RNA sequencing of chronic GVHD skin lesions defines shared and unique inflammatory pathways characterizing lichen planus and morphea

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Key Points

- RNA sequencing suggests the potential role of TREM-1 in lichen planus cutaneous cGVHD pathogenesis, thus providing new treatment target.

- The results were confirmed at a protein level by immunohistochemical testing and real-time quantitative PCR.

Cutaneous involvement of chronic graft-versus-host disease (cGVHD) has a wide range of manifestations including a lichenoid form with a currently assumed mixed Th1/Th17 signature and a sclerotic form with Th1 signature. Despite substantial heterogeneity of innate and adaptive immune cells recruited to the skin and of the different clinical manifestations, treatment depends mainly on the severity of the skin involvement and relies on systemic, high-dose glucocorticoids alone or in combination with a calcineurin inhibitor. We performed the first study using RNA sequencing to profile and compare the transcriptome of lichen planus cGVHD (n = 8), morphea cGVHD (n = 5), and healthy controls (n = 6). Our findings revealed shared and unique inflammatory pathways to each cGVHD subtype that are both pathogenic and targetable. In particular, the deregulation of IFN signaling pathway was strongly associated with cutaneous cGVHD, whereas the triggering receptor expressed on myeloid cells 1 pathway was found to be specific of lichen planus and likely contributes to its pathogenesis. The results were confirmed at a protein level by performing immunohistochemistry staining and at a transcriptomic level using real-time quantitative polymerase chain reaction.

Introduction

Graft-versus-host disease (GVHD) is a severe complication and a major cause of nonrelapse mortality following allogenic hematopoietic stem cell transplantation.[1-3] Cutaneous chronic GVHD (cGVHD) includes a lichenoid form (lichen planus [LP]-like eruptions and poikiloderma) and a sclerotic form
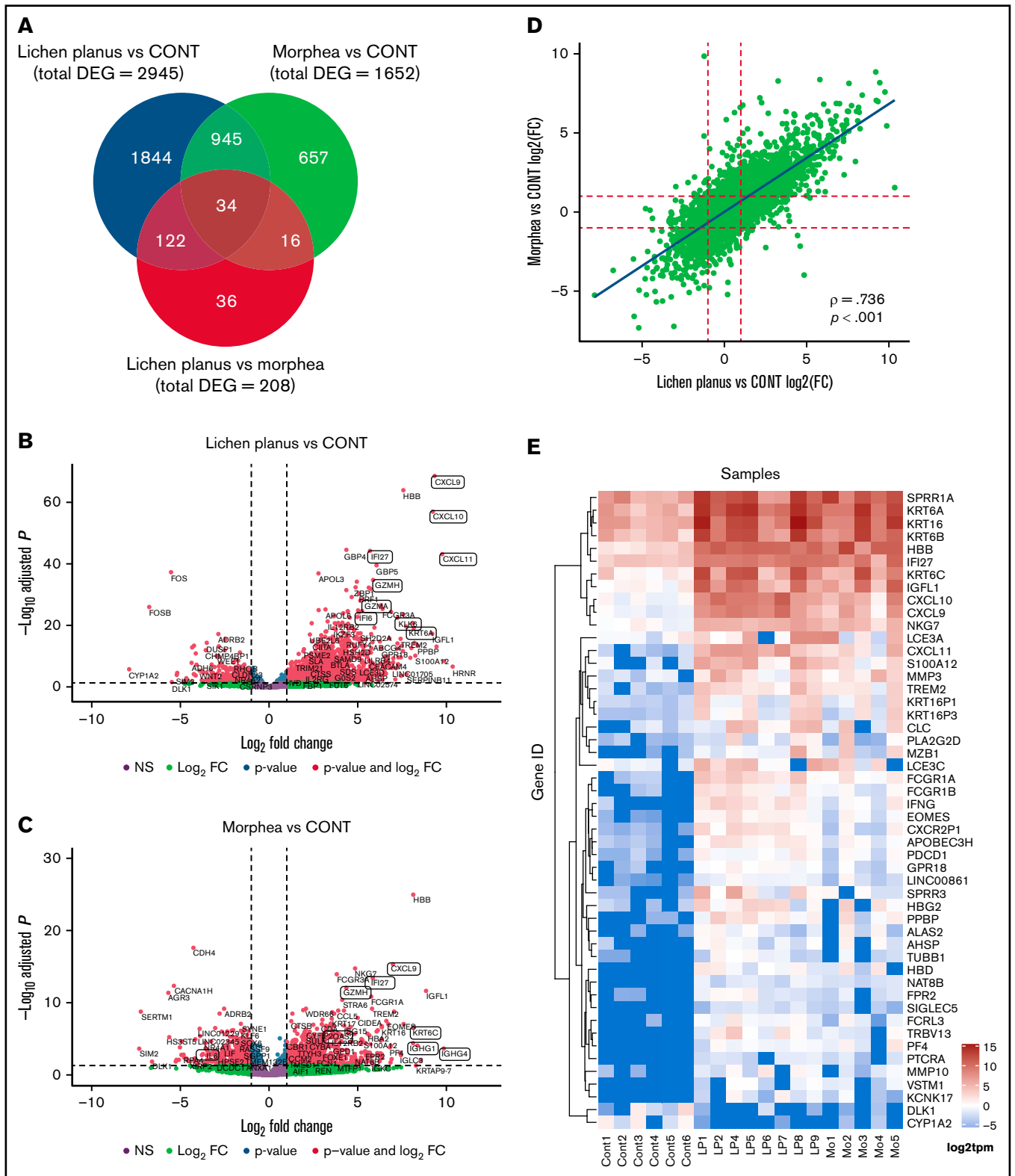
Figure 1. Transcriptomic signature of LP and morphea cGVHD skin lesions. (A) Venn diagram showing the overlap between DEGs in lesional skin of cGVHD as compared with healthy controls (CONT). LP vs CONT in blue, morphea vs CONT in green, and LP vs morphea in red. (B-C) Volcano plot with log2FC and −log10 P values for the DEGs between LP (n = 8) (B) and morphea (n = 5) (C) cGVHD skin lesions vs CONT (n = 6). The most upregulated genes are toward the right, the most downregulated genes are toward the left, and the most statistically significant genes are toward the top. Genes of interest are encircled. (D) Scatterplot of the expression FCs of DEGs in lesional cGVHD. (E) Heatmap of the 50 most upregulated DEGs shared between LP and morphea, defined by contrasting lesional skin of cGVHD and CONT.

(morphea-like, diffuse sclerosis or lichen sclerosus–like features).[4] Cutaneous cGVHD is mediated by the activation of alloreactive donor T cells, a defect of CD4$^+$Foxp3$^+$CD25$^+$ regulatory T cells (Tregs),[5] a profound disruption of B-cell homeostasis, and pathological tissue repair with fibrosis.[3] Although lichenoid cGVHD has been associated with a mixed Th1/Th17 signature and sclerotic cGVHD with a Th1 signature, other T-cell subsets may contribute to its pathogenesis.[6,7] Despite pathogenic and clinical heterogeneity, treatment of cGVHD relies mainly on the severity of the skin involvement. Standard first-line therapy for moderate to severe cutaneous cGVHD relies on systemic, high-dose glucocorticoids alone or in combination with a calcineurin inhibitor. Therefore, studies providing new insights into the mechanisms of skin cGVHD subtypes are mandatory. To address this issue, we performed an RNA sequencing study of the transcriptomes of LP and morphea cGVHD skin lesions. The results were confirmed at a protein level by performing immunohistochemistry staining and at a transcriptomic level using real-time quantitative polymerase chain reaction (RT-qPCR).

Methods

Refer to supplemental Methods for details.

RNA sequencing

RNA from 13 patients with cGVHD and 6 healthy controls (CONT) were extracted and sequenced on the Illumina sequencing platform.

Immunohistochemical testing

Archival skin samples from LP cGVHD (n = 3), morphea cGVHD (n = 3), and CONT (n = 3) were processed for immunohistochemical testing to detect triggering receptor expressed on myeloid cells 1 (TREM-1) signaling. Abdominal aorta atherosclerosis (n = 3) was used as positive control.

RT-qPCR

RT-qPCR was performed to measure TREM-1 messenger RNA (mRNA) expression in LP cGVHD (n = 6), morphea cGVHD (n = 5), and CONT (n = 5).

Data analysis

Differential expression (DE) analyses were conducted using DESeq2 R package in R (Benjamini-Hochberg adjusted *P*-value <5% and |log2 (fold-change FC)| ≥ 1). Functional analyzes were performed using gene set enrichment analysis (GSEA) on all genes and ingenuity pathway analysis (IPA) software on differentially expressed genes (DEGs).

Study approval

This study was reviewed and approved by the local ethics committee at Comité de Protection des Personnes (CPP) Paris Ile de France IV (Paris, France) with informed consent obtained from all patients (Saint-Louis Hospital, Paris, France, between January 2017 and February 2019).

Results and discussion

Skin biopsies were obtained from patients diagnosed with LP (n = 8) and morphea (n = 5) cGVHD according to the National Institutes of Health consensus criteria with histologic confirmation[8] and were compared with CONT (n = 6). At the time of skin sampling, patients had no immunosuppressive treatment (n = 7) or low residual immunosuppressive treatment background (n = 6) (see supplemental Table 1 for complete clinical and molecular features collected for each patient/sample).

Although both the first 2 and the first 3 principal component analysis of the whole transcriptome could separate the CONT from the lesional skin samples, neither of them could provide perfect separation between the 2 subtypes of cutaneous cGVHD (supplemental Figure 1), suggesting that these 2 manifestations of cutaneous cGVHD have significant overlap in their molecular composition.

LP cGVHD transcriptome analysis

The DE analysis for LP cGVHD vs CONT identified 2945 DEGs (up/down = 2284/661; Figure 1A-B; supplemental Table 2A). Toll-like receptors (TLRs) such as TLR2, TLR7, and TLR8 were found upregulated. TLRs are known to play a fundamental role in the activation of innate immunity and in the initiation phase of cGVHD through production of interferon (IFN).[2,9] Upregulated DEGs also included genes associated with IFN signaling and Th1 pathway such as STAT4, Tbet, several members of the TNFα superfamily, and their receptors, as well as IL12B and IL12 receptors. Notably, 3 relevant chemokine DEGs (CXCL9, CXCL10, and CXCL11) were highly expressed (log2FC > 9). These IFNγ-inducible chemokines are known to induce a chemotactic response in Th1 and natural killer cells expressing CXCR3, which was also found highly upregulated (log2FC = 4.718).[10-12] CXCL9 has been proposed as a serum prognostic biomarker in cGVHD and was found to be expressed in lesional tissues.[13,14] Expression of TNFSF13B (also known as BAFF) was also found to be upregulated. Increased serum BAFF level is associated with B-cell dysregulation in cGVHD.[15-17] Macrophage surface markers such as IFN-γ–inducible allograft inflammatory factor-1 (AIF1), CD163, and CD68 were found to be upregulated, suggesting that macrophages are increased in dermis of lichenoid cGVHD.

Morphea cGVHD transcriptome analysis

When comparing morphea cGVHD vs CONT, 1652 DEGs were identified (up/down = 1281/371; Figure 1A-C; supplemental Table 2B). DEGs involved in the IFN signaling (CXCL9, CXCL10, IFNG, MX1, and IFI27), T-cell activation (PRKCQ, CD3G, CD3D, and ITGAL), and Th1 pathway (STAT1, IL12RB1, and IL12RB2), as well as genes encoding for Th1 chemokines (CCL5), were highly expressed.

Common and specific molecular features of LP and morphea cGVHD

IFN signature in cutaneous cGVHD. Type 2 and type 2 IFNs play a role in the initiation and persistence of cGVHD.[18] To confirm

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Figure 1 (continued) Levels of expression for most of the DEGs differed in the 2 pathologies. Data are presented as log2 transcripts per million (TPM) expression value. Expression values are depicted according to the color scale. TPM values were estimated for each experimental dataset. A pseudo-count of 0.001 was added to each transcript before log2 transformation. Annotations are colored based on the TPM property, ranging from blue for 0, through to white for the mean TPM, up to red for the highest TPM for any gene in the sample.
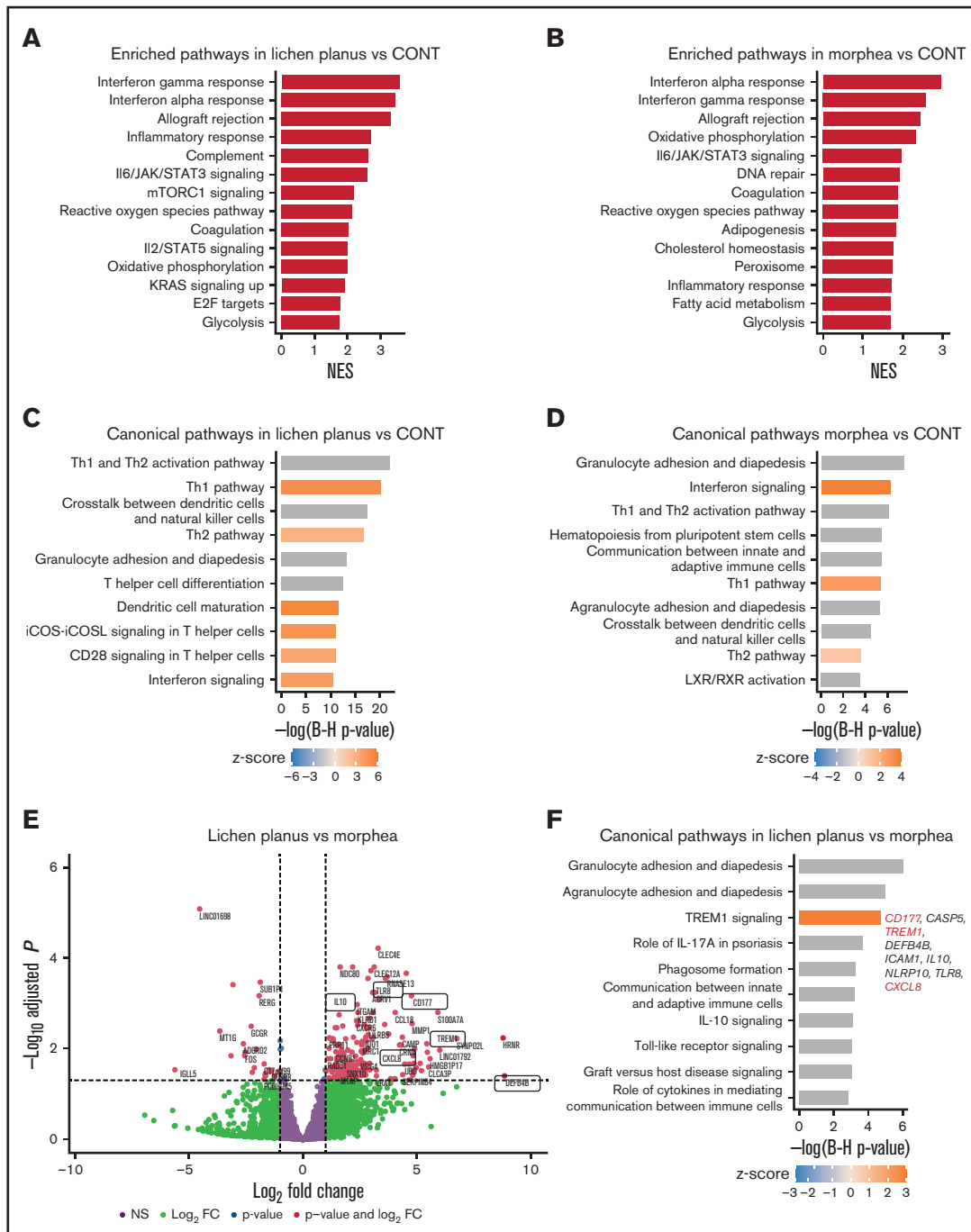
Figure 2. Common and specific molecular features of LP and morphea cGVHD. GSEA for "hallmark gene set" for LP (n = 8) vs healthy controls (CONT, n = 6) (A) and for morphea (n = 5) vs CONT (n = 6) (B). (C-D) Relevant canonical pathways using IPA differentiating (C) LP (n = 8) vs control (CONT) and (D) morphea (n = 5) cGVHD skin lesions VS CONT (n = 6). Pathways identified are represented on the y-axis. The x-axis corresponds to the −log of the *P* value. Orange, z-score value is positive (>0), pathway is predicted to be activated; blue, z-score value is negative (<0), pathway is predicted to be inhibited; gray, no activity pattern available. (E) Volcano plot with log2FC and −log10 *P* values for the DEGs comparing LP (n = 8) vs morphea (n = 5) chronic cGVHD skin lesions. The most statistically significant genes are toward the top and are depicted in red. Genes of interest are encircled. (F) Relevant canonical pathways using IPA differentiating LP (n = 8) vs morphea (n = 5) cGVHD skin lesions, with upregulated DEGs in the TREM1 signaling pathway. Pathways identified are represented on the y-axis. The x-axis corresponds to the −log of the *P* value. Orange, z-score value is positive (>0), pathway is predicted to be activated; blue, z-score value is negative (<0), pathway is predicted to be inhibited; gray, no activity pattern available.

the role of IFN signature in cutaneous cGVHD, we looked at the shared genes between LP and morphea cGVHD. We identified 979 DEGs shared between LP and morphea defined by contrasting lesional skin of cGVHD and CONT (Figure 1A; supplemental Table

2C), suggesting that these 2 manifestations of cutaneous cGVHD have significant overlap in their molecular signature. Furthermore, the comparison between the magnitudes in dysregulation in the lesional skin in both subtypes showed a strong correlation (Figure 1D,
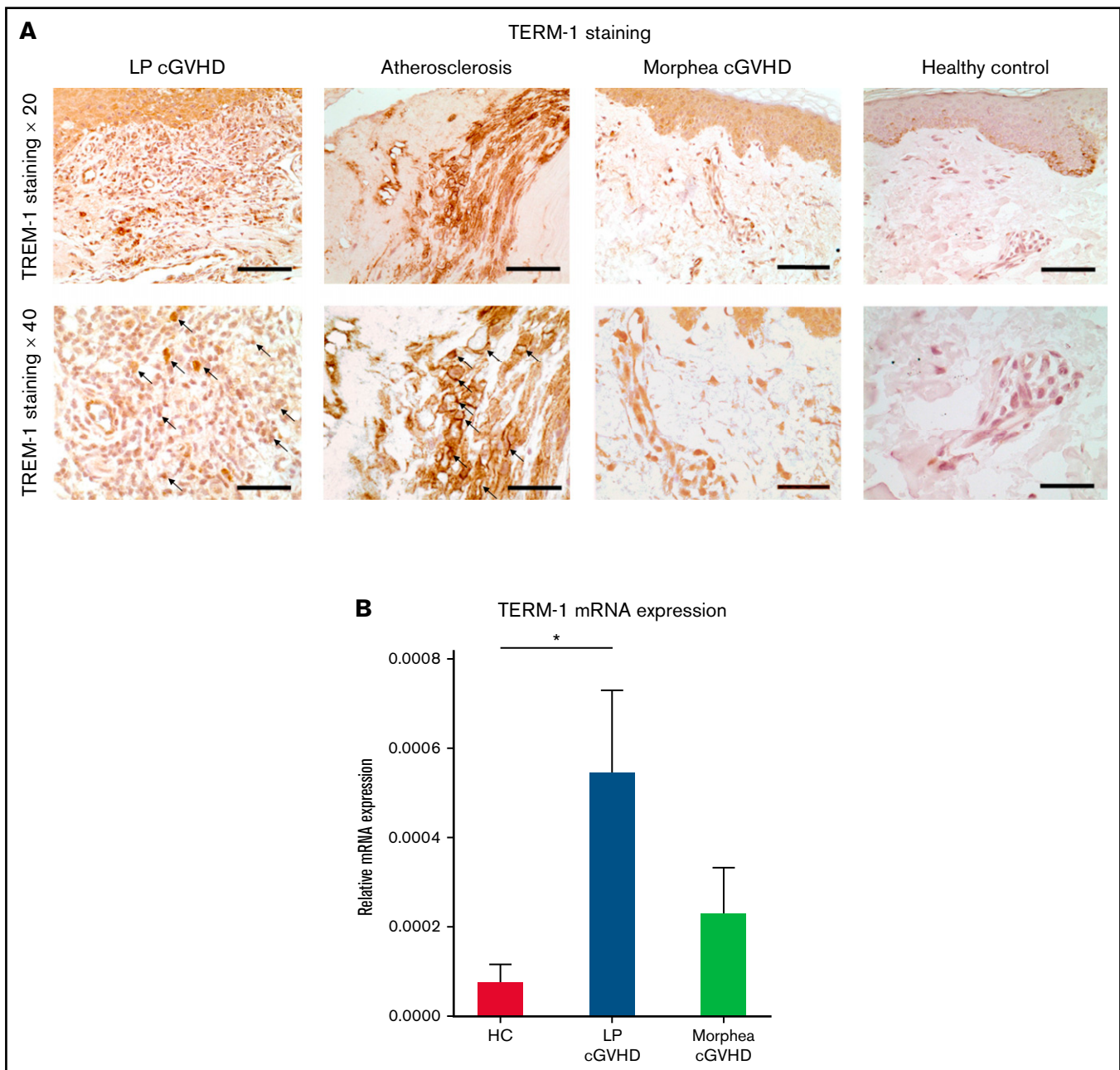
Figure 3. IHC testing and RT-qPCR in patients with LP and morphea cGVHD. (A) TREM-1 IHC labeling of skin biopsies of LP (left panels) chronic graft-versus-host disease (cGVHD), morphea cGVHD (middle right panels), and healthy controls (right panels). Abdominal aorta atherosclerosis was used as positive control. Scale bar, 200 mm for panels at ×20; scale bar, 100 mm for panels at ×40; arrows indicate some of the TREM-1⁺ macrophages. (B) Bar graph showing TREM-1 mRNA expression by using RT-qPCR in healthy controls (n = 5), lichen cGVHD (n = 6), and morphea cGVHD (n = 5). *$P < .05$.

Spearman $\rho = .736$, $P < .001$). Among the shared genes, a robust upregulation of specific type 1 IFN-induced genes (eg, CXCL9, CXCL10, CXCL11, and MX1) and of IFN-induced genes related to antigen processing and presentation (MSR1, PSMB9, and PSME2), antiviral and antibacterial function (APOL1 and IFI35), and IFN receptor signaling (STAT1) were found (Figure 1E).

GSEA analyses of normalized data based on the normalized enrichment score (≥1.5) identified 2 gene sets enriched in both LP and morphea that are related to IFNγ and α responses (Figure 2A-B). IFNγ expression and a Th1/Th17 polarization has been reported in human cGVHD.[6]

Most of the canonical pathways identified in both datasets (using IPA) were involved in the immune response and inflammation. In LP cGVHD, IFN signaling as well Th1 and Th2 pathways were predicted to be activated (Figure 2C). Although lichenoid cGVHD is currently defined by a mixed Th1/Th17 signature, a recent study

suggested that the Th2 pathway may also be involved.[6,7] In morphea cGVHD, IFN signaling as well as Th1 and Th2 pathways were also predicted to be activated (Figure 2D).

Identification of TREM-1 signaling pathway in LP cGVHD.

A total of 208 DEGs were identified when comparing LP to morphea (up/down = 183/25) (Figure 2E). Among the 10 most significant canonical pathways identified using IPA (Figure 2F), TREM-1 signaling pathway was predicted to be activated with upregulation of CASP5, TREM-1, CXCL8, DEFB4B, ICAM1, IL10, NLRP10, and TLR8. TREM-1 (CD354) is a cell surface receptor mainly expressed on myeloid cells (monocytes, macrophages, and granulocytes), known to amplify the inflammatory response by acting synergistically with TLRs.[19,20] TREM-1 constitutively associates with DAP12[21] for induction of intracellular signals leading to production of proinflammatory cytokines (eg, IL-8/CXCL8 and TNFα), chemokines, and cell-surface molecule.[22] CD177 that has been previously suggested as a potential ligand for TREM-1 was found to be upregulated in our DEGs (with a log2FC of 4.77) when comparing LP to morphea.[22,23] Several other candidates were suggested as potential ligands such as HMGB, Hsp70, and PGLYRP1.[23]

IHC testing and RT-qPCR in patients with LP and morphea cGVHD.

To further evaluate TREM-1 activation in LP cGVHD, we performed immunohistochemical (IHC) testing for TREM-1 on archival skin lesion samples from LP, morphea, and CONT (Figure 3A). Abdominal aorta atherosclerosis was used as positive control. In LP skin lesion samples, TREM-1 was detected in several mononuclear cells among inflammatory cells in the dermis. The labeled cells were consistent with macrophages' morphology. TREM-1 was also strongly expressed in macrophages of the lipid core in atherosclerosis. In comparison, no TREM-1 signal was detected in biopsies obtained from morphea cGVHD and healthy controls. Overexpression of TREM-1 in LP cGVHD may be due to either higher abundance of TREM-1$^+$ macrophages, without induction of TREM-1 expression on per cell level, or due to associated enhanced expression of TREM-1 gene.

Furthermore, increased expression of TREM-1 in LP cGVHD was validated by means of RT-qPCR. Although TREM-1 showed increased mRNA expression in morphea cGVHD, significantly higher expression ($P = .044$) was observed in LP cGVHD (Figure 3B; supplemental Table 3).

Increased expression of TREM-1 has been reported in psoriasis and atopic dermatitis.[24,25] Psoriasis patients showed a decrease in TREM-1 expression following narrow-band ultraviolet B, anti-TNF, and anti-IL17 treatments.[24] Similarly, in atopic dermatitis patients subjected to a 12-week treatment with cyclosporine, a significant decrease in TREM-1 mRNA levels was observed.[25] Preclinical studies showed that TREM-1 inhibition, via synthetic soluble TREM-1 protein mimickers (LP17 and LR12), is effective in treating or preventing inflammatory disorders.[23] Altogether, these data suggest that TREM-1 could be a promising therapeutic target in LP cGVHD.

Our findings suggest that despite distinct clinical features of LP and morphea, these manifestations of cutaneous cGVHD have significant overlap in their molecular transcriptional structure. Additional studies using samples from posttransplant patients without cGVHD for comparison might provide further information about the molecular pathogenesis of cutaneous cGVHD. Finally, important distinctions were drawn between LP and morphea. We have uncovered the potential novel involvement of TREM-1 in the pathogenesis of LP cGVHD, thus providing new insights for future treatment targets.

Authorship

Contribution: H.Z. analyzed the data and participated in manuscript preparation; J.L. collected the samples, carried out the experiments, and participated in manuscript preparation; A.C. analyzed the data, interpreted the results, and had a leading role in manuscript preparation; C.B., H.l.B., L.M., and M.M. provided advice and technical assistance and participated in manuscript preparation; C.G. carried out some of the experiments and commented on the manuscript; D.M., G.D., M.R., A.d.M., R.A., C.C., T.M., A.B., M.R., F.S.d.F., R.P.d.l.T., P.B., H.A.-O., M. Battistella., M.J., M. Bagot, and G.S. provided patient samples and commented on the manuscript; and J.-D.B., G.S., and J.-F.D. coordinated the study, developed the study design, analyzed the results, and participated in manuscript preparation.

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