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Sclerotic-Type Cutaneous Chronic Graft-Versus-Host Disease Exhibits Activation of T Helper 1 and OX40 Cytokines

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CONFLICT OF INTEREST

PF has served as a consultant for Eli Lilly. EG-Y has served as a consultant for AbbVie, Almirall, Amgen, Allergan, Asana Bioscience, Aslan Pharmaceuticals, AstraZeneca, Biolojic Design, Boerhinger-Ingelhiem, Bristol Meyers Squibb, Cara Therapeutics, Celgene, Concert, Connect Pharma, Dermira, DBV Technologies, DS Biopharma, EMD Serono, Escalier, Evidera, Galderma, Gate Bio, Genentech, Glenmark, Incyte, Inmagene, Janssen Biotech, Kyowa Kirin, LEO Pharmaceuticals, Lilly, Merck, Mitsubishi Tanabe, Novartis, Pfizer, Q32 Bio, RAPT, Regeneron, Sanofi, SATO, Siolta, Target, UCB, Union Therapeutics, and Ventyx; a member of advisory boards of AbbVie, Allergan, Asana Bioscience, Celgene, DBV, Dermavant, Dermira, Dexcel, Escalier, Galderma, Glenmark, Kyowa Kirin, LEO Pharma, Lilly, Novartis, Pfizer, Regeneron, and Sanofi; and a recipient of research grants from AbbVie, Amgen, AnaptysBio, AntibioTx, Asana Bioscience, Aslan, Boehringer-Ingelheim, Bristol Meyers Squibb, Cara Therapeutics, Celgene, Concert, DBV, Dermavant, DS Biopharma, Galderma, Glenmark, GSK, Incyte, Innovaderm, Janssen Biotech, Kiniksa Pharma, Kyowa Kirin, LEO Pharmaceuticals, Lilly, Medimmune, Sanofi, Sienna Biopharmaceuticals, Novan, Novartis, Ralexar, RAPT, Regeneron, Pfizer, UCB, and Union Therapeutics. VM-P was a member of advisory boards of Sanofi, Pfizer, and Pharma and a recipient of a research grant from Pfizer. The remaining authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2023.08.026.

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Abstract

Sclerotic-type cutaneous chronic graft-versus-host disease is a severe complication of allogeneic hematopoietic stem cell transplantation, with profound morbidity. A dearth of effective, targeted treatment options necessitates further investigation into the molecular mechanisms underlying this T-cell–mediated disease. In this study, we compared the transcriptome in skin biopsies from pediatric and young adult (aged <25 years) patients with sclerotic-type cutaneous chronic graft-versus-host disease (n = 7) with that in demographically matched healthy controls (n = 8) and patients with atopic dermatitis (n = 10) using RNA sequencing with RT-PCR and immunohistochemistry validation. Differential expression was defined as fold change > 1.5 and false discovery rate < 0.05. Sclerotic-type cutaneous chronic graft-versus-host disease exhibited strong and significant T helper (Th)1 skewing through key related cytokines and chemokines (*CXCL9/10/11, IFNG/IFN-γ*, STAT1/signal transducer and activator of transcription 1). Several markers related to the TSLP–OX40 axis were significantly upregulated relative to those in both controls and lesional atopic dermatitis, including *TNFSF4/OX40L, TSLP*, and *IL33*, as well as fibroinflammatory signatures characterized in a prior study in systemic sclerosis. Gene set variation analysis reflected marker-level findings, showing the greatest enrichment of the Th1 and fibroinflammatory pathways, with no global activation identified in Th2 or Th17/Th22. Cell-type deconvolution revealed a significant representation of macrophages and vascular endothelial cells. Sclerotic-type cutaneous chronic graft-versus-host disease in young patients may therefore be characterized by strong Th1-related upregulation with a unique TSLP–OX40 signature, suggesting new therapeutic avenues for this devastating disease.

INTRODUCTION

Sclerotic-type cutaneous chronic graft-versus-host disease (ScGvHD) is a severe complication of allogeneic hematopoietic stem cell transplantation, characterized by T-cell–mediated inflammation and subsequent fibrosis that most commonly affects the skin (Martires et al, 2011). Progression of the cutaneous manifestations of ScGvHD is associated with significant morbidity, including joint contractures, neuropathy, poor wound healing, and infections (Martires et al, 2011; Wolff et al, 2021). Depending on the severity of the disease and the need for graft-versus-tumor effect, first-line pharmacologic treatment of cutaneous ScGvHD is currently limited to topical and systemic corticosteroids with or without additional immunosuppression, which have multiple side effects and no consensus for disease management in steroid-refractory cases (Strong Rodrigues et al, 2018; Wolff et al, 2021). Therefore, there remains a highly unmet need for safer and more targeted therapies for this devastating disease. Prior human studies examining T-cell subsets and the phenotype of sclerotic-type and morphea-like graft-versus-host disease (GvHD) skin lesions demonstrated enrichment of several immune pathways and mast cell populations (Brüggen et al, 2014; Zouali et al, 2022) as well as increased IFN-inducible cytokines in circulating monocytes (Hakim et al, 2016). However, these reports focused on changes in older adults (aged >40 years). Another study that included 3 adolescent-to-young adult patients with ScGvHD demonstrated increases in a panel of profibrotic genes and upregulation of select

proangiogenetic proteins (Greenberger et al, 2022). Nevertheless, to our knowledge, a broader characterization of the ScGvHD molecular phenotype has yet to be performed in the skin of younger patients, who may require special considerations for treatment, given the adverse effects of long-term systemic corticosteroids on growth and development (Flowers and Martin, 2015). This is particularly relevant to optimizing emerging therapeutics because age group–specific inflammatory patterns have been found in other T-cell–mediated diseases such as atopic dermatitis (AD) (Del Duca et al, 2023; Renert-Yuval et al, 2021; Zhou et al, 2019).

To characterize the skin phenotype of ScGvHD earlier in life, we performed a comprehensive transcriptomic and proteomic analysis in a cohort of pediatric and young adult patients (aged <25 years) with ScGvHD versus demographically matched healthy controls and patients with AD. Our findings demonstrate immune abnormalities in ScGvHD consistent with prior reports published with older adults and additionally suggest the OX40 axis as an avenue of therapeutic investigation.

RESULTS

We enrolled 7 children, adolescents, and young adult patients with ScGvHD (aged 11.5–24 years) and a group of healthy controls (n = 8) matched by age and sex assigned at birth. For appropriate comparisons of the immune and barrier phenotype of ScGvHD, we also included an age-matched group of 10 patients with moderate-to-severe AD, a well-characterized disease (Brunner et al, 2018; Del Duca et al, 2023; Esaki et al, 2016, 2015; Renert-Yuval et al, 2021; Zhou et al, 2019). All patients identified as White, with no significant differences in age or sex (Table 1). Patients were diagnosed with ScGvHD per National Institutes of Health consensus criteria (mean time from hematopoietic stem cell transplantation to chronic GvHD diagnosis of 9 months). Patients presented with skin severity scores of either 2 (n = 1) or 3 (n = 6), and all were classified as globally severe. At the time of sampling, patients were either on no immunosuppression (n = 3) or on a low residual immunosuppressive regimen (n = 4). Further clinical information on the ScGvHD cohort is available in Supplementary Table S1. No patients with AD were on systemic therapies at the time of sampling (Supplementary Materials and Methods). Principal coordinate analysis showed distinct clustering of the ScGvHD cohort from controls and both AD lesional and nonlesional samples (Figure 1a). Differentially expressed genes (DEGs) were defined by a fold change (FCH) >1.5 and false discovery rate (FDR) <0.05. Our analysis captured 2,803 DEGs (1,767 upregulated; 1,036 downregulated) in the ScGvHD transcriptome (ie, DEGs in ScGvHD vs controls), of which 372 (242 upregulated, 129 downregulated) overlapped with lesional AD and 404 (246 upregulated, 140 downregulated) overlapped with nonlesional AD, summarized in Figure 1b.

ScGvHD demonstrates significant T helper 1 and OX40/TSLP activation with more modest T helper 2 skewing

Using previously published immune gene sets (Chiricozzi et al, 2011; Dhingra et al, 2014; He et al., 2020a), we examined the inflammatory profile of ScGvHD in comparison with that of controls. We observed significant (FDR < 0.05) enrichment of T helper (Th)1

markers (eg, *IFNG*, STAT1/signal transducer and activator of transcription 1, *CXCL9*/*10*/*11*, *CCL5*) (FCH = 2.97–32.5, FDR < 0.05). We also observed significant upregulation of genes related to the OX40 pathway in ScGvHD, including *TNFSF4*/OX40L (FCH = 4.55), *TSLP*, and *IL33* (FCH > 1.5 for both), in addition to other OX40-associated signatures (eg, *MS4A1*/CD20, *TRAF5*, *PRF1*, *LRRC32*, *JAK2*) (FCH > 1.5 for all) (Figure 2 and Supplementary Data S1). Noting that the OX40/OX40L axis plays a role in Th1 and Th2 pathway activation and in memory T-cell formation as well as in the pathogenesis of AD (Furue and Furue, 2021; Guttman-Yassky et al, 2023, 2019b; Nakagawa et al, 2020), we included in our analysis a comparison with AD, a classic Th2 disease with variable Th1 and Th17 dysregulation (Esaki et al, 2016; Fujita et al, 2011).

Juxtaposing these diseases highlighted a clear distinction in Th pathway modulation patterns. Although Th2 skewing was significantly greater in lesional and even nonlesional AD than in ScGvHD (eg, *IL4R*, *IL13*, *CCR4*, *CCL13/17/18/22/26*), many of these genes were either nonsignificant or even downregulated in ScGvHD relative to that in the controls (eg, *CCL17/22*). *GATA3* and *CCR4*, also associated with the Th2 pathway, were significantly downregulated in ScGvHD compared with those in AD tissues. Modulation of Th17/Th22 markers was also significantly higher in lesional and some even in nonlesional AD than in ScGvHD (eg, *IL19*, *IL22*, *S100A7/8/9*, *LCN2*, *PI3*) (FCH > 6).

Several key OX40-associated markers were differentially expressed in ScGvHD versus controls and were also significantly (FDR < 0.05) upregulated in ScGvHD compared with those in lesional AD (eg, *TNFSF4*/OX40L, *IL33*, *TSLP*) (FCH > 2). Although ScGvHD and AD shared upregulation of the regulatory markers *CCL7* and *IL10* (FDR < 0.05), ScGvHD exhibited downregulation of other regulatory markers, such as *TRAF3* and *CTLA4* (FDR < 0.01), compared with lesional AD.

To assess these changes on a pathway level, we performed a gene set variation analysis using previously published immune gene sets (Chiricozzi et al, 2011; Dhingra et al, 2014; He et al., 2020a). Consistent with marker-level findings, ScGvHD showed clear upregulation of the Th1 axis relative to controls ($P < .01$) with limited Th2, Th17, and Th22 modulation (Figure 3a–d), in contrast to lesional AD, which exhibited broad activation across the Th axes ($P < .01$ for all).

ScGvHD exhibits enrichment of fibroinflammatory signatures in systemic sclerosis

Given the clinical similarities between ScGvHD and systemic sclerosis (SSc), we also compared our ScGvHD transcriptome (diseased vs control skin) with a previously published lesional SSc geneset (“affected SSc vs controls”) (Assassi et al, 2015). We found 334 DEGs shared between ScGvHD and SSc (308 upregulated, 26 downregulated) (Figure 4a and Supplementary Data S2). Among the top 50 DEGs by FDR (all <0.01), presented in Figure 4b, were Th1-associated markers (*CXCL9*, *EOMES*, *GZMA*, and *TNFSF4*/OX40L) (all FCH > 4.5); innate immune markers such as *NOX4*, *CARD8*, *FCGR1A*/CD64 (all FCH > 2); and many fibrosis- (eg, *IGFBP7*, *PRSS23*, *PAPPA*) and angiogenesis- (eg, *NRP1*, *AKAP12*, *SCG2*) related products (Bale et al, 2022; Beck et al, 2011; Benhadou et al, 2020; Benz et al, 2020; Gao et al, 2022; Ko et al, 2022; Li et al, 2013; Luo et al, 2020; Moon et al, 2016; Tabib et al, 2021; Wu et al, 2022; Zhang et al, 2022, 2018). Many of these markers

also overlapped with the SSc fibroinflammatory signature (Assassi et al, 2015), which additionally included several collagen-related DEGs (eg, *COL4A1/2*, *COL8A1*, *COL10A1*) (FCH > 2.9, FDR < 0.01 for all) (Supplementary Data S3). In our gene set variation analysis, the SSc fibroinflammatory pathway was also significantly upregulated ($P < .05$) in ScGvHD relative to controls (Figure 4c).

RT-PCR validates Th1- and OX40-axis activation

To validate our RNA-sequencing results, using RT-PCR, we evaluated the mRNA expression of a large panel of 58 immune and barrier genes, many of which show low detection levels on RNA sequencing (Dhingra et al, 2014; Guttman-Yassky et al, 2019a) (Supplementary Figure S1). We observed consistent, significant ($P < .05$) upregulation of Th1 markers *CXCL9*/*10*, *STAT1*/signal transducer and activator of transcription 1, and *IFNG* (FCH > 2) as well as of *TNFSF4*/OX40L, *IL10*, and *IL4R*. Although AD showed some decreases in barrier genes, such as *FLG* and loricrin gene *LOR*, and negative regulators *IL34* and *IL37* compared with controls, these markers were largely not downregulated in ScGvHD, except for *CLDN8* ($P < .05$). Similar and even more accentuated differences (FCH > 6) were seen in Th2 (*IL13*, *CCR4*, *CCL17*/*22*) ($P < .05$) and Th17/Th22 (*IL17A*/*F* [$P < .1$] and *IL22* [$P < .01$]) as well as general inflammatory marker matrix metalloproteinase 12 between lesional AD and ScGvHD. Tregulatory cell marker *FOXP3* was also decreased in ScGvHD compared with that in lesional AD skin (FCH < −3, $P < .1$).

Immunohistochemistry supports OX40-ligand enrichment in ScGvHD

To extend and validate our transcriptomic findings, we evaluated OX40 and OX40L protein expression through immunohistochemistry (Figure 5). In line with our gene expression results, OX40+ cell counts were significantly higher in lesional AD than in controls ($P < .05$) but not significantly higher in ScGvHD than in controls. OX40L+ cell counts were significantly enriched in ScGvHD relative to the enrichment in controls as well as in lesional AD versus controls; however, the difference in the latter comparison was not significant ($P < .1$).

Cell-type deconvolution highlights macrophage predominance in ScGvHD

For insight into the cellular composition of our samples, we performed a cell-type deconvolution analysis of our bulk RNA-sequencing data with CIBERSORTx (Newman et al, 2019) with previously published single-cell RNA-sequencing data from healthy skin (He et al., 2020b) and LM22, a validated signature matrix representing 22 leukocyte phenotypes (Newman et al, 2015). With the skin reference profile (Supplementary Figure S2a and Supplementary Table S2), we observed greater proportions of vascular endothelial cells in both ScGvHD and lesional AD than in controls ($P < .05$ for both) and of macrophages in ScGvHD than in lesional AD ($P < .05$) and controls ($P < .1$). Dendritic cells (DCs) were increased in lesional AD relative to those in controls and ScGvHD ($P < .05$) but not in ScGvHD versus controls.

We next focused on the relative representation of immune cells (Supplementary Figure S2b and Supplementary Table S3), noting significantly greater proportions of M2 macrophages in ScGvHD than in controls and nonlesional AD and of M1 macrophages in ScGvHD than

in all other groups. Activated DC fractions were significantly greater in AD than in controls and ScGvHD, and Tregulatory cell fractions were significantly lower in ScGvHD than in all other groups.

Enrichment analysis reveals immune and profibrotic signaling pathways in ScGvHD

For an unbiased functional evaluation of the ScGvHD transcriptome, we also performed an enrichment analysis of all DEGs in comparison of ScGvHD with the controls using validated gene ontology databases (Canonical/Kyoto Encyclopedia of Genes and Genomes/Reactome/BioCarta pathways) (Supplementary Data S4). In the upregulated DEGs (Supplementary Figure S3), we observed significant (FDR < 0.05) representation of many canonical pathways related to integrin signaling and angiogenesis as well as enrichment in extracellular matrix, collagen, Th1 (IL-12- and CXCR3-mediated), Th2 (CXCR4-, IL-5-mediated), and DC (IL-3-mediated) gene ontologies. Downregulated DEGs were primarily represented in pathways related to cell cycle and proliferation.

DISCUSSION

We present a study examining, to our knowledge, previously unreported skin profiles of pediatric and young adult patients with ScGvHD in comparison with those of demographically matched controls and those with AD, a reference Th2 disease with increasing availability of targeted and effective treatment options (Bieber, 2022; Chovatiya and Paller, 2021), including for pediatric populations (Pfizer, 2020; United States Food and Drug Administration, 2017). Our data demonstrated strong Th1 activation in ScGvHD, in line with the results of a few studies primarily focusing on older adults (Brüggen et al, 2014; Zouali et al, 2022), as well as upregulation of key OX40-associated cytokines relative to that in both controls and lesional AD.

Characterization of the unique phenotype of ScGvHD in younger patients is important because better targeted treatments for these age groups have emerged for inflammatory skin diseases such as AD, which could inform the development of specific therapeutic strategies that are safer than current nonspecific therapies for ScGvHD with considerable long-term side effects (Flowers and Martin, 2015). Prior studies have shown enrichment of some morphogen pathways in ScGvHD with therapeutic potential but serious limitations. The TGF-β cytokine, although a key player in fibrotic disease, is difficult to target owing to its heterogeneous effects on downstream signaling pathways in different tissues (Banovic et al, 2005; Wolff et al, 2021). Drugs modulating other pathways associated with pathologic tissue remodeling, such as Hedgehog, have been associated with significant toxicity (DeFilipp et al, 2017; Radojcic et al, 2021). Targeting contributory immune pathways may be a more promising approach as evidenced by recent studies with Jak inhibitors in steroid-refractory acute and chronic GvHD (Spoerl et al, 2014; Zeiser et al, 2020).

OX40/TNFRSF4, primarily expressed on effector T cells, binds its ligand (OX40L/*TNFSF4*), predominantly expressed on antigen-presenting cells (eg, DCs, macrophages, and activated B cells), to promote Th1 and Th2 cell expansion and CD4+ T-cell memory (Furue and Furue, 2021). These costimulatory immune checkpoint molecules are promising therapeutic targets for several inflammatory skin diseases, most notably moderate-to-severe

AD, as demonstrated in recent randomized placebo-controlled clinical trials with GBR 830 and rocatinlimab, mAbs against OX40, and amlitelimab, an anti-OX40L antibody (Guttman-Yassky et al, 2023, 2019b; Iriki et al, 2023; Nakagawa et al, 2020; Weidinger et al, 2023).

In addition to strong OX40-axis upregulation in ScGvHD, there was a clear Th1 signal in both our RNA-sequencing and RT-PCR data as well as significant upregulation of the plasmacytoid DC marker *IL3RA* on RT-PCR and increases in M1 and M2 macrophages. Expression of OX40L in DCs has been proposed to be activated by NK cell–derived IFN-g and is key to the expansion of OX40+ Th1 effector cells (Gajdasik et al, 2020; Kopf et al, 1999). Different subsets of DCs have been shown to promote both alloreactivity and tolerance in the setting of GvHD (Yu et al, 2019). Although DC estimates were not increased in our ScGvHD samples, *IL3RA* is primarily expressed in circulating DCs (Wu et al, 2021), and it is possible that a stronger contribution of DCs would be seen in the blood compartment.

On the other hand, macrophages, which also express OX40L (Cai et al, 2022; Gwyer Findlay et al, 2014), have been implicated in cGvHD pathogenesis in murine models (Alexander et al, 2014; Du et al, 2017). M1 macrophages are key players in promoting Th1 inflammation (Mills et al, 2000; Shapouri-Moghaddam et al, 2018), whereas M2 macrophages play important roles in fibrosis and angiogenesis (Kishore and Petrek, 2021; Shapouri-Moghaddam et al, 2018). Our results may therefore suggest a Th1 profile in ScGvHD driven by OX40/OX40L signaling, paralleling observations that the anti-OX40 antibody GBR 830 was shown to attenuate the Th1 axis, in addition to other immune pathways (Guttman-Yassky et al, 2019b). Th1 activation may, in turn, engage in a pathogenic interplay with M1 and M2 macrophage dysregulation to promote sclerotic changes, with possible contributions from aberrant DC activity. Validation with single-cell and/or flow cytometry, especially of OX40L+ antigen-presenting cells, would be valuable.

Although ScGvHD lacked the broad Th2 activation observed in the AD cohort, we were able to capture activation of several molecules linked to Th2 and/or atopy with RNA-sequencing and/or RT-PCR, including *IL33*, *IL10*, and *IL4R*, as well as enrichment in Th2-related gene ontologies. This is relevant to the comparison of our ScGvHD transcriptome with the lesional SSc transcriptome published by Assassi et al (2015) because increased OX40/OX40L signaling has been well-described in SSc (Boleto et al, 2018; Bossini-Castillo et al, 2011; Elhai et al, 2019), as has Th2's role in promoting the characteristic fibrosis of the disease (Jin et al, 2022; MacDonald et al, 2015). We indeed noted shared upregulation of OX40L and several Th1 markers and profibrotic signatures as well as a significant pathway-level upregulation in the fibroinflammatory gene set (Assassi et al, 2015) in the ScGvHD cohort and significant representation in extracellular matrix and integrin-signaling pathways, which have been linked to both immune chemotaxis and fibrosis (Margadant and Sonnenberg, 2010; McEver, 2015). The increases in fibrosis and angiogenesis-related products as well as in vascular endothelial cells observed in our data were additionally in line with a prior report in ScGvHD (Greenberger et al, 2022). Finally, Th17/Th22 modulation was mostly unremarkable in ScGvHD relative to that in controls, which may reflect the role of OX40 and IFN-γ in attenuating the expression of Th17 cytokines (Jin et al, 2022; Xiao et al, 2016).

The limitations of our study include a small sample size owing, at least in part, to the rarity of ScGvHD and our focus on pediatric patients as well as the unavailability of lichenoid or acute GvHD comparisons. Our patients were additionally all White and predominantly male, potentially limiting generalizability to other races and to those assigned female at birth. We also note that although ScGvHD can be associated with high morbidity, at least some degree of GvHD in the form of a graft-versus-tumor effect can be beneficial in certain patients with a history of malignancy and that OX40 agonism has shown utility in promoting antitumor T-cell activity (Hong et al, 2022; Peng et al, 2019), which are important considerations in the therapeutic contextualization of our findings.

In conclusion, we captured strong Th1 upregulation in pediatric to young adult patients as well as robust OX40L–TSLP signaling with more modest features of Th2 activation, in line with findings of transcriptomic studies in SSc. We also captured strong activation of the OX40/OX40L/TSLP/IL-33 axis, which may be an efficacious therapeutic target in several atopic diseases, including AD and asthma (Guttman-Yassky et al, 2023; Kaur and Brightling, 2012). Because safe and effective, long-term treatment for cutaneous ScGvHD remains elusive, specific agents modulating this pathway can be considered for young patients with this disease. Similarly, specific, systemic Jak inhibitors that are now approved or in late-stage studies for AD (United States Food and Drug Administration, 2022a, 2022b, 2019; Zhao et al, 2021) may present another beneficial approach. Future therapeutic studies, ideally with biomarker evaluation, should clarify the potential utility of these agents in cutaneous ScGvHD, including in more diverse patient cohorts and at different stages of disease progression.

MATERIALS AND METHODS

Patient characteristics and sample collection

This study was reviewed and approved by local institutional review boards at Mount Sinai and Hadassah Medical Center. All subjects (if aged ≥12 years) and parents (if aged <18 years) provided institutional review boards–approved, written, and informed consent at their respective institutions. Demographic and clinical information were collected, and 3–4 mm biopsy specimens were obtained from the upper arm in control or nonlesional skin and in location-matched lesions when possible.

RNA sequencing

RNA was extracted from the skin biopsy with Qiagen miRNAeasy mini kits, from which libraries were generated using Illumina TruSeq Stranded mRNA Library Prep kits, and Next-generation sequencing was performed with NovaSeq 6000 (single-read sequencing, 100 cycles) per manufacturer’s instructions. RNA-sequencing quality was assessed with FastQC (Wingett and Andrews, 2018) and MultiQC (Ewels et al, 2016). Samples were aligned to the human reference genome GRCh37 with STAR RNA-seq aligner (Dobin et al, 2013), and aligned sequences were assigned genomic features with the featureCounts algorithm (Liao et al, 2014).

RT-PCR and immunohistochemistry

A total of 100 ng of RNA per sample underwent reverse transcription, preamplification, and RT-PCR with TaqMan Low-Density Array cards (Thermo Fisher Scientific), as previously described (Sanyal et al, 2019). The primers are listed in Supplementary Table S4. Ct values were normalized to *RPLP0* by negatively transforming Ct values to −dCt. Undetected −dCt values of each gene were imputed with 20% of the minimal unlogged expression over the detection limit across all samples.

Immunohistochemistry was performed on frozen human tissue sections using mAbs, listed in Supplementary Table S5. Positive cells per millimeter on images taken at ×100 magnification were counted manually using computer-assisted image analysis software (ImageJ 1.42, National Institutes of Health), as previously described (Renert-Yuval et al, 2023; Suárez-Fariñas et al, 2011).

Cell-type deconvolution

Relative fractions of cell types were estimated with the CIBERSORTx platform (Newman et al, 2019) using a matrix of bulk RNA-sequencing gene expression in unlogged transcripts per million, along with a signature matrix from single-cell RNA-sequencing data from healthy skin (He et al., 2020b) and the LM22 signature matrix (Newman et al, 2015). Comparisons of mean proportions were assessed with unpaired or paired *t*-tests, where applicable.

Statistical analysis

Statistical analysis was performed with R software (www.R-project.org) and packages available through Bioconductor (www.bioconductor.org

Aligned RNA-sequencing data were normalized and $\log_2$ transformed with the voom package (Law et al, 2014). Gene expression was modeled with linear regression, and mean estimation and hypothesis testing were conducted using the limma package (Ritchie et al, 2015). *P*-values from the moderated (paired) *t*-test were corrected for multiple comparisons using the Benjamini–Hochberg procedure. DEGs were defined with FCH > 1.5 and FDR < 0.05, exploratory criteria used in prior transcriptomic studies with biopsies (Glickman et al, 2021; Renert-Yuval et al, 2023). Hierarchical clustering of samples was performed with a McQuitty agglomeration algorithm.

Immunohistochemistry counts and RT-PCR expression values were analyzed in $\log_2$ scale and modeled by linear mixed-effect models, with group (combined factor of disease and tissue type) as a fixed effect and a random effect for each patient. Group means were estimated with emmeans, and comparisons of interest were tested with contrasts. Unsupervised clustering of group means was performed with Euclidean distance and average agglomeration criteria.

Gene set variation analysis of unsupervised sample-wise enrichment was performed with the GSVA package (Hänzelmann et al, 2013) using the z-score method. Gene ontology enrichment analysis was performed with XGR software across the canonical/Kyoto Encyclopedia of Genes and Genomes/Reactome/Biocarta pathways (Fang et al, 2016).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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EGY is designated a guarantor for this work.

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DATA AVAILABILITY STATEMENT

The data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through Gene Expression Omnibus Series accession number GSE230200 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE230200).

Abbreviations:

AD	atopic dermatitis
DC	dendritic cell
DEG	differentially expressed gene
FCH	old change
FDR	false discovery rate
GvHD	graft-versus-host disease
ScGvHD	sclerotic-type cutaneous chronic graft-versus-host disease
SSc	systemic sclerosis
Th	T helper

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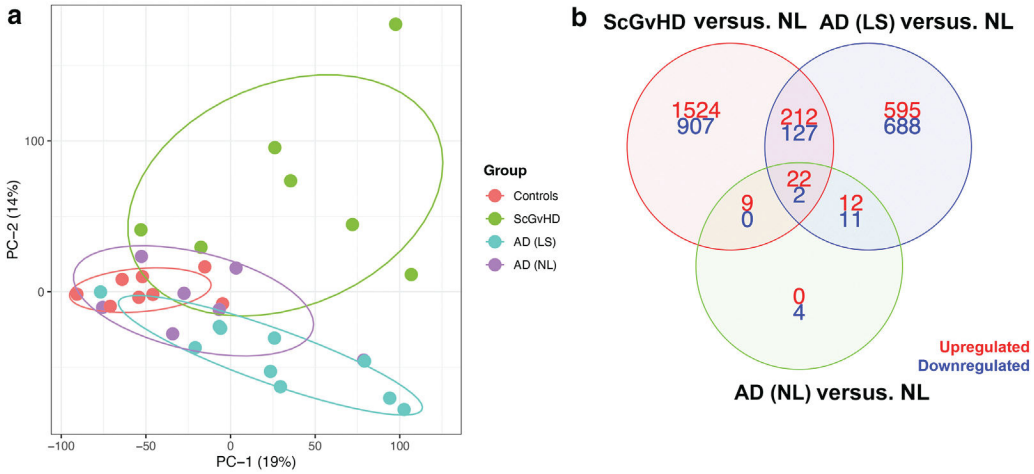

Figure 1. Overview of ScGvHD versus AD and controls.

(**a**) Principal coordinate analysis plot with ScGvHD, LS AD, NL AD, and control samples. (**b**) Venn diagram summarizing DEGs detected between ScGvHD and controls, LS AD and controls, and NL AD and controls. Red text denotes upregulated DEGs, and blue text denotes downregulated DEGs. AD, atopic dermatitis; DEG, differentially expressed gene; LS, lesional; NL, nonlesional; PC-1, principal component 1; PC-2, principal component 2; ScGvHD, sclerotic-type cutaneous chronic graft-versus-host disease.

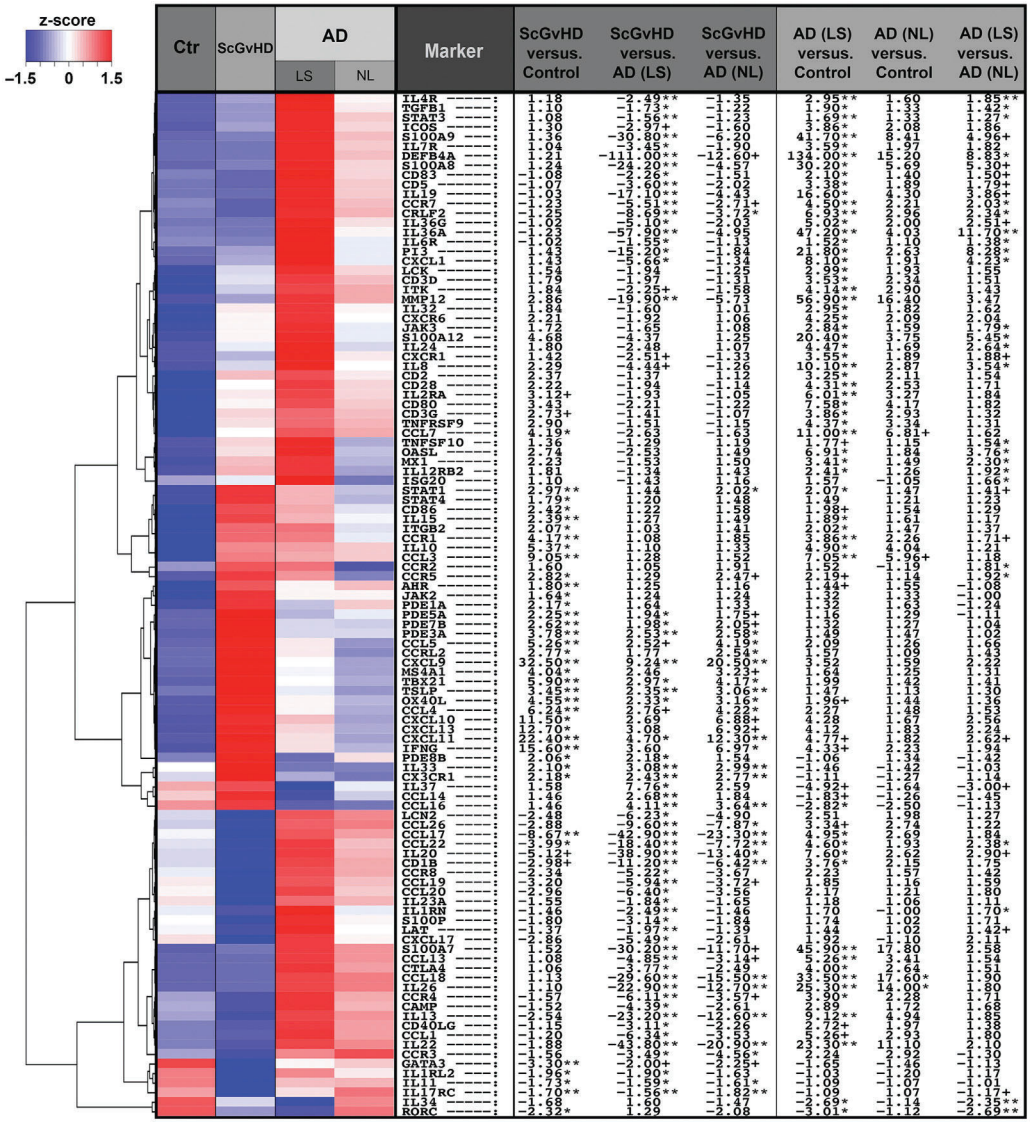

Figure 2. ScGvHD exhibits strong Th1 and OX40/OX40L skewing.

Heatmap representing the mean expression of differentially expressed immune genes in (from left to right) Ctrl, ScGvHD, LS AD, and NL AD samples. Criteria for differential expression are FCH > 1.5 and FDR < 0.05. The adjacent table displays fold changes in ScGvHD versus Ctrls, ScGvHD versus lesional AD, ScGvHD versus NL AD, LS AD versus Ctrls, NL AD versus Ctrls, and LS AD versus NL AD. **FDR < 0.01, *FDR < 0.05, and +FDR < 0.1. AD, atopic dermatitis; Ctr, controls; FCH, fold change; FDR, false discovery rate; LS, lesional; NL, nonlesional; ScGvHD, sclerotic-type cutaneous chronic graft-versus-host disease; Th, T helper.

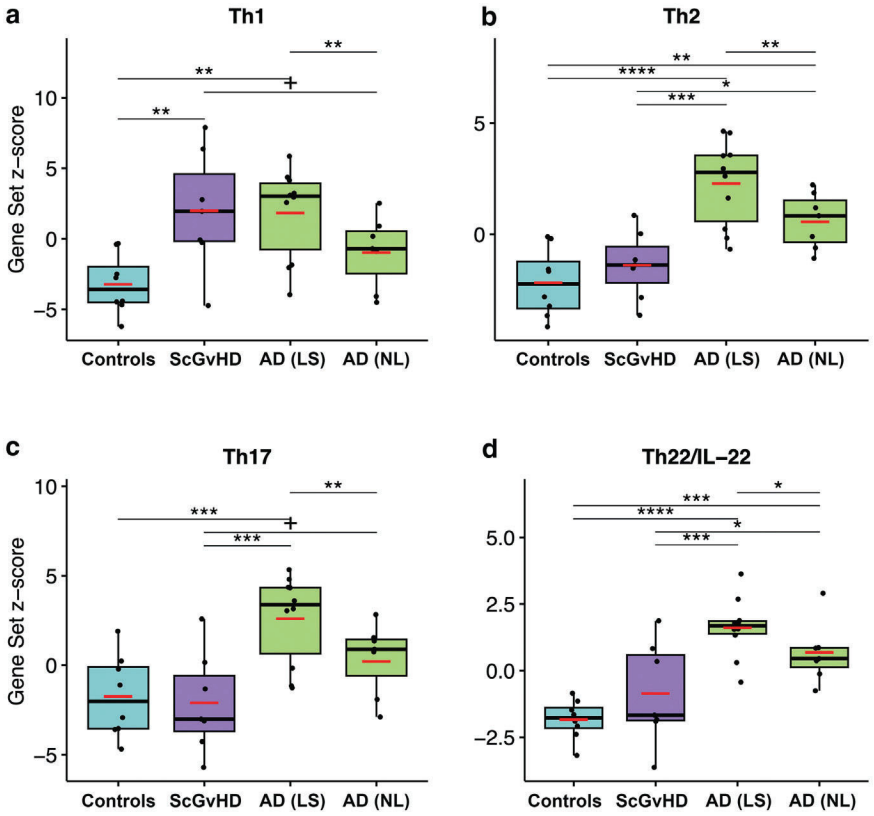

Figure 3. GSVA highlights the activation of the Th1 pathway in ScGvHD.

Boxplots of mean z-score depicting the pathways of genes related to (**a**) Th1, (**b**) Th2, (**c**) Th17, and (**d**) Th22/IL-22. Red bars represent the average z-scores of their respective groups. ****$P < .0001$, ***$P < .001$, **$P < .01$, *$P < .05$, and +$P < .1$. AD, atopic dermatitis; GSVA, gene set variation analysis; LS, lesional; NL, nonlesional; SSc, systemic sclerosis; ScGvHD, sclerotic-type cutaneous chronic graft-versus-host disease; Th, T helper.

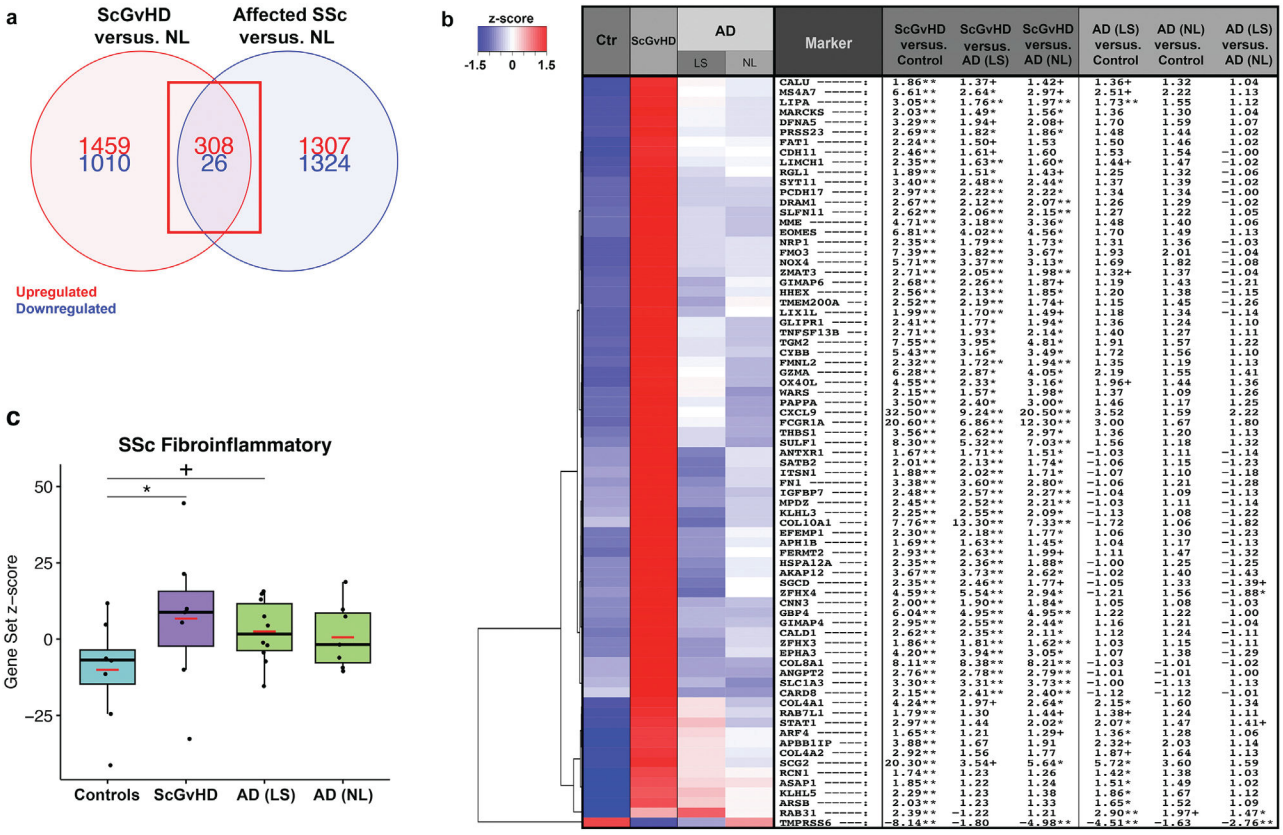

Figure 4. ScGvHD exhibits shared enrichment of fibroinflammatory signatures with lesional systemic sclerosis.

(**a**) Venn diagram summarizing DEGs detected between ScGvHD and Ctrls overlapping with the affected SSc gene set published in Assassi et al (2015). (**b**) Heatmap representing the top 75 DEGs in ScGvHD versus Ctrls by FDR overlapping with the affected SSc gene set. Criteria for differential expression are FCH > 1.5 and FDR < 0.05. The heatmap shows the mean expression of Ctrls, ScGvHD, LS AD, and NL AD samples. The adjacent table displays FCHs in ScGvHD versus Ctrls, ScGvHD versus LS AD, ScGvHD versus NL AD, LS AD versus Ctrls, NL AD versus Ctrls, and LS AD versus NL AD. **FDR < 0.01, *FDR < 0.05, and +FDR < 0.1. (**c**) Boxplots of mean z-scores of the SSc fibroinflammatory gene set published in Assassi et al (2015). *P < .05 and +P < .1. AD, atopic dermatitis; Ctr, control; DEG, differentially expressed gene; FCH, fold change; FDR, false discovery rate; LS, lesional; NL, nonlesional; SSc, systemic sclerosis; ScGvHD, sclerotic-type cutaneous chronic graft-versus-host disease.

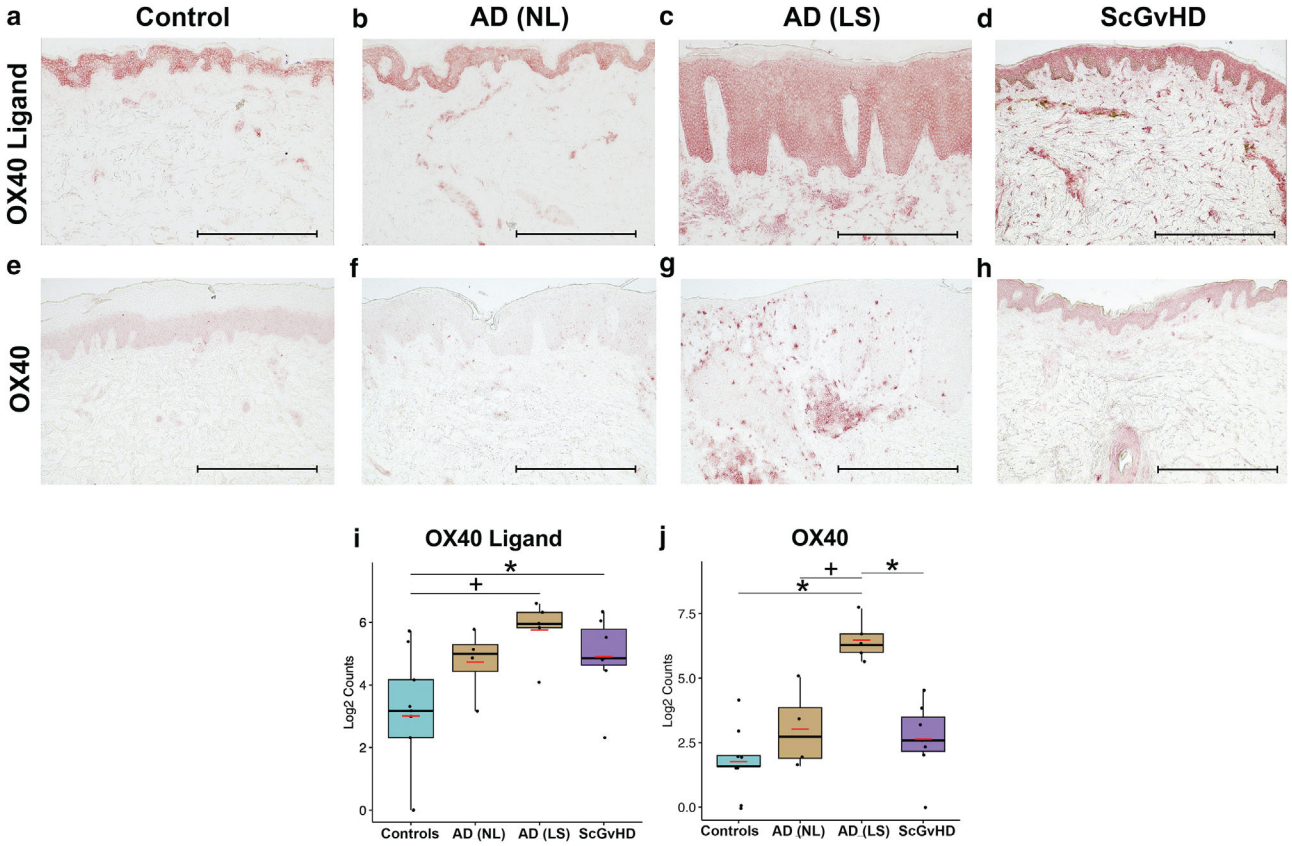

Figure 5. Immunohistochemistry demonstrates OX40L upregulation in ScGvHD.

Representative immunohistochemistry stainings of (**a–d**) OX40L and (**e–h**) OX40 in (**a, e**) Ctrls, (**b, f**) NL AD, (**c, g**) LS AD, and (**d, h**) ScGvHD. Boxplots comparing $\log_2$ (i) OX40L and (j) OX40 counts between Ctrls, ScGvHD, LS AD, and NL AD at ×100 magnification. Bars = 0.4 mm. $*P < .05$ and $+P < .1$. AD, atopic dermatitis; Ctr, control; LS, lesional; NL, nonlesional; ScGvHD, sclerotic-type cutaneous chronic graft-versus-host disease.

Table 1.

Patient Demographics

	Controls		ScGvHD			AD			
Demographics	**n = 8**		**n = 7**		***P*-Value[1]**	**n = 10**		***P*-Value[1]**	***P*-Value[2]**
Age, y					0.70			0.46	0.79
Mean (SD)	17.1 (3)		17.9 (4.4)			18.4 (3.9)			
Range	11.9–21		11.5–24			11.6–23			
Sex	n	%	n	%	0.57	n	%	1.00	0.74
Male	5	63%	6	85.7%		7	70%		
Female	3	38%	1	14.3%		3	30%		
Race	n	%	n	%	—	n	%	—	—
White	8	100%	7	100%		10	100%		
SCORAD					—			—	—
Mean (SD)	—		—			60 (21)			
Range	—		—			31.4–91.2			

Abbreviations: AD, atopic dermatitis; ScGvHD, sclerotic-type cutaneous chronic graft-versus-host disease; SCORAD, SCOring Atopic Dermatitis.

Quantitative comparisons between disease and control were evaluated with Student's t-tests and across all groups with 1-way ANOVA tests. Analogous comparisons of sex distribution were assessed with Fisher's exact tests.

[1]Comparison of group versus control.

[2]Comparison across all groups.