primary analytical pipeline for the scRNA-seq analysis followed the instructions from 10X Genomics. Briefly, we demultiplexed raw base call files generated by Illumina sequencers into FASTQ files, upon which alignment to the appropriate reference transcriptome (GRCh38-3.0.0), filtering, barcode counting, and unique molecular identifier counting were performed using 10X Cell Ranger software, version 3.1.0. The company protocol uses the Chromium cell barcode to generate feature-barcode matrices encompassing all cells captured in each library. The secondary statistical analysis was performed using an R package in Seurat (version 4.2.1), which performs quality control and subsequent analyses on the feature-barcode matrices produced by Cell Ranger. In Seurat, data were first normalized and scaled after basic filtering for minimum gene and cell observance frequency cut offs. We then closely examined the data and performed further filtering on the basis of a range of metrics to identify and exclude possible multiplets (ie, instances where more than 1 cell was present and sequenced in a single emulsified gel bead). The removal of further technical artifacts was performed using regression methods to reduce noise.

After quality control procedures and integration were complete, we performed linear dimensional reduction calculating principal components using the most variably expressed genes in our dataset. Library size and/or the numbers of genes expressed across subsets of cells may necessitate the restriction of cells upon which the variably expressed genes were selected for inclusion when calculating principal components. Significant principal components for downstream analyses were determined through methods mirroring those implemented by Macosko et al (2015), and these principal components were carried forward for 2 main purposes: to perform cell clustering and to enhance visualization (Macosko et al, 2015; Satija et al, 2015). Cells were grouped into an optimal number of clusters for de novo cell-type discovery using Seurat's FindNeighbors and FindClusters functions. Graph-based clustering approaches with visualization of cells were achieved using Uniform Manifold Approximation and Projection technique, which reduced the information captured in the selected significant principal components to 2 dimensions.

Differential expression of relevant cell marker genes was visualized on Uniform Manifold Approximation and Projection plot to reveal specific individual cell types. Additional downstream analyses included examining the cellular distribution of a priori genes of interest, closer examination of genes associated with cell clusters, and the refined clustering of cells to identify further resolution of cell types, in addition to comparing differences between experiments of different states. Combining multiple libraries relies on the integration strategy and allows for calculation of differential expression, not only between clusters but also within clusters across libraries (Stuart et al, 2019). Gene expression plots were created using Seurat's DoHeatmap, VlnPlot, DotPlot, and FeaturePlot functions. Gene ontology analysis was conducted with ShinyGO 0.76.3 (Ge et al, 2020).

Immunofluorescence staining

Formalin-fixed, paraffin-embedded tissue was obtained from archived specimens of patients with cutaneous cGVHD. Anonymized, unaffected excess skin from surgical excision ellipses of patients without hematopoietic stem cell transplantation were used as normal controls. Tissue was sectioned at 5-μm thickness onto positively charged glass slides and stained using a typical manual immunofluorescence staining protocol. Briefly, formalin-fixed, paraffin-embedded slides were baked for 30 minutes at 60 °C and deparaffinized in xylene. Antigen retrieval was performed using a steamer for 25 minutes using Tris-EDTA (pH 9) buffer. Slides were fixed in 10% neutral buffered formalin for 15 minutes. A single slide for each sample was stained for 1 hour at room temperature with a pool of fluorescently conjugated antibodies against K16 (Novus Biological, catalog number NBP3-08692AF647) and S100A7 (Novus Biological, catalog number NB100-56559AF532) and a syto-13 dye (Thermo Fisher Scientific, catalog number S7575) to visualize DNA within nuclei. Stained slides were scanned on the GeoMx DSP instrument (NanoString Technologies) in scan-only mode (ie, no collection) Shown are the scanned images for K16 and S100A7 in their individual channels.

ETHICS STATEMENT

The study was approved by the Duke University Medical Center Institutional Review Board. Written, informed consent was obtained from all patients included in this study. Participants consented to the publication of the clinical image.

DATA AVAILABILITY STATEMENT

The datasets presented in this study are available in the National Center for Biotechnology Information repositories (accession number GSE191335) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE191335).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: ARC, JYZ; Data Curation: YJJ, EH, HBP, AM, MAS; Formal Analysis: AJP, VJ; Funding Acquisition: ARC, JYZ; Investigation: AJP, ARC; Methodology: ARC, YJJ, SG, JYZ; Project Administration: ARC, JYZ; Resources: ARC, SG, JYZ; Software: AJP, VJ; Supervision: ARC, SG, JYZ; Validation: AJP, JYZ; Visualization: AJP, VJ; MAS; Writing — Original Draft Preparation: AJP; Writing — Review and Editing: ARC, JYZ

DECLARATION OF GENERATIVE ARTIFICIAL INTELLIGENCE (AI) OR LARGE LANGUAGE MODELS (LLMs)

The author(s) did not use AI/LLM in any part of the research process and/or manuscript preparation.

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