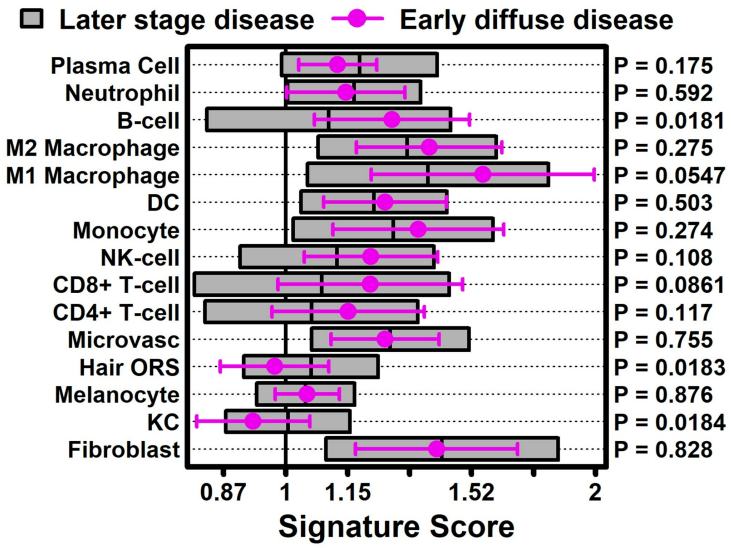
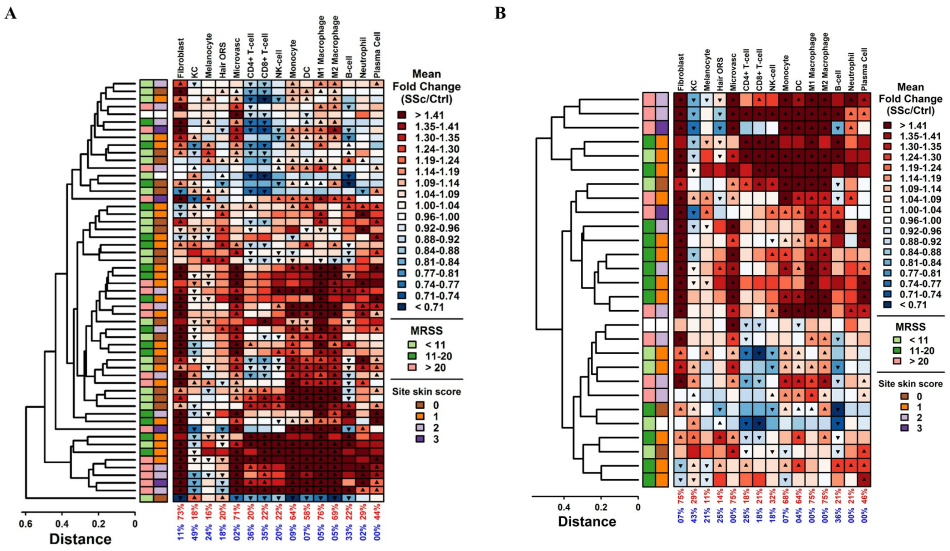
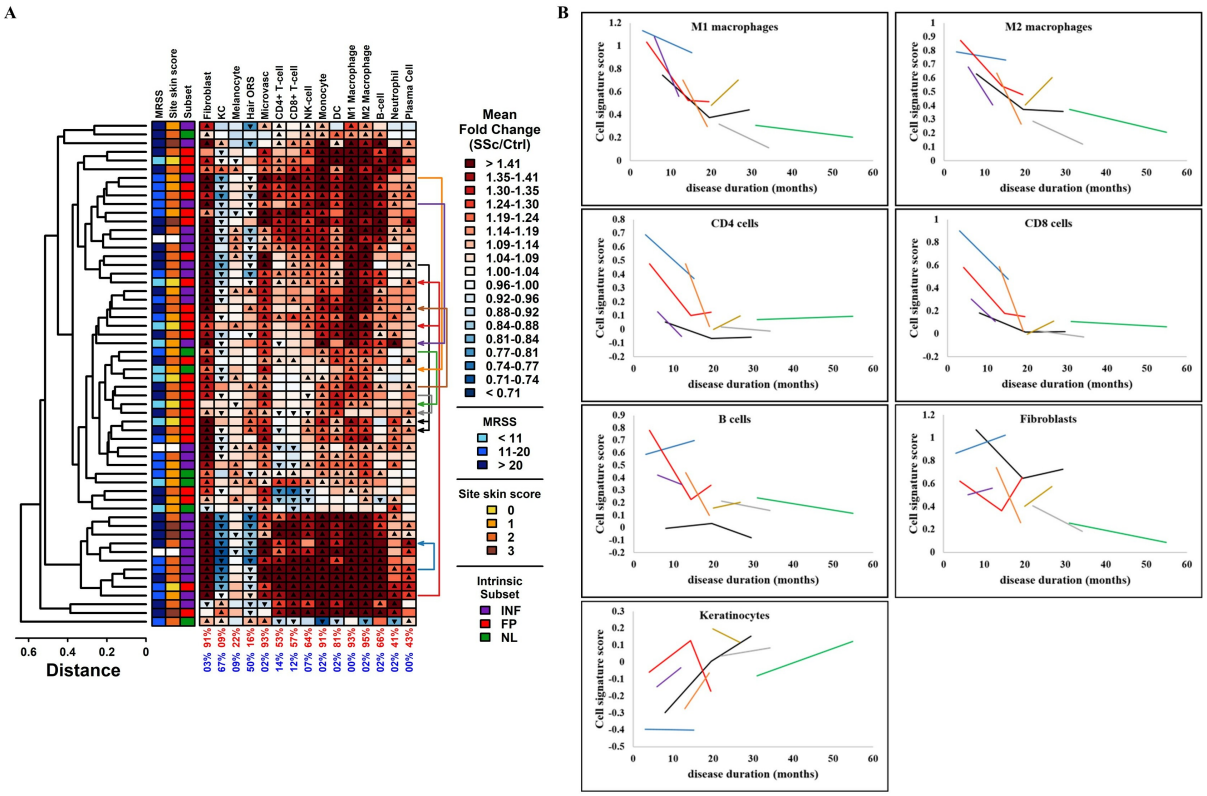
Supplementary Figure 1

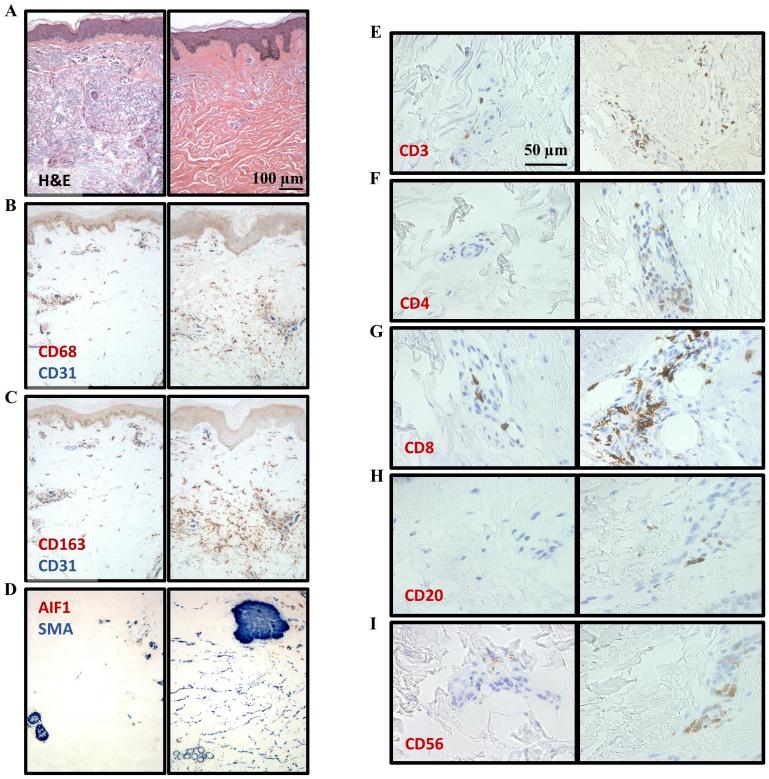


Supplementary Figure 2



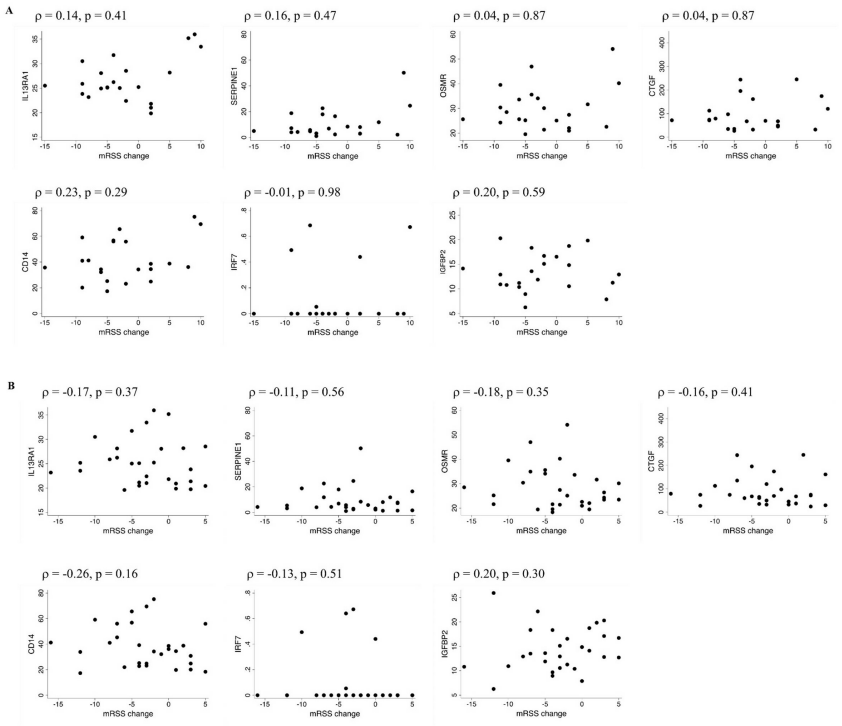


Supplementary Figure 3

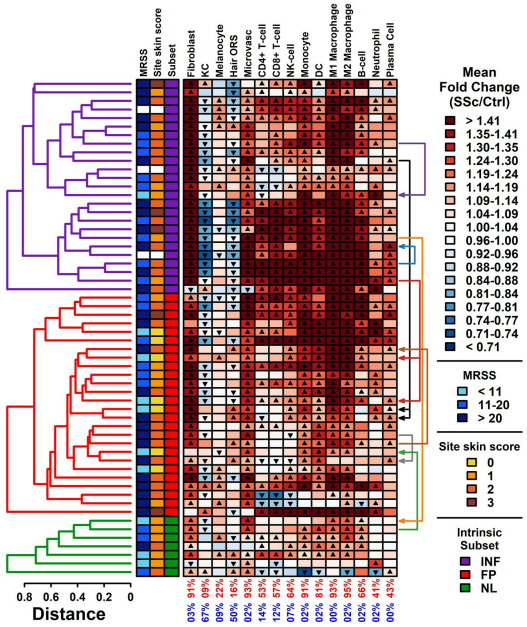


Supplementary Figure 4

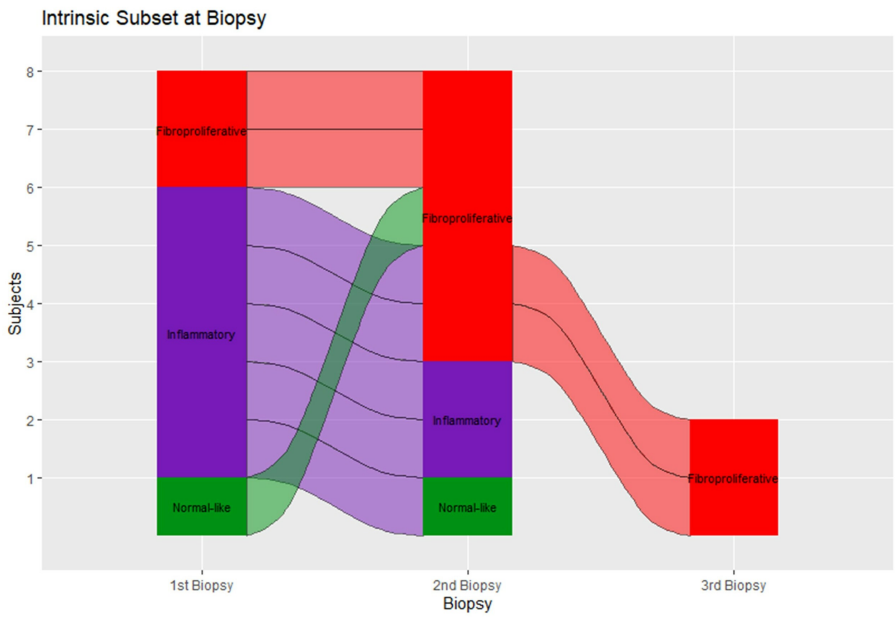
Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7



Supplementary material

Global skin gene expression analysis of early diffuse cutaneous systemic sclerosis shows a prominent innate and adaptive inflammatory profile

METHODS

Skin biopsy and RNA sequencing

One to two 3- or 4-millimeter punch biopsies were obtained from the forearm skin. One was immersed in RNAlater solution (Qiagen), and the other in formalin when a second biopsy was obtained. RNA later samples were frozen and shipped on dry ice to the University of Texas Houston (UTH) study site. RNA was extracted using miRNeasy Mini kits (Qiagen). Data from the PRESS cohort were compared to similarly-obtained data from the GENISOS cohort that included SSc patients with longer disease duration at the time of biopsy[1]. Although microarray technology was used for gene expression profiling in the previously published study, we performed RNA sequencing in these GENISOS samples (n = 55) and matched healthy controls (n = 33) for the present study in order to avoid heterogeneity resulting from methodological differences.

RNA integrity was measured on an Agilent 2100 Bioanalyzer (Agilent Technologies). For the PRESS biopsies, cDNA libraries were prepared using the Illumina TruSeq stranded Total RNA Library Prep Gold kit according to the manufacturer's protocol. Briefly, ribosomal RNA (rRNA)-depleted RNAs were fragmented and reverse transcribed to cDNAs. The cDNAs were converted to double stranded cDNAs, then subjected to end-repair, A-tailing, and adapter ligation. The constructed libraries were amplified by 8 cycles of PCR. cDNA library quality was measured on an Agilent 2200 TapeStation (Agilent Technologies), and its quantity was measured using KAPA Library Quantification Kit (KAPA Biosystems) prior to sequencing. The libraries were loaded on cBot (Illumina) at a final concentration of 10 pM to perform cluster generation, followed by 2 x 76 bp paired-end sequencing on HiSeq 2500 (Illumina), generating on average around 50 million reads per sample. For the GENISOS biopsies, cDNA libraries were also prepared using the Illumina TruSeq stranded Total RNA Library Prep Gold kit. 52 bp single end sequencing was performed, using HiSeq 2000 (Illumina).

RNA Sequencing data analysis

Raw reads were mapped to the human genome hg38, downloaded from the University of California Santa Cruz Genome Bioinformatics site (http://genome.ucsc.edu), with no more than two mismatches for each read, using TopHat v2.1.11.[2] Expression level was calculated as fragments per kilobase per million reads (FPKM) with Cufflinks v2.2.1,[3] and transcript count value was obtained using htseq-count,[4] using default parameters. The annotation file for transcripts is from gencode v22 comprehensive gene annotation gtf file (http://www.gencodegenes.org).[5] Those transcripts with FPKM values >0 in at least 20% of total samples (including patients and healthy controls) were used for further analysis. The R Bioconductor package edgeR[6] was applied to identify differentially expressed transcripts between SSc patients and healthy controls with a false discovery rate cutoff of 0.05 and fold change cutoff of >1.5 or <0.67. Long non-coding RNA

(lncRNA) transcripts were identified based on GENCODE annotations[7]. Pearson's correlation coefficients and Spearman's rank order correlation coefficients between transcript expression and mRSS or FVC were analyzed using R. Differentially expressed genes were modeled using Ingenuity Pathways Analysis software (Qiagen) to identify putative upstream regulators of the observed gene expression differences, as previously described.[1]

Ingenuity Pathway Analysis

Genes that were differentially over- or under-expressed on average in SSc compared to HC by 1.5-fold at a false discovery rate of <0.05 were uploaded to Ingenuity Pathway Analysis (Qiagen). Expression analysis was performed using "Ingenuity Knowledge Base (Genes Only)" as reference set, including "direct and indirect relationships," with Confidence set to "Experimentally observed" only. The top ten canonical pathways are reported in the manuscript (Figure 1B). The ten upstream regulators as well as cytokines/growth factors with the highest activation z-score are reported in the manuscript (Figure 1C-D).

Analysis of cell type-specific expression

We performed cell-type specific gene expression analysis using the method we have used previously.[1 8] In our earlier study, we assembled a gene expression database with samples from 14 cell types, where each sample had been generated using the Affymetrix Human Genome U133A array platform[1]. This database was modified slightly for the current analysis to provide finer resolution of macrophage expression by including separate M1- and M2-polarized macrophage signatures, yielding a total of 15 skin-associated cell types (fibroblast, keratinocyte, melanocyte, hair outer root sheet, microvasculature, CD4 T-cell, CD8 T-cell, NK-cell, monocyte, M1 macrophage, M2 macrophage, dendritic cell, B-cell, neutrophil, plasma cell). The final database included 276 samples, with an average of 18.4 samples per cell type (range: 2-30 per cell type). To identify cell type-specific genes, linear models with moderated t-statistics were used to compare expression in each cell type to the other 14 cell types (R package: limma).[9] The 250 genes with lowest p-values and increased expression in the target cell type were identified, and of these, we selected the 125 genes with highest fold-change (target cell type / 14 other cell types). This provided a ranked set of 125 cell type-specific signature genes for each of the 15 cell types, which were used to calculate signature scores for SSc patient biopsies. To calculate signature scores, raw mapped counts for protein-coding transcripts were normalized using the voom algorithm,[10] generating $\log_2$-normalized expression values. For each gene, the average expression difference between SSc and HC samples was estimated, yielding $\log_2$-scaled fold-change estimates. For a given cell type, the signature score was equal to the (weighted) average $\log_2$-scaled fold-change estimate (SSc/HC) among the 125 signature genes. As described previously,[8] the average was calculated with greater weight assigned to those signature genes more strongly elevated in the target cell type as compared to the other 14 cell types. For a given patient and cell type, a signature score was significantly elevated if fold-change estimates for the 125 signature genes were higher than those of non-signature genes ($p < 0.05$, Wilcoxon rank sum test). The cell type signature calculation was completed separately for the PRESS and GENISOS cohorts. Of note, for each data set their own matched healthy control samples were used to calculate the cell type signature scores, decreasing the potential impact of batch effect. Lastly, signature scores for

corresponding cell types were compared between the two cohorts using two-sample t-tests, with raw p-values obtained across the 15 cell types corrected using the Benjamini-Hochberg method.

Immunohistochemistry

Hematoxylin and eosin (H&E) staining and immunohistochemical analyses of formalin-fixed paraffin embedded sections of dermal biopsies were performed using standard methods that have previously been described.[11] Sections were stained by JLB and AMSB using antibodies to macrophage markers CD68 (Abcam 199000) and CD163 (Abcam 189915) and counterstained with endothelial cell marker CD31 (Abcam 182981 with CD68 and Thermo MA5-13188 with CD163). AIF (IBA1, Millipore ABN67) staining (another marker of macrophages) was combined with smooth muscle actin (Dako mAb 1A4). Scoring of these samples was performed by JLB and AMSB using a 0-3 scale, with the ranges being set based on the most extreme examples. Macrophage scoring was focused on the extra-vascular compartment, as the density of perivascular monocytes/macrophages was relatively constant across samples. A semi-quantitative score reflecting relative collagen thickness, ranging from 0-3, was assigned to each sample using H&E-stained sections. Staining of sections for CD3, CD4, CD8, CD20, and CD56 was performed at the UTH study site using the following antibodies from Agilent: CD3 (GA50361-2), CD4 (IR64961-2), CD8 (IR62361-2), CD20 (GA60461-2), and CD56 (IR62861-2). MW and NW counted a few slides together for training, then counted all samples while blinded to all clinical information and the marker used for staining. Every positive cell was counted except for exclusion of epidermis, hair follicles, and intravascular cells. After the initial counts, any sample with a coefficient of variation of >25% and an overall difference of 5 or more cells between observers was re-counted by both observers until consensus (CV <25% or overall difference <5) was achieved. Then the average count was used for analyses.

Statistical Analysis

Differences in histologic features between SSc patients and healthy controls were analyzed by Wilcoxon rank sum test. Differences in immune cell signatures between baseline and follow up biopsies were analyzed by Wilcoxon signed-ranks test.

Patient and Public Involvement

This study was supported by the Scleroderma Foundation; the study design was discussed at a Scleroderma Foundation meeting that included patients with scleroderma.

SUPPLEMENTARY TABLES

Supplementary Table 1: immune cell signatures in male vs female patients

Cell type signature	Male (n = 18)	Female (n = 30)	p value*
CD8 T cell, mean (SD)	0.30 (0.35)	0.26 (0.27)	0.59
CD4 T cell, mean (SD)	0.22 (0.30)	0.19 (0.21)	0.62
NK cell, mean (SD)	0.29 (0.25)	0.27 (0.20)	0.70
B cell, mean (SD)	0.42 (0.28)	0.30 (0.23)	0.10
M1 macrophage, mean (SD)	0.69 (0.38)	0.61 (0.35)	0.46
M2 macrophage, mean (SD)	0.50 (0.29)	0.44 (0.20)	0.37

***t-test**

Supplementary Table 2: immune cell signatures in RNA Polymerase III vs Topoisomerase-I antibody-positive patients

Cell type signature	RNA Pol III (n = 17)	Topoisomerase-I (n = 12)	p value*
CD8 T cell, mean (SD)	0.38 (0.28)	0.22 (0.25)	0.15
CD4 T cell, mean (SD)	0.28 (0.25)	0.19 (0.20)	0.31
NK cell, mean (SD)	0.35 (0.20)	0.24 (0.16)	0.15
B cell, mean (SD)	0.41 (0.23)	0.34 (0.24)	0.42
M1 macrophage, mean (SD)	0.75 (0.33)	0.59 (0.29)	0.20
M2 macrophage, mean (SD)	0.55 (0.30)	0.45 (0.22)	0.33

***t-test**

Supplementary Table 3: Multivariable regression analyses of key clinical variables with cell type specific signatures in pooled PRESS and GENISOS datasets, with adjustment for cohort.

	Coefficient	95% CI	P Value
CD8 T cell*			
Disease duration	-0.020	-0.039 – -0.001	0.04
mRSS	0.005	-0.002 – 0.013	0.17
FVC % pred	-0.001	-0.005 – 0.002	0.46
No immunosuppression	0.119	-0.040 – 0.277	0.14
PRESS cohort	0.111	-0.072 – 0.293	0.23
CD4 T cell*			
Disease duration	-0.016	-0,031 – 0.000	0.05
mRSS	0.003	-0.003 – 0.010	0.32
FVC % pred	-0.001	-0.004 – 0.002	0.42
No immunosuppression	0.078	-0.055 – 0.211	0.25
PRESS cohort	0.082	-0.071 – 0.236	0.29
NK cell*			
Disease duration	-0.017	-0.030 – -0.003	0.02
mRSS	0.004	-0.001 – 0.010	0.14
FVC % pred	-0.001	-0.004 – 0.001	0.35
No immunosuppression	0.095	-0.020 – 0.210	0.11
PRESS cohort	0.044	-0.089 – 0.177	0.51
B cell*			
Disease duration	-0.020	-0.036 – -0.003	0.02
mRSS	0.002	-0.005 – 0.008	0.64
FVC % pred	-0.001	-0.004 – 0.002	0.44
No immunosuppression	0.001	-0.135 – 0.138	0.99
PRESS cohort	0.070	-.087 – 0.228	0.38
M1 macrophage*			
Disease duration	-0.007	-0.027 – 0.012	0.45
mRSS	0.012	0.004 – 0.020	<0.01
FVC % pred	-0.002	-0.005 – 0.002	0.45
No immunosuppression	0.063	-0.101 – 0.226	0.31
PRESS cohort	0.106	-0.083 – 0.295	0.27
M2 macrophage*			
Disease duration	0.002	-0.013 – 0.017	0.84
mRSS	0.013	0.007 – 0.020	<0.01
FVC % pred	-0.001	-0.004 – 0.002	0.57
No immunosuppression	0.014	-0.112 – 0.141	0.82
PRESS cohort	0.045	-0.101 – 0.190	0.55
Fibroblast*			
Disease duration	-0.004	-0.022 – 0.014	0.67
mRSS	0.016	0.009 – 0.024	<0.01
FVC % pred	0.001	-0.002 – 0.004	0.50
No immunosuppression	0.009	-0.142 – 0.160	0.91
PRESS cohort	-0.089	-0.263 – 0.086	0.32

*Cell type transcript signature used as the dependent variable in the multivariable model

mRSS = modified Rodnan skin score, FVC = forced vital capacity

Supplementary Table 4: changes in cell type signatures in follow up biopsies

	Median (IQR)	Correlation with ΔmRSS from initial biopsy to follow up biopsy
ΔM1 macrophage	-0.25 (-0.46, -0.15), p = 0.0499*	0.52 (0.183)
ΔM2 macrophage	-0.22 (-0.32, -0.11), p = 0.0499*	0.69 (0.058)
ΔCD4 cell	-0.14 (-0.34, -0.004), p = 0.0499*	0.29 (0.493)
ΔCD8 cell	-0.18 (-0.43, -0.05), p = 0.036*	0.29 (0.493)
ΔB cell	-0.07 (-0.23, -0.01), p = 0.093	0.71 (0.047)*
Δfibroblast	-0.07 (-0.28, 0.11), p = 0.33	0.76 (0.028)*
Δkeratinocyte	0.08 (-0.04, 0.21), p = 0.16	-0.45 (0.26)
ΔmRSS	-4.5 (-9.5, -0.5), p = 0.08	

Middle column: Change in each cell signature score or mRSS (bottom row) is shown, using the final biopsy for each patient (n = 8) in comparison to the baseline biopsy (for samples with 3 biopsies, the third biopsy was compared to the baseline). Difference between the follow up biopsy and baseline biopsy was analyzed by Wilcoxon matched-pairs signed-ranks test. **Right column:** Correlation between change in cell type signatures and change in mRSS from baseline to follow up biopsy. Shown are the Spearman's rank order correlation coefficients for each association, followed by the p value in parentheses. * indicates $p < 0.05$. IQR = interquartile range, mRSS = modified Rodnan skin score

Supplementary Table 5: Demographics of SSc patients and healthy controls whose skin biopsies were examined by immunohistochemical staining and for collagen thickness

	HC (n = 12)	SSc (n = 15)
Age, mean (SD)	44.1 (13.1)	48.0 (15.0)
Female, n (%)	7 (58.3)	7 (46.7)
Race		
White, n (%)	12 (100)	11 (73.3)
Black, n (%)	0	3 (20.0)
Asian, n (%)	0	1 (6.7)

SSc = systemic sclerosis, HC = healthy control, SD = standard deviation

Supplementary Table 6: Comparisons of histologic features between healthy controls and PRESS systemic sclerosis patients, collagen thickness and IHC staining of myofibroblasts and macrophages

	SSc	HC	p value*
Collagen thickness, mean (SD)	2.0 (1.2)	0.1 (0.3)	<0.01
α-SMA, mean (SD)	1.0 (1.1)	0 (0)	<0.01
CD68, mean (SD)	1.6 (0.8)	1.1 (0.3)	0.04
CD163, mean (SD)	1.8 (0.8)	1.1 (0.3)	0.01
AIF1, mean (SD)	1.7 (0.8)	0.6 (0.5)	<0.01

*Wilcoxon rank sum test.

SSc = systemic sclerosis, HC = healthy control, SD = standard deviation

Supplementary Table 7: Abundance of immune cell IHC staining positivity in PRESS SSc patients and HCs

	SSc	HC	p value*
CD3, median (IQR)	101.5 (43.0, 167.5)	48 (28.25, 71.5)	0.01
CD4, median (IQR)	20.5 (6.0, 46.0)	14.5 (8.5, 17.75)	0.38
CD8, median (IQR)	56.0 (32.5, 89.5)	26.5 (17.75, 47.25)	0.04
CD20, median (IQR)	2.0 (1.0, 10.5)	0.0 (0.0, 1.25)	0.03
CD56, median (IQR)	5.0 (2.5, 18.0)	2.25 (0.5, 4.25)	0.10

*Wilcoxon Rank Sum test

Supplementary Table 8: Correlations between histologic and clinical features in PRESS systemic sclerosis patients

	age	Disease duration	Local skin score	Total mRSS
Collagen thickness	0.03 (0.92)	0.35 (0.25)	0.67 (0.01)*	0.57 (0.04)*
α-SMA (myofibroblasts)	0.02 (0.95)	-0.18 (0.57)	0.64 (0.02)	0.44 (0.14)
CD68	0.40 (0.18)	-0.08 (0.79)	-0.04 (0.90)	0 (1.0)
CD163	0.50 (0.08)	-0.29 (0.34)	-0.04 (0.90)	0.16 (0.60)
AIF1	0.41 (0.16)	-0.21 (0.49)	-0.04 (0.90)	0.13 (0.67)
CD3	-0.16 (0.58)	-0.72 (<0.01)*	0.12 (0.66)	0.23 (0.42)
CD4	-0.15 (0.59)	-0.50 (0.06)	0.11 (0.70)	0.25 (0.36)
CD8	0.08 (0.78)	-0.47 (0.08)	0.16 (0.57)	0.23 (0.40)
CD20	0.08 (0.78)	-0.13 (0.65)	0.52 (0.049)*	0.57 (0.03)*
CD56	-0.17 (0.54)	-0.06 (0.82)	-0.18 (0.51)	0.15 (0.60)

Shown for each association is the Spearman rank order correlation coefficient followed by the p value in parentheses. * indicates p <0.05

Supplementary Table 9: Longitudinal mRSS, FVC, and immunosuppressive medication use in PRESS SSc patients 6 or 12 months after skin biopsy

Change in mRSS 6 months after biopsy, median (IQR), n = 22	-3.5 (-6.0, 2.0)
Change in mRSS 12 months after biopsy, median (IQR), n = 29	-3.0 (-6.0, 1.0)
Change in FVC 12 months after biopsy, median (IQR), n = 20	0.5 (-4.0, 7.5)
Mycophenolate use 12 months after biopsy, n (%)	19 (67.9)*
Methotrexate use 12 months after biopsy, n (%)	3 (10.7)*
Cyclophosphamide use 12 months after biopsy, n (%)	0 (0)

*Indicates the percentage positive of those with an mRSS recorded 12 months after skin biopsy and in whom immunosuppression use was recorded (n = 28)

SSc = systemic sclerosis, HC = healthy control, mRSS = modified Rodnan skin score, FVC = forced vital capacity

Supplementary Table 10: Associations between cell type signatures and course of skin and lung disease in PRESS patients

	Skin thickness progression rate preceding biopsy (n = 45)	Change in mRSS 6 months after biopsy (n = 22)	Change in mRSS 12 months after biopsy (n = 29)	Change in FVC 12 months after biopsy (n = 20)
M1 macrophage	0.39 (0.01)*	0.13 (0.55)	-0.12 (0.54)	0.06 (0.81)
M2 macrophage	0.39 (0.01)*	0.09 (0.69)	-0.21 (0.27)	0.04 (0.87)
CD4 cell	0.39 (0.01)*	-0.07 (0.75)	-0.07 (0.71)	0.11 (0.62)
CD8 cell	0.40 (0.01)*	-0.05 (0.83)	-0.11 (0.56)	0.09 (0.70)
B cell	0.32 (0.03)*	0.04 (0.87)	0.01 (0.97)	0.08 (0.74)
NK cell	0.36 (0.01)*	0.08 (0.72)	-0.10 (0.62)	0.10 (0.69)
Fibroblast	0.33 (0.03)*	0.27 (0.22)	-0.13 (0.52)	-0.19 (0.42)

Shown are the Spearman's rank order correlation coefficients for each association, followed by the p value in parentheses. * indicates $p < 0.05$.

mRSS = modified Rodnan skin score, FVC = forced vital capacity

Supplementary Table 11: Regression of mRSS change 6 or 12 months after biopsy with intrinsic subsets in PRESS patients, with reference to normal-like subset

	All patients			Patients taking immunosuppressive medication at follow up#		
	mean difference	**95% CI**	**p value**	**mean difference**	**95% CI**	**p value**
6-month ΔmRSS			**0.71***			**0.39***
Inflammatory	3.8	-6.9 – 14.5	0.47	4.5	-6.0 – 15.0	0.38
Fibroproliferative	2.1	-8.8 – 13.0	0.69	0.43	-10.4 – 11.3	0.93
12-month ΔmRSS			**0.13***			**0.47***
Inflammatory	-1.5	-8.1 – 5.1	0.65	-1.2	-7.7 – 5.4	0.72
Fibroproliferative	2.7	-4.0 – 9.3	0.42	1.7	-5.2 – 8.5	0.62

*p value for the overall model
#includes mycophenolate, methotrexate, or cyclophosphamide
mRSS: modified Rodnan Skin Score

Supplementary Table 12: Regression of pre-biopsy STPR with intrinsic subsets in PRESS patients, with reference to normal-like subset

	mean difference	95% CI	p value
pre-biopsy STPR			**0.003***
Inflammatory	28.3	9.1 – 47.4	<0.01
Fibroproliferative	8.2	-11.0 – 27.5	0.39

*p value for the overall model
STPR: skin thickness progression rate

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SUPPLEMENTARY FIGURES

Supplementary Figure 1: Cell type signatures in skin of early diffuse SSc patients (PRESS) compared to later stage diffuse SSc patients in GENISOS

Signature scores for PRESS patients (n = 48) were compared to those of GENISOS patients with diffuse cutaneous involvement and >3 years disease duration (n = 28). The mean PRESS score is represented by round symbols with error bars spanning ± 1 standard deviation. The mean GENISOS score is represented by the midline for each grey box with boxes spanning ± 1 standard deviation. Right margin p-values were obtained from a two-sample t-test of PRESS vs. GENISOS scores.

Supplementary Figure 2: Cell type-specific signatures in GENISOS patients

(A) Cell type signatures in all GENISOS patients (n = 55). (B) Cell type signatures in GENISOS patients with diffuse SSc and disease duration > 3 years (n = 28).

Supplementary Figure 3: changes in cell type-specific signatures in PRESS follow up skin biopsies

(A) Cell type signatures in PRESS patients (n = 48) including follow up biopsies (n = 10 total follow up biopsies in 8 patients). Follow up biopsies are indicated to the right of the heatmap by colored arrows pointing from the original biopsy to the follow up biopsy or biopsies. (B) Changes in cell type signatures from baseline to follow up biopsy. Each color represents a patient and corresponds to the colored arrows to the right of the heatmap in (A). Each data point represents a biopsy.

INF = inflammatory subset, FP = fibroproliferative subset, NL = normal-like subset

Supplementary Figure 4: Representative staining of skin biopsies for collagen thickness, markers of macrophages, myofibroblasts, and adaptive immune cells

A. Collagen thickness was scored from 0 (normal) to 3 (very thickened). Shown are representative samples with scores of 0 (left) and 3 (right). Scale bar is 100 μm. **B.** CD68 stained in brown, CD31 in blue. Non-perivascular CD68 abundance was scored from 0 (none) to 3 (densely populated). Shown are representative samples with scores of 1 (left) and 3 (right). **C.** CD163 stained in brown, CD31 in blue. Non-perivascular CD163 abundance was scored from 0 (none) to 3 (densely populated). Shown are representative samples with scores of 1 (left) and 3 (right). **D.** AIF1 stained in brown, α-SMA in blue. AIF1 abundance was scored from 0 (none) to 3 (densely populated). α-SMA was scored from 0 (none) to 3 (more than one band across biopsy or extensively wide region). The dense blue areas are arrector pili muscles and were not counted. Shown are representative samples with scores of 0 and 0 for AIF1 and α-SMA, respectively (left) and of 2 and 3, respectively (right). **E-I.** CD3, CD4, CD8, CD20, or CD56 stained in brown as noted in the Figures. All positively-staining cells in each tissue section, excluding epidermis, hair follicles, and intravascular cells, were counted. Shown are representative samples from a healthy control (left) and an SSc patient (right). Images were captured at 100x magnification.

Supplementary Figure 5: Association of seven genes with mRSS change 6 or 12 months after biopsy.
A. Expression level of each gene (based on FPKM value from RNA sequencing) is plotted on the y-axis. mRSS change from the time of initial biopsy to 6 months later (A) or 12 months later (B) is plotted on the x-axis. Spearman's rank order correlation coefficient and significance value is shown.

Supplementary Figure 6: Cell type signatures in PRESS patients, including longitudinal biopsies, organized by intrinsic subset.

Supplementary Figure 7: Intrinsic subset assignments at first, second, and third biopsy in PRESS patients who underwent longitudinal biopsies.