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Expansion of Fcγ Receptor IIIa–Positive Macrophages, Ficolin 1–Positive Monocyte-Derived Dendritic Cells, and Plasmacytoid Dendritic Cells Associated With Severe Skin Disease in Systemic Sclerosis

Dan Xue,[1] Tracy Tabib,[2] Christina Morse,[2] Yi Yang,[3] Robyn T. Domsic,[2] Dinesh Khanna,[4] and Robert Lafyatis[2]

Objective. In this study, we sought a comprehensive understanding of myeloid cell types driving fibrosis in diffuse cutaneous systemic sclerosis (dcSSc) skin.

Methods. We analyzed the transcriptomes of 2,465 myeloid cells from skin biopsy specimens from 12 dcSSc patients and 10 healthy control subjects using single-cell RNA sequencing. Monocyte-derived dendritic cells (mo-DCs) were assessed using immunohistochemical staining and immunofluorescence analyses targeting ficolin-1 (FCN-1).

Results. A t-distributed stochastic neighbor embedding analysis of single-cell transcriptome data revealed 12 myeloid cell clusters, 9 of which paralleled previously described healthy control macrophage/DC clusters, and 3 of which were dcSSc-specific myeloid cell clusters. One SSc-associated macrophage cluster, highly expressing Fcγ receptor IIIA, was suggested on pseudotime analysis to be derived from normal CCR1+ and MARCO+ macrophages. A second SSc-associated myeloid population highly expressed monocyte markers FCN-1, epiregulin, S100A8, and S100A9, but was closely related to type 2 conventional DCs on pseudotime analysis and identified as mo-DCs. Mo-DCs were associated with more severe skin disease. Proliferating macrophages and plasmacytoid DCs were detected almost exclusively in dcSSc skin, the latter clustering with B cells and apparently derived from lymphoid progenitors.

Conclusion. Transcriptional signatures in these and other myeloid populations indicate innate immune system activation, possibly through Toll-like receptors and highly up-regulated chemokines. However, the appearance and activation of myeloid cells varies between patients, indicating potential differences in the underlying pathogenesis and/or temporal disease activity in dcSSc.

INTRODUCTION

Systemic sclerosis (SSc, also known as scleroderma), is a heterogeneous autoimmune disease with an unknown etiology characterized by fibrosis, vasculopathy, and immune dysfunction. Current treatment remains ineffective for controlling many complications, resulting in high morbidity and mortality (1). Diffuse cutaneous SSc (dcSSc) is a subtype of SSc characterized by widespread skin fibrosis. Myeloid cells, including macrophages and dendritic cells (DCs), heterogeneous immune cells in human skin, have been implicated in initiating and perpetuating SSc by releasing or activating profibrotic and proinflammatory factors;

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[1]Dan Xue, MD: University of Pittsburgh, Pittsburgh, Pennsylvania, and Xiangya Hospital, Central South University, Changsha, China; [2]Tracy Tabib, MS, Christina Morse, BS, Robyn T. Domsic, MD, MPH, Robert Lafyatis, MD: University of Pittsburgh, Pittsburgh, Pennsylvania; [3]Yi Yang, MD: Xiangya Hospital, Central South University, Changsha, China; [4]Dinesh Khanna, MD: University of Michigan, Ann Arbor.

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Address correspondence to Robert Lafyatis, MD, 200 Lothrop, BST S720, Pittsburgh, PA 15260. Email: lafyatis@pitt.edu.

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however, the precise mechanisms that contribute to SSc pathogenesis remain poorly understood (2,3).

Infiltration of macrophages in SSc skin was first described in the 1990s (4,5). More recent studies have shown increased expression of specific macrophage markers in dcSSc skin, including Siglec-1 (CD169), a type I interferon (IFN)–inducible gene, and CD163 and CD204, markers of activated M2 macrophages (6,7). Other studies have implicated several chemokines in SSc pathogenesis: CCL2, CCL5, CCL18, CCL19, CXCL9, and CXCL13 (8). CCL19, in particular, is a strong macrophage chemoattractant (8,9). Further supporting the idea that macrophages play an important role in SSc pathogenesis, expression of macrophage markers CD14, MS4A4A, CD163, and CCL2 correlate with clinical disease severity, as assessed using the modified Rodnan skin score (MRSS), and are prognostic biomarkers of progressive skin disease in dcSSc (10,11). Macrophage and DC genes were also detected by bulk gene expression analysis in a subset of inflamed skin biopsy specimens (12,13).

Recent studies indicate that DCs may also play important roles in the pathophysiology of SSc skin disease (3,14). The role of myeloid or conventional DCs (cDCs) in SSc skin is uncertain, since markers used in previous studies are not specific to these cells (3). On the other hand, several studies have shown increased levels of plasmacytoid dendritic cells (pDCs) in the skin of SSc patients (14–16), and studies have also shown that circulating SSc pDCs secrete CXCL4, as well as IFNα (14,16).

Despite these multiple studies, altered myeloid cell numbers and functions in SSc remain poorly understood, in part due to the complexity of macrophage and DC subsets. Here, we compare recently detailed transcriptomes of macrophage and DC subsets in normal skin (17) to discern alterations in myeloid cell subsets in dcSSc skin.

PATIENTS AND METHODS

Single-cell RNA sequencing (RNA-seq). For single-cell RNA-seq analyses, 3-mm skin punch biopsy specimens from the dorsal mid-forearm were obtained after receiving informed consent under protocols approved by the University of Pittsburgh or University of Michigan institutional review boards. Skin was digested enzymatically, and cells were loaded into the Chromium instrument (10x Genomics), as described (18). Data analysis was performed with the R package Seurat version 2.3.4. Differential gene expression analysis comparing dcSSc cells to healthy control cells for each cluster was performed using the Wilcoxon rank sum test with a cutoff $P < 0.05$, a fold change >1.5, and further requiring the expression of genes from >25% of denoted cells. All single-cell RNA-seq data, including a gene/cell unique molecular identifier matrix and a BAM file containing aligned reads, are available at the GEO (accession no. GSE138669).

Staining of paraffin-embedded skin biopsy specimens. Immunohistochemical (IHC) and immunofluorescence staining using tyramide signal amplification was performed with monoclonal mouse anti–ficolin-1 (anti–FCN-1) on formalin-fixed paraffin-embedded human skin tissue from 38 SSc patients and 16 healthy control subjects.

Microarray analysis. Microarray RNA gene expression data from 64 SSc skin biopsy specimens and 15 healthy control skin biopsy specimens were analyzed using Cluster 3.0 software and were visualized using TreeView 1.1.6 software. Additional methods used in this study are described in detail in the Supplementary Methods (see the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract).

RESULTS

Single-cell transcriptome profiles of myeloid cells from dcSSc and healthy control skin. Using single-cell RNA-seq analysis, we examined gene expression of all enzymatically digested skin cells obtained from 12 patients with dcSSc and 10 healthy control subjects. All subjects have been included in another analysis of fibroblasts in fibrotic skin (19). Patients with dcSSc and control subjects were balanced across sex and age (Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). Transcriptomes of 28,216 cells from healthy control subjects and 36,983 cells from dcSSc patients were grouped and analyzed together. Similar numbers of cells were included from healthy control and dcSSc skin (mean 2,822 cells per biopsy specimen and mean 3,082 cells per biopsy specimen, respectively) (Table 1).

Combined cell/gene count matrices were analyzed by t-distributed stochastic neighbor embedding (t-SNE) dimensional reduction, visualization, and clustering. Twenty-eight distinct clusters were aligned to expected cell types according to the top highly expressed genes in each cluster as described previously (18) (Supplementary Figure 1A and Supplementary Tables 2 and 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). All clusters included cells from multiple subjects and were unbiased from V1 and V2 chemistries (Supplementary Figures 1B, 2A, and 2B). Cluster 9 was identified as myeloid cells, showing specific LIN(CD3D/CD79A/NKG7)–HLA–DQ+ and expression of myeloid-specific genes (*ITGAM*, *ITGAX*, *CD14*, *CSF1R*, *CD68* and *CD209*); and verified by expression of MS4A4A, CD1C and CLEC9A, markers for macrophage, cDC2 and cDC1, respectively (Supplementary Figures 3A–C, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract) (17).

Table 1. Proportions of each macrophage/dendritic cell subpopulation in skin samples from SSc patients and healthy controls*

	Total no. of cells	No. of myeloid cells	Macrophages and DCs, % among all cells	No. of MS4A4A+ macrophages	MS4A4A+ macrophages, % among all cells	No. of FCGR3A+ macrophages	FCGR3A+ macrophages, % among myeloid cells	No. of FCN-1+ cells	FCN-1+ cells, % among myeloid cells	No. of proliferating macrophages	Proliferating macrophages, % among myeloid cells
Individual sample											
Healthy control											
SC1nor	1,488	51	3.43	23	1.55	0	0.00	1	1.96	0	0.00
SC4nor	3,887	56	1.44	21	0.54	4	7.14	4	7.14	0	0.00
SC18nor	3,183	154	4.84	68	2.14	1	0.65	8	5.19	1	0.65
SC32nor	1,956	100	5.11	35	1.79	0	0.00	2	2.00	0	0.00
SC33nor	2,156	173	8.02	92	4.27	0	0.00	4	2.31	0	0.00
SC34nor	2,118	24	1.13	6	0.28	2	8.33	1	4.17	0	0.00
SC50nor	3,670	54	1.47	19	0.52	0	0.00	1	1.85	0	0.00
SC68nor	2,116	64	3.02	33	1.56	4	6.25	1	1.56	0	0.00
SC124nor	4,552	99	2.17	36	0.79	3	3.03	0	0.00	0	0.00
SC125nor	3,090	62	2.01	21	0.68	0	0.00	0			
SSc patient									0.00	0	0.00
SC2ssc	1,608	10	0.62	4	0.25	0	0.00	1	10.00	0	0.00
SC5ssc	4,120	158	3.83	100	2.43	2	1.27	11	6.96	1	0.63
SC19ssc	1,920	127	6.61	51	2.66	4	3.15	12	9.45	0	0.00
SC49ssc	3,091	15	0.49	1	0.03	0	0.00	2	13.33	0	0.00
SC60ssc	1,785	38	2.13	10	0.56	0	0.00	10	26.32	0	0.00
SC69ssc	2,407	169	7.02	79	3.28	44	26.04	11	6.51	1	0.59
SC70ssc	2,714	25	0.92	9	0.33	0	0.00	4	16.00	0	0.00
SC86ssc	3,663	88	2.40	31	0.85	1	1.14	2	2.27	0	0.00
SC119ssc	4,696	35	0.75	12	0.26	0	0.00	1	2.86	1	2.86
SC185ssc	3,443	262	7.61	124	3.60	2	0.76	5	1.91	0	0.00
SC188ssc	3,993	219	5.48	88	2.20	2	0.91	59	26.94	13	5.94
SC189ssc	3,543	482	13.60	267	7.54	155	32.16	68	14.11	10	2.07
Combined group, mean											
SSc patients	3,082	136	4.41	65	2.11	18	13.24	16	11.76	2	1.47
Healthy controls	2,822	84	2.98	35	1.24	1	1.19	2	2.38	0	0.00
P†	0.3139	0.3857	0.4614	0.404	0.4106	0.3342	0.416	0.0048	0.001	0.0831	0.0654

* SSc = systemic sclerosis; FCGR3A = Fcγ receptor IIIa; FCN-1+ = ficolin-1–positive.
† By Wilcoxon's rank sum test.

The percentage of myeloid cells of the total cells analyzed for each subject was strikingly variable, ranging from 0.49% of total cells (15 myeloid cells of 3,091 cells for patient SC49ssc) to 13.60% of total cells (482 myeloid cells of 3,543 cells for patient SC189ssc), and the percentage of myeloid cells showed a trend toward an increased percentage of myeloid cells in SSc, ranging from 2.98% in healthy control skin to 4.41% in dcSSc skin (Table 1). The percentage of myeloid cells in SSc patients showed a weak negative correlation with disease duration but was not statistically significant ($R = -0.26$) (Supplementary Figure 3B, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). Notably, the shared correlation strength calculated based on gene expression of all cells from each subject in a canonical correlation analysis showed that SC188, SC189, and SC69 patient samples deviated from other samples (Supplementary Figure 4).

All myeloid cell clusters identified in healthy skin were preserved in dcSSc skin. Reanalysis by t-SNE dimensions of 2,465 myeloid cells from dcSSc and healthy control skin showed 12 clusters of macrophage/DCs (Figure 1A). Each cluster was composed of cells from ≥6 subjects (Figure 1C and Supplementary Table 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). Nine of these 12 clusters paralleled previously described macrophage/DC clusters in healthy human skin (17).

The myeloid cell clusters were highly preserved in dcSSc skin (Figure 1B), identified by the top 50 differentially expressed genes in each subcluster (Supplementary Table 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). Subclusters 1 and 3 highly expressed CD1c and represented 2 cDC2 populations, each highly expressing either markers mucolipin-2 (MCOLN-2) or

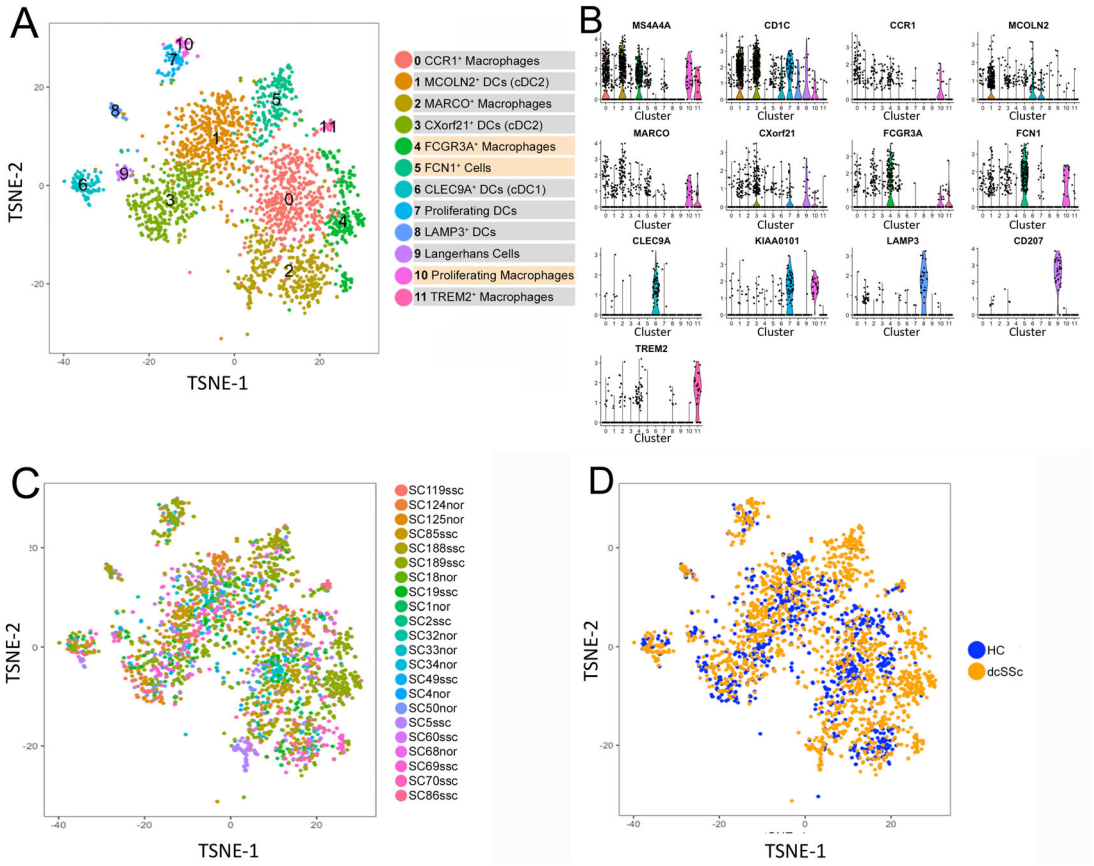

Figure 1. Single-cell RNA sequencing analysis of myeloid cell populations from healthy control subjects (HC) and patients with diffuse cutaneous systemic sclerosis (dcSSc). **A**, The 2-dimensional t-distributed stochastic neighbor embedding (t-SNE) cluster plot shows clustering of myeloid cells from all 22 healthy control and dcSSc patient skin samples, identified by cell type. Each point represents a single cell, with results illustrating the dimensional reduction in cell transcriptomes. Cells were assigned a color in the k-nearest neighbor graph according to the Euclidean distance in a principal components analysis, using a smart local moving algorithm to iteratively group cells. **B**, Violin plots of marker gene expression distinguish each cluster of myeloid cells. Values below the plots represent the cluster numbers shown in **A**. Each point represents a single cell. **C** and **D**, Cells were grouped using t-SNE according to individual sample identity number (**C**) or health status (**D**). MCOLN-2 = mucolipin-2; DCs = dendritic cells; cDC2 = type 2 conventional DCs; MARCO = macrophage receptor with collagenous structure; FCGR3A = Fcγ receptor IIIa; FCN-1 = ficolin-1; CLEC9A = C-type lectin domain containing 9A; LAMP-3 = lysosome-associated membrane protein 3; TREM-2 = triggering receptor expressed on myeloid cells 2. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract.

CXorf21 (MCOLN2+ DCs and CXorf21+ DCs). These populations correspond closely to the DC2 and DC3 circulating DCs (20) we previously described in healthy skin (17). Cells in subcluster 6 highly expressed CLEC9A and other cDC1 markers and were identified as cDC1 cells, corresponding closely to circulating DC1 cells (17,20). The cells in myeloid cluster 7 highly expressing CD1c and proliferation genes, such as *MKI67*, *KIAA0101*, *TYMS*, and *PTTG1*, were identified as proliferating cDC2. Cells in subcluster 8 highly expressing lysosome-associated membrane protein 3 (LAMP-3), CCL17, and BIRC-3 were identified as a mature subpopulation of cDC (LAMP-3+ DCs), and cells in subcluster 9 highly expressing CD207, FCGBP, and HLA–DQB2 were identified as Langerhans' cells (Figure 1B and Supplementary Figures 5 and 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). The cells in these myeloid clusters showed no noticeable shift in the phenotype of the clusters between healthy controls and dcSSc (Figure 1D).

Three previously described macrophage clusters in healthy skin, selectively expressing CCR1, macrophage receptor with collagenous structure (MARCO), or triggering receptor expressed on myeloid cells 2 (TREM-2), were also detected (Figures 1A and B and Supplementary Table 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). The specific expression of *MS4A4A*, as well as other macrophage marker genes, and the absence of DC markers in subclusters 0, 2, 4, 10, and 11, indicated that these are macrophage clusters (Figure 1A and Supplementary Figure 5). The percentage of all macrophage populations increased from 1.24% in healthy control skin to 2.11% in dcSSc skin (Table 1). CCR-1 and MARCO, which we previously described as good markers for 2 major macrophages subsets in healthy control skin (17), were somewhat more highly expressed by cells in clusters 0 and 2, respectively (Figure 1B and Supplementary Figure 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract), but were not observed to be discrete markers in this combined analysis of dcSSc and healthy control skin, apparently because the disease altered expression of these markers. However, comparing the top 15 genes distinguishing clusters in the combined database with healthy control clusters indicated that other markers of these 2 healthy control macrophages were preserved. *HMOX1*, *MMP9*, *MMP19*, *CCL2*, *CEBPB*, *CTSL*, *CREM*, *THBD*, and *EIF4E* were still highly expressed by cells in subcluster 0 (CCR-1+ macrophages) and *SEPP1*, *CCL13*, *FOLR2*, and *C1QA* were still highly expressed by cells in subcluster 2 (MARCO+ macrophages) (Supplementary Table 5 and Supplementary Figure 5B, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract).

A distinct third macrophage population seen in both healthy control subjects and SSc patients was distinguished by high expression of TREM-2, fatty acid binding protein 4 (FABP-4), and FABP-5, parallel to the TREM-2+ macrophages, which we previously identified in healthy skin (17) (Figure 1B and Supplementary Figure 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). In contrast to the 9 myeloid populations common to healthy control and dcSSc skin samples, 3 other myeloid populations (clusters 4, 5, and 10) were detected almost exclusively in dcSSc skin (Figures 1A–D).

We reanalyzed the data set using Harmony to correct for potential batch effects (21). Harmony analysis detected the same clusters shown above (Figure 1 and Supplementary Figure 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract), except it failed to identify a discrete cluster for proliferating macrophages (described further below) and indicated an additional population of macrophages (see cluster 4 in Supplementary Figure 6A). Although this additional macrophage cluster included cells from healthy skin (Supplementary Figure 6C), this population was not seen previously when analyzing only healthy skin (17).

Differential gene expression of dcSSc myeloid populations. Mean values for differential gene expression in cells within each myeloid cluster were compared between dcSSc skin and healthy control skin (Supplementary Table 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). We then analyzed the Gene Ontology (GO) pathways activated in each myeloid population in SSc skin after selecting differentially expressed genes with a fold change >1.5, detectable expression of each gene in >25% of SSc cells in the cluster, and an uncorrected *P* value <0.05 (Supplementary Table 7). CCR-1+ macrophages in dcSSc skin showed several statistically significant up-regulated pathways, including genes associated with the IFNγ pathway, *ICAM1*, *IFIT3*, *CCL13*, and *MT2A* (22), and innate immune response pathways (genes not shown). SSc MARCO+ macrophages showed up-regulated genes in several pathways, including the following: pathways associated with leukocyte chemotaxis (*IL10*, *CXCL1*, *CCL4*, *GPR183*, *CXCL8*, *S100A9*, *IL1B*, and *CCL3*), innate immune response pathways (genes not shown), pathways associated with response to type 1 IFN (*EGR1*, *ISG15*, and *IFITM3*), and pathways associated with response to IFNγ (*CCL3*, *CCL4*, *HLA-DRB5*, and *IFITM3*), as well as other pathways. Both SSc cDC2 subsets showed up-regulation of similar genes, but the only significant GO pathway was associated with immune response genes (*C1QC*, *HLA–DRB5*, *CRIP1*, *IFITM3*, *LTB*, *PKM*, *IFITM2*, *S100B*, and *CST7*). SSc cDC1 showed up-regulated genes in several pathways, including the Fcγ receptor signaling pathway involved in phagocytosis (*ACTR1*, *ACTR2*, *ACTR3*, *ACTB*) and the pathway associated with *CDC42* and response to wounding (*ANXA1*, *TNFAIP3*, *CXCR4*, *GRN*, *ACTG*, and *KLF4*). However,

macrophage-related genes previously detected as highly expressed in dcSSc skin by bulk RNA expression analysis in the skin (23) were not found to be up-regulated in these clusters (Supplementary Figure 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract).

SIGLEC1 (CD169), a marker of IFN in SSc skin and a marker of disease severity (6,11), was expressed only in the macrophage/DC cluster and was up-regulated in this cluster. On subsetting this cluster, SIGLEC1 was found mainly on subclusters 0, 1, 2, 4, and 11 (all macrophage clusters except cluster 1) and was up-regulated in SSc, mainly on clusters 0, 1, 4, and 10 (see Supplementary Table 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract).

Fcγ receptor IIIa–positive (FCGR3A+) macrophages and potential progenitor populations in dcSSc skin. Cells in subcluster 4 (FCG3RA+ macrophages) highly expressed macrophage markers, including MS4A4A and FCGR3A encoding CD16A (Figures 1A and B and Supplementary Figures 5 and 6). These cells also selectively expressed SLC40A1, IFI27, macrophage scavenger receptor 1 (MSR1), RCAN1, and OLR1, suggesting specialized functions of FCGR3A+ macrophages (Figure 2A). Most of the cells in this cluster were from only 2 of the 12 dcSSc subjects: patient SC69ssc (44 cells) and patient SC189ssc (155 cells), whereas the remaining 25 cells were from 5 healthy control subjects (14 cells) and 5 dcSSc patients (11 cells) (Figures 1C and D and Table 1), suggesting that this subpopulation is highly expanded, but only in select dcSSc patients.

The differential gene expression in FCGR3A+ macrophages, determined by assessing gene expression in dcSSc skin relative to healthy control skin, showed only a few genes that were significantly differentially expressed in SSc skin (Supplementary Table 7, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). We therefore compared gene expression by cells in this cluster with all the other macrophage/DCs from healthy control clusters (Supplementary Table 8), showing many significantly up-regulated genes. Genes encoding chemokines CCL18, CXCL1,

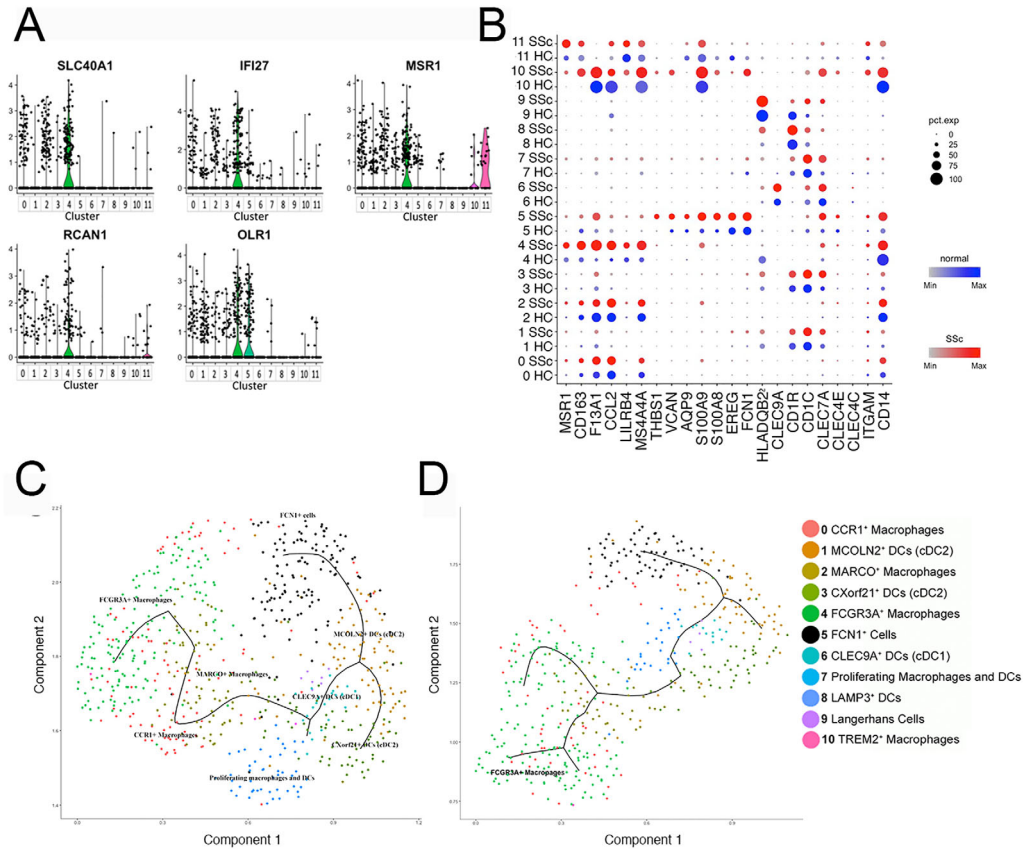

Figure 2. Gene signatures and putative functions of cluster 4 FCGR3A+ macrophages. **A**, Violin plots show marker gene expression in cluster 4. Each point represents a single cell; colors correspond to the cell subtypes defined in **D**. **B**, Dot plots show genes selectively expressed by FCGR3A+ macrophages in the skin of dcSSc patients and healthy controls. **C** and **D,** Pseudotime analysis tracking the relationship between transcriptomes of all myeloid cells from dcSSc and healthy control skin samples (**C**) and myeloid cells from a single dcSSc skin sample (patient SC189) (**D**) showing FCGR3A+ macrophages trajectory from CCR1+ and MARCO+ macrophages, and FCN-1+ monocyte-derived DC trajectory from MCOLN2+ cDC2. Each dot represents an individual cell. pct. exp = percent expressing (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract.

CCL8, CCL3, CCL4, CCL2, CCL13, and CXCL2 were highly expressed in this cluster. FCGR3A+ macrophages also showed up-regulated expression of genes correlated with the severity of dcSSc, such as *MSR1*, *CD163*, *F13A1*, *CCL2*, *LILRB4*, and *MS4A4A* (11,23) (Figure 2B). Based on the highly expressed genes of FCGR3A+ macrophages, GO analysis revealed significantly enriched, relatively broad functions such as "immune system process" and "immune response," but also several more specific enriched functions, including multiple pathways involved in chemotaxis, and Toll-like receptor (TLR)/innate immune activation, including "response to lipopolysaccharide (LPS)" and "TLR-6:TLR-2 signaling pathway" (Supplementary Table 9, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract).

FCGR3A+ macrophages expressed many of the most highly differentially expressed genes of CCR1+ cells (subcluster 0) and MARCO+ macrophages (subcluster 2) at moderate levels, suggesting a close relationship between these macrophage subpopulations (Figure 2B and Supplementary Figure 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). To further explore the relationship between FCGR3A+, CCR1+, and MARCO+ macrophages, we analyzed the data set trajectory using pseudotime analysis, an algorithm that tracks the relationship between transcriptomes of single cells (24). Pseudotime analysis of myeloid cells from all subjects showed that FCGR3A+ macrophages very closely related to and even admixed with CCR1+ macrophages but were also closely adjacent to MARCO+ macrophages (Figures 2C and D).

Increased numbers of FCN-1+ myeloid cells in dcSSc skin. The number of FCN-1+ myeloid cells was significantly increased in dcSSc skin compared with healthy control skin ($P = 0.001$), indicating that this cluster is specifically expanded in dcSSc (Table 1). These cells were unevenly distributed across different dSSc samples, with many of the cells coming from 2 patients: SC188ssc and SC189ssc (Figure 1 and Table 1). Cells in this cluster (cluster 5, FCN-1+ mo-DCs) selectively showed up-regulated expression of *FCN1*, *EREG*, *S100A8*, *AQP9*, *VCAN*, and *THBS1*, and expressed macrophage markers *CD163*, *F13A1*, and *MS4A4A* at only moderate levels (Figure 2B and 3A and Supplementary Figure 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). As FCN-1 and EREG are monocyte markers, relative signature scores were calculated for the expression levels of CD14 monocyte and FCGR3A+ monocyte marker genes, previously described by single-cell RNA-seq analyses of human peripheral blood mononuclear cells (PBMCs) (25). FCN-1+ cells showed some similarity to CD14 monocytes, but not FCGR3A+ monocytes (Supplementary Figures 7A and B, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). Among the FCN-1+ myeloid cells, the 50 top differentially expressed genes, *FCN1*, *S100A8*, *AQP9*, *VCAN*, *CD300E*, *S100A9*, *FPR1*, *SOD2*, and *C5AR1*, are markers of classic monocytes (Supplementary Table 5); however, other monocyte marker genes, *ITGAM/CD11B*, *ITGB2/CD18*, *TLR2*, and *CLEC7A*, were not enriched in this cluster (Figure 2B).

In SSc skin, FCN-1+ cells maintained expression of CD14, CD13/ANPEP, CD172a/SIRPA, as well as S100A8 and S100A9, all described as markers of mo-DCs (Supplementary Figure 11A, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract) (26), with only low expression of CD1c, CD1a, and IFN regulatory factor 4 (IRF-4), distinguishing these cells from DCs, and no expression of MS4A4A and CD163, distinguishing these cells from macrophages (Figure 2B). In a pseudotime analysis, FCN-1+ cells were on a trajectory closest to cDC2 (Figures 2C and D), further supporting their designation as mo-DCs (26).

Based on the differentially expressed genes of FCN-1+ mo-DCs, GO pathway analyses revealed significantly enriched, relatively nonspecific terms such as "inflammatory response," "defense response," "response to stress," and "immune response." These and many other terms overlapped with those seen in FCGR3A+ macrophages, including pathways involved in chemotaxis and response to LPS (Supplementary Table 9, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract).

To verify the associations of FCN-1+ and FCGR3A+ myeloid cells with SSc skin, we compared 15 additional dcSSc biopsy specimens from another cohort to the same 10 healthy control biopsy specimens. FCN-1+ but not FCGR3A+ cells were again identified as a discrete cluster in a t-SNE plot of myeloid cells (subcluster 4) (Supplementary Figures 8 and 9, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract), were significantly increased in dcSSc skin (Supplementary Table 10), and correlated with the total number of myeloid cells in biopsy specimens (Figure 3B).

Perivascular localization of FCN-1+ myeloid cells in dcSSc skin. IHC analysis of dcSSc skin showed FCN-1+ myeloid cells distributed primarily in perivascular regions in dcSSc skin (Figure 3C). Immunofluorescence staining of FCN-1+ cells in 16 healthy control and 39 dcSSc skin biopsy specimens showed an increased number of FCN-1+ cells in dcSSc patients compared with healthy control subjects ($P = 0.0141$) (Figure 3D, Supplementary Figure 10, and Supplementary Table 11, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). More FCN-1+ myeloid cells were seen in dcSSc skin biopsy specimens from patients with a higher MRSS (>20) compared with a lower MRSS (<20) ($P = 0.0005$) (Figure 3E), FCN-1+ myeloid cells were also significantly correlated with the MRSS ($P < 0.05$) (Supplementary

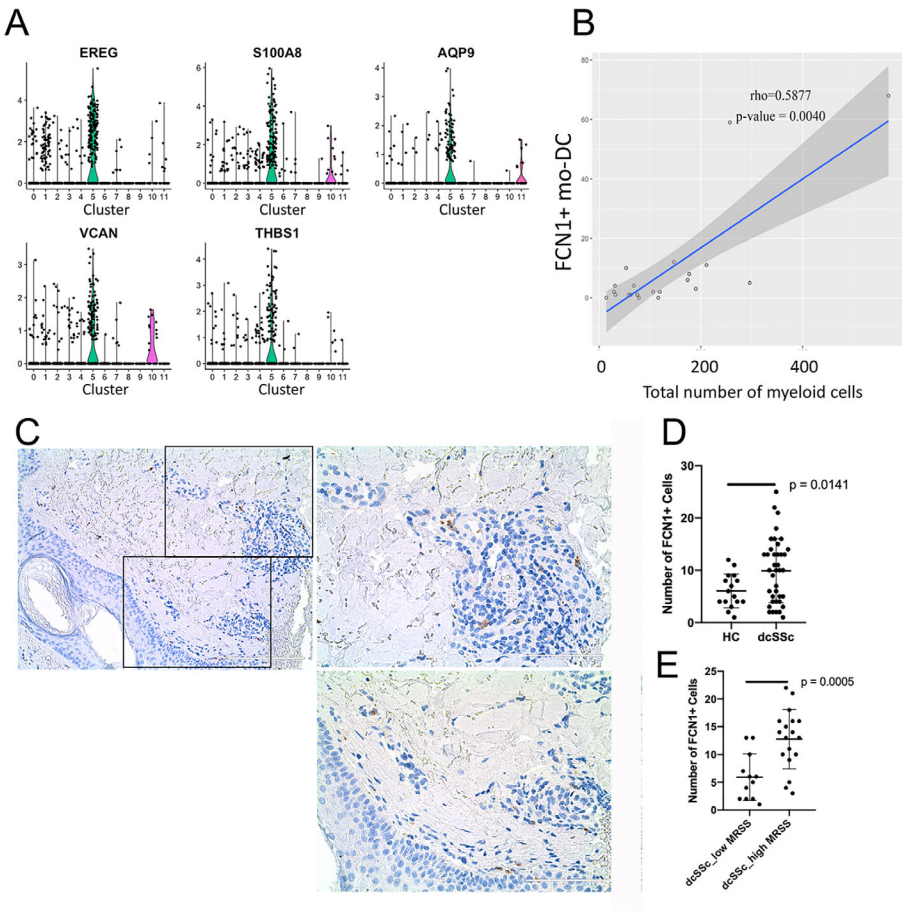

Figure 3. Gene signatures and putative functions of cluster 5 FCN-1+ monocyte-derived DCs (mo-DCs). **A**, Violin plots show marker gene expression in cluster 5 FCN-1+ cells. Each point represents a single cell; colors correspond to the cell subtypes defined in Figure 1A. **B**, The correlation between the total number of myeloid cells and the number of FCN-1+ mo-DCs in dcSSc skin samples was assessed using Spearman's rho. **C** and **D**, Staining shows the perivascular distribution of FCN-1+ cells in a representative skin sample from a patient with dcSSc (**C**) (immunofluorescence is shown in Supplementary Figure 10, http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract), with the results revealing more FCN-1+ cells in dcSSc skin compared to healthy control skin (**D**). In **C**, the right panels show higher-magnification views of the boxed areas (original magnification × 10 on left; × 40 on right). **E**, Immunofluorescence staining of skin biopsy specimens from patients with dcSSc shows that the number of FCN-1+ cells differed according to whether the patients had either a low or high modified Rodnan skin thickness score (MRSS) (with high MRSS being defined as >20). *P* values in **D** and **E** were determined by Wilcoxon's rank sum test. Symbols represent individual patients; horizontal lines with bars show the mean ± SD. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract.

Figure 10B, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract).

Identification of FCN-1+ myeloid cell markers in bulk microarray analyses of messenger RNA expression in the skin of patients with dcSSc. To extend our single-cell RNA-seq observations, we examined bulk microarray data from 64 SSc and 15 HC skin biopsy specimens. Several genes, including *LILRA5*, *FCN1*, *SERPINA1*, *FPR1*, *LILRB2*, and *TREM1*, were expressed by myeloid cells (cluster 9 in all cells shown in a t-SNE cluster plot) and were most highly expressed by FCN-1+ myeloid cells (myeloid subcluster 5) (Supplementary Figures 11A and B, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract) and not by other skin cells or myeloid cell populations (Supplementary Figure 12). The expression of these genes from bulk sequencing analysis confirms the emergence of FCN-1+ mo-DCs in a subset of dcSSc patients (Figure 4A). Most of the genes in these clusters were selectively expressed in FCN-1+ mo-DCs (*FCN1*, *LILRA5*, *SERPINEA1*, *FPR1*, *LILRB2*, and *TREM1*), were selectively expressed in macrophages (*CD14* and *CD163*), or were more broadly expressed by myeloid cells (*AIF1* and *PLEK*) (Figure 4B). Moreover, the MRSS scores for SSc patients expressing markers of FCN-1+ cells were higher than those of dcSSc patients not expressing these markers ($P = 0.0019$) (Figure 4C), consistent with a general increase in macrophage/inflammatory cell infiltration in patients who have FCN-1+ mo-DCs, as shown by histologic findings and by the observed correlation between FCN-1+ mo-DCs and numbers of myeloid cells (Figure 4A).

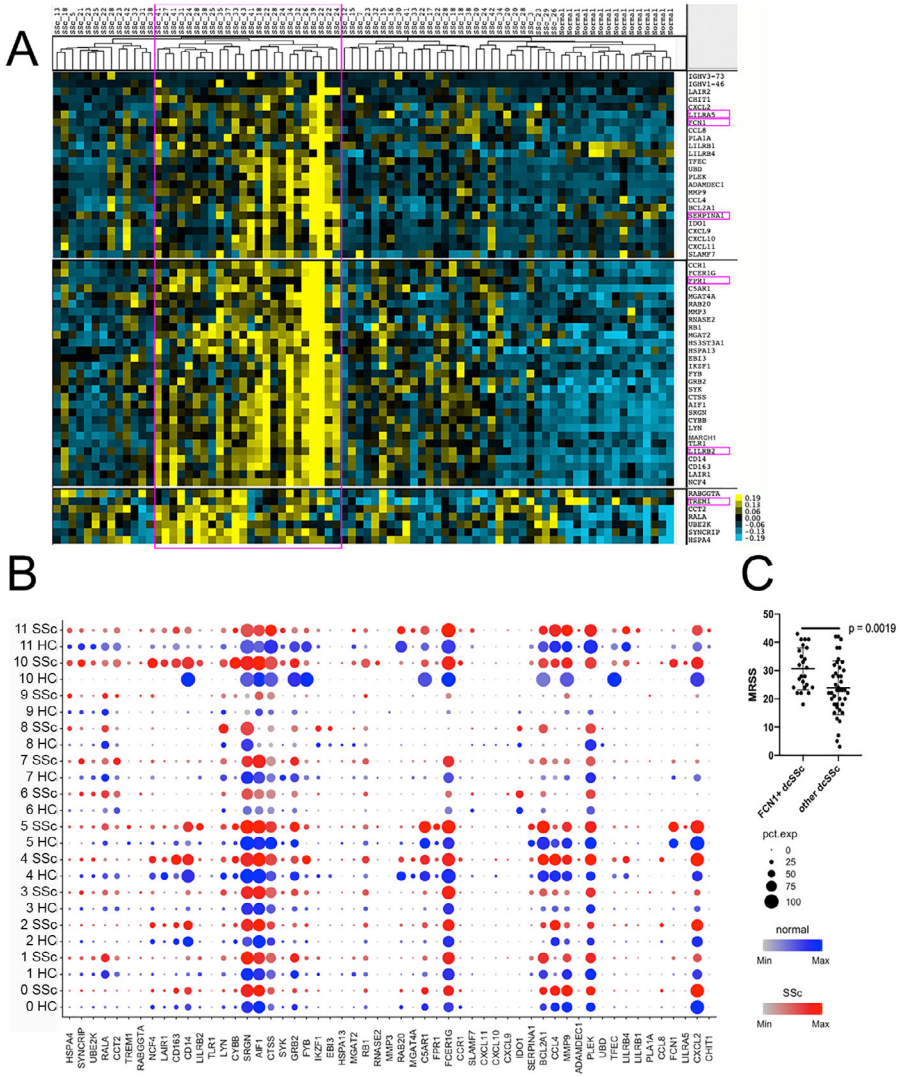

Figure 4. **A**, Heatmaps from hierarchical clustering analyses of bulk sequencing data reveal clusters of genes showing increased expression in myeloid cells, macrophages, and FCN1+ monocyte-derived DCs (mo-DCs) from patients with dcSSc (n = 64) relative to healthy controls (n = 15). The pink boxes highlight those gene markers with increased expression in FCN-1+ cells, which were used to identify cluster 5. **B**, Dot plots show gene expression in each myeloid cell type based on the results of a single-cell RNA sequencing analysis of skin samples from dcSSc patients and healthy controls. **C**, The modified Rodnan skin thickness score (MRSS) was compared between dcSSc patients in gene cluster 5 (high gene expression in FCN-1+ cells) and dcSSc patients having different gene cluster profiles ($P = 0.0019$, by Wilcoxon's rank sum test). Symbols represent individual patients; horizontal lines with bars show the mean ± SD. pct. exp = percent expressing (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract.

Proliferating macrophages and DCs in dcSSc skin. Proliferating myeloid cells (subclusters 7 and 10), predicted to be in the G_2/S phase by a cell phase analysis (Supplementary Figure 13A, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract), were clustered separately by their uniquely high expression of genes associated with active cell proliferation, including *KIAA0101*, *MKI67*, *TYMS*, *PTTG1*, *CDK1*, and *PCNA* (Supplementary Figure 6 and Supplementary Table 5). Cells in the lower corner in subcluster 7 were identified as proliferating cDC2 cells, as we have previously described in healthy skin, expressing both *KIAA0101* and *CD1c* (Supplementary Figure 13B). These were found in similar numbers in dcSSc and healthy control skin (Figure 1D). In addition, we identified a group of proliferating macrophages based on coexpression of cell proliferation genes and MS4A4A (subcluster 10) (see Supplementary Figure 13C, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). Cells making up this cluster were mainly from patient SC188ssc (13 of 25 cells) and patient SC189ssc (10 of 25 cells), the same patient samples showing the most FCN-1+ mo-DCs (Supplementary Table 4).

To track which macrophage subpopulations were proliferating, we examined expression of single marker genes in each macrophage cluster and module scores of each cluster based on the

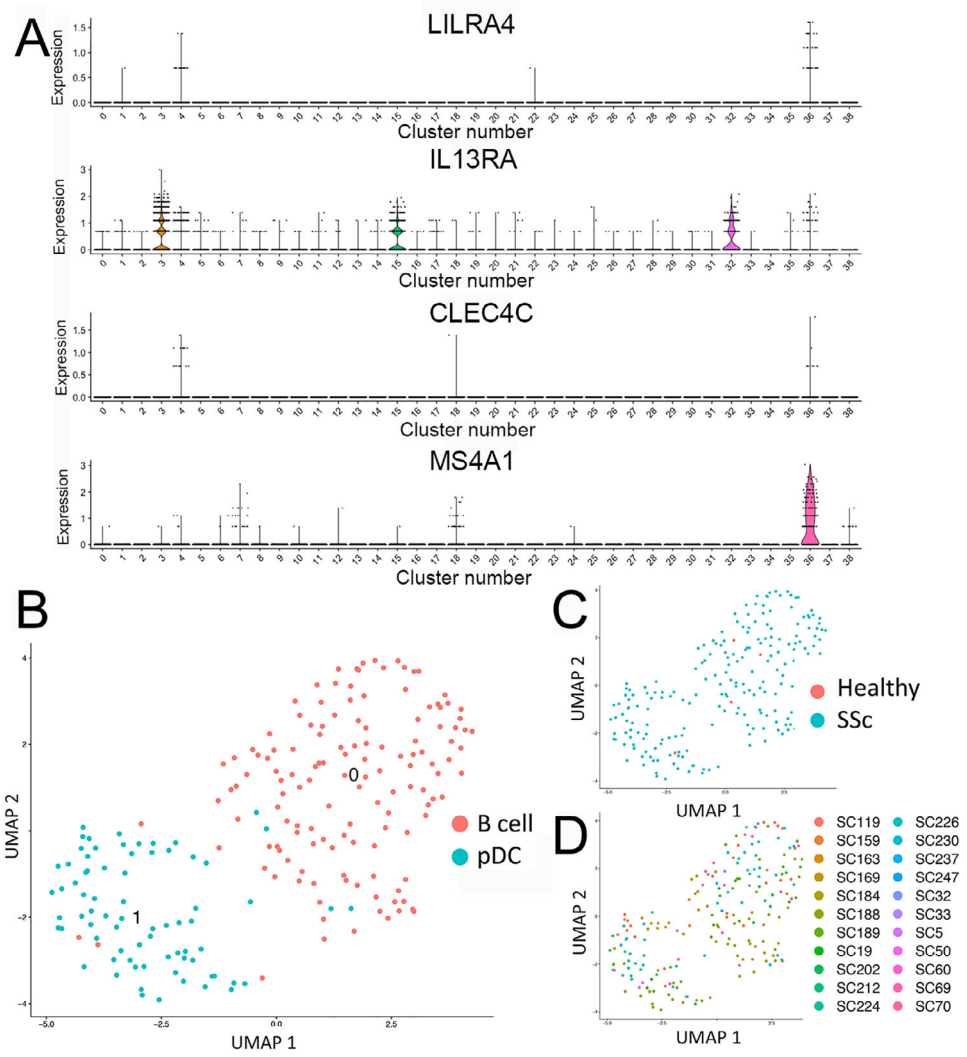

Figure 5. Gene cluster analyses of plasmacytoid DCs (pDCs) in dcSSc and healthy control skin. **A**, Violin plots show expression of pDC markers *LILRA4*, *IL3RA* (*CD123*), and *CLEC4C* (*BDCA2*), as well as B cell marker *MS4A1* (*CD20*) in 27 dcSSc skin samples and 10 healthy control samples in cluster 36. **B**–**D**, Dot plots show reclustered cells in cluster 36 according to the subsets of B cells or pDCs (**B**), health status (**C**), or sample origin (**D**). Each dot represents an individual cell. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract.

top 10 most highly expressed genes in each macrophage cluster in the proliferating cell cluster (Supplementary Figure 14, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). It appeared that all the macrophage subsets, as well as FCN-1+ mo-DCs, were represented in the proliferating cell cluster.

Clustering of pDCs with B cells and increased pDCs in dcSSc skin. Because we did not find pDCs in our initial clustering, we clustered all 27 dcSSc samples with the 10 normal skin samples (data not shown) and searched again for markers of these cells. *LILRA4*, *IL3RA*, and *CLEC4C* were coexpressed in a cluster comprising mostly B cells (Figure 5). Reclustering these cells resulted in the detection of 2 clusters, 1 prominently expressing B cell markers (*MS4A1A*, *CD79A*, and Ig genes) and the other expressing markers of pDCs (*LILRA4*, *IL3RA*, and *CLEC4C*), described previously as circulating DC6 (17,20). The identified pDCs came nearly exclusively from dcSSc patients (73 cells of 74 total cells versus 1 of 74 total cells from the 10 healthy controls). We detected up-regulated expression of transcription factors that are important in pDC differentiation: IRF-4, IRF-7, IRF-8, and ZEB-2 compared to other skin cells or B cells, but not expression of IFNA1–11 or CXCL4 (Supplementary Figure 15 and Supplementary Table 12, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract).

DISCUSSION

Using bulk RNA analyses, we have previously implicated markers of macrophages in both the severity and progression of SSc skin disease (11,23,27). Single-cell RNA-seq studies here

provide a comprehensive analysis of myeloid cell populations in dcSSc skin. While all myeloid clusters detected in healthy skin were preserved in dcSSc skin, 4 dcSSc-specific myeloid populations were discovered: FCGR3A+ macrophages, FCN-1+ mo-DCs, pDCs, and proliferating macrophages. The transcriptomes of each of these populations revealed gene signatures and putative altered functions of each cluster. Importantly, FCN-1+ mo-DCs were perivascularly distributed along with other inflammatory cells, were found at a higher frequency in dcSSc, and were associated with the severity of skin disease.

Fibrotic SSc skin preserved all of the myeloid populations that we previously reported in healthy skin, including CCR1+, MARCO+, and TREM2+ macrophages, as well as 6 clusters of DCs: cDC1, 2 cDC2 populations, Langerhans' cells, a mature subpopulation of LAMP-3+ cDCs, and a population of proliferating cDC2 cells (17). Up-regulated genes in these clusters indicated pathways of innate immune and TLR activation and responses to both type I and type II IFNs, consistent with several previous studies (6,10,28,29).

The highly increased frequency of FCN-1+ mo-DCs in dcSSc skin was verified by single-cell RNA-seq, microarray analysis, IHC, and immunofluorescence staining for FCN-1. The expression of monocyte marker genes *FCN1*, *S100A8*, and *S100A9* by these cells indicates that these cells are recruited from circulating monocytes. A trajectory analysis, as well as low-level expression of CD1a and CD1c, indicated that FCN+ cells are most closely related to mo-DCs (30). Cells with very similar gene expression profiles have been identified in blister fluid of saline and house dust mite–stimulated human skin (31). In humans, mo-DCs are one of several myeloid cell types capable of cross-presentation (26). The association of these cells with perivascular inflammation and high expression of multiple chemokines suggests that they may promote the migration of other inflammatory cells.

THBS1 and *FPR1*, which are both pharmacodynamic biomarkers for the extent of skin disease and are prognostic markers for progressive skin disease in dcSSc (11,23), were predominantly expressed by FCN-1+ mo-DCs (Supplementary Figure 15, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41813/abstract). FCN-1+ mo-DCs were also shown to be significantly associated with dcSSc severity (MRSS >20), suggesting a key role in disease pathogenesis. In addition, these cells have likely played a prominent role in previous bulk RNA SSc skin expression studies, which have described a subset of patients with dcSSc (12,32), as patients overexpressing FCN-1+ markers also overexpressed both IFN-regulated and Ig genes (Supplementary Figure 16).

FCGR3A+ macrophages expressed most other macrophage markers, though they failed to express monocyte markers, and trajectory analysis suggested that CCR1+ macrophages and/or MARCO+ macrophages are the progenitors of these cells. As CCR1+ and MARCO+ macrophages are present in normal skin and proliferate in SSc skin, it is likely that these are resident cell populations that differentiate the FCGR3A+ macrophages in SSc skin. Enriched GO terms suggest that FCGR3A+ macrophages are activated through TLR signaling. Several recent studies have placed TLR signaling upstream of inflammatory and profibrotic changes in SSc (33–35). Potentially, TLR stimulates FCGR3A+ macrophages that highly express many cytokines and chemokines that attract and activate other immune cells, including CCL18 and interleukin-6 (IL-6). We have previously reported that *CCL18*, the gene shown to have the highest increase in expression (4.4-fold increase), is up-regulated in SSc skin and SSc-related interstitial lung disease (ILD) (8,10). Serum levels of CCL18 were rapidly reduced to normal levels, and CCL18 expression in the skin was blocked upon inhibition of IL-6, suggesting that IL-6 may play a role in the differentiation/activation of FCGR3A+ macrophages in select patients demonstrating infiltration with this macrophage population (36).

We identified proliferating macrophages in dcSSc skin, but not normal skin. It appeared that all macrophage subsets and FCN-1+ mo-DC cells were proliferating in some dcSSc patients. The majority of the proliferating cells were from patient SC188ssc and patient SC189ssc, the same patients who contributed most of cluster 5 of FCN-1+ mo-DCs. We have recently characterized proliferating macrophages in idiopathic pulmonary fibrosis and have seen similar cells in SSc-associated ILD (37,38), suggesting that common cytokine signals may drive macrophage proliferation in both SSc skin and the SSc lung.

Previous studies have detected perivascular pDCs in the affected skin of SSc patients, but not the skin of control subjects, by co-staining of CD123 and in situ *IFNA* expression (15), or by expression of *CLEC4* (BCDA-2) (16). Consistent with these studies, we found that pDCs were very rare in normal skin (1 of 20,073 cells) and were increased in numbers but still rare in dcSSc skin (73 of 74,607 cells). We were unable to detect expression of *IFNA1–IFNA11* or *CXCL4* genes by PDCs or any other cell type in SSc skin, the former classically secreted by these pDCs and the latter described as up-regulated in SSc pDCs (16) and implicated in the coactivation of TLR-9 (14,39). This likely represents the relatively low-level expression of these genes, though we were able to easily detect CXCL4 in platelets in a different data set (Lafyatis R, et al: unpublished observations). Although rare, these pDCs are likely the main source of type I IFNs in SSc skin and the resulting effects on multiple other cell types. The functional importance of these cells in skin fibrosis is supported by markedly ameliorated bleomycin-induced fibrosis in skin upon pDC depletion (14). Our data confirm the expression of IRF-8 in dermal pDCs, previously shown to be up-regulated in pDCs in SSc PBMCs (14), as well as up-regulated expression of IRF-4 and IRF-7 compared to other dermal myeloid cells or their closest transcriptome relative, B cells. The clustering of SSc pDCs with B cells is intriguing in terms of the recent understanding that pDCs can differentiate along a myeloid or lymphoid pathway with a

common B cell and pDC progenitor (40,41). The clustering of these cells with B cells in SSc skin suggests that pDCs differentiate exclusively along the lymphoid pathway from an IL-7R+FLT-3+ lymphoid progenitor.

Not all SSc patients demonstrate dramatic myeloid expansion, so it remains possible that myeloid cells drive pathogenesis in only a subset of patients, consistent with bulk microarray studies (32). As macrophage subsets are also involved in repair, it is also possible that their presence reflects repair processes in the skin (42). The lack of local skin disease score data is a limitation in this study.

In summary, our data reveal several populations of myeloid cells in dcSSc that are likely driving SSc vascular pathology and skin fibrosis. This more profound understanding of differing subsets of patients with inflammatory dcSSc provides new insights into stratifying patients for targeted therapies and can possibly be used for predicting responses to immunosuppressive treatment.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Lafyatis had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Xue, Tabib, Morse, Domsic, Khanna, Lafyatis.

Acquisition of data. Xue, Tabib, Morse, Yang, Domsic, Khanna, Lafyatis.

Analysis and interpretation of data. Xue, Tabib, Morse, Yang, Domsic, Khanna, Lafyatis.

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