MATERIALS AND METHODS SUPPLEMENT

Bleomycin-induced lung injury and CSD peptide treatment – This procedure was approved by the MUSC Institutional Animal Care & Use Committee. The CSD peptide (amino acids 82–101 of caveolin-1) and a scrambled control peptide were synthesized as fusion peptides to the C terminus of the *Antennapedia* internalization sequence and dissolved at 10 mM in DMSO (1). Ten-week old, male CD1 mice (Charles River, Boston, MA) received daily i.p. injections of 100 µl of CSD or control peptide diluted to 0.1 mM in PBS throughout the entire experiment from the day prior to bleomycin treatment until the day of sacrifice. Mice to be treated with bleomycin (Calbiochem, La Jolla, CA) were sedated using isoflurane, attached to a tilting table at a 60° angle, and their tongues were carefully extended as recently described (2). Then 50 µl of bleomycin (2 units per ml in PBS) or PBS vehicle was pipetted into their oral cavities. Mice aspirate the fluid directly into their lungs. Either 3 or 7 days after bleomycin treatment, lungs were removed and paraffin sections prepared and stained with primary antibodies (rat anti-mouse Mac-3 [BD Pharmingen 550292, San Diego, CA] to identify monocytes/macrophages and rat anti-mouse Gr-1 to identify neutrophils [BD Pharmingen 553124]), AlexaFluor 555-labeled secondary antibody, and the nuclear stain DAPI (Invitrogen, Carlsbad, CA). Images were collected using an Olympus IX71 fluorescence microscope.

Mouse leucocyte staining – From some of these mice, peripheral blood was collected and leucocytes harvested by cytospin. Slides were fixed and stained using antibodies against Mac-3 and Gr-1 (see above) and caveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA), then with appropriate AlexaFluor 488 or 555-labeled secondary antibodies and with DAPI. Images were acquired using a Zeiss 510SML Laser Confocal Microscope with an oil-immersion objective (63x /1.4) and analyzed using ImageJ141 software to quantify caveolin-1 staining in Mac-3-positive and Gr-1-positive cells.

Human Monocyte and PMN Isolation and Western Blotting – In aggregate, blood cells were isolated (3-6) from eighteen scleroderma patients (Supplemental Table 1) and eighteen control subjects. The number of subjects used per experiment is indicated in each Figure Legend. 40 ml of blood was drawn, treated with anticoagulant, diluted 1:2 with HBSS, and centrifuged on a density 1.083 Histopaque cushion (Sigma, St Louis, MO). Peripheral blood mononuclear cells (PBMC) at the interface were collected and monocytes purified by adhesion to tissue culture plastic for 1 h; non-adherent lymphocytes were discarded, and adherent cells were washed twice with HBSS. The resulting cell population was about 75% Mac-1[BD Pharmingen 553308]-positive monocytes as assessed by both flow cytometry and fluorescent microscopy of cells collected by cytospin. In an alternative approach, mononuclear cells from the interface were enriched by immunodepletion using a Dynal Monocyte Negative Isolation Kit (Invitrogen, Carlsbad, CA) resulting in a cell population that was about 95 % Mac-1-positive monocytes as assessed by both flow cytometry and fluorescent microscopy.

To isolate polymorphonuclear cells (PMNs), the bottom Histopaque layer was diluted with two volumes of HBSS/ 1.5 % dextran (Sigma), incubated 1 h RT, and centrifuged 10 min, 1300 rpm. Erythrocytes in the PMN-enriched upper layer were lysed in ice-cold 0.15 M ammonium chloride/ 0.01 M potassium chloride. The surviving PMNs were collected by centrifugation and washed with HBSS.

Cytokine and CSD peptide treatment of monocytes are described in the Figure Legends. For Western blotting, cells were directly dissolved in boiling SDS-PAGE sample buffer and boiled for three minutes. Western blots were probed with the following antibodies: Caveolin-1 (sc-894), Cox-2 (sc-1745), CXCR4 (sc-9048) and activated JNK (sc-12882) from Santa Cruz Biotechnology (Santa Cruz, CA); ERK 1/2 (9102), activated ERK 1/2 (9106), JNK (9252), p38 (9212), and activated p38 (9211) from Cell Signaling (Beverly, MA); actin (MAB1501) from Millipore (Temecula, CA), and β-actin (A5441) from Sigma (St. Louis, MO).

Human Leucocyte Staining - Cells were collected by cytospin and stained (7) using the indicated primary antibodies and AlexaFluor 555-tagged secondary antibodies. For monocytes and PMNs, nuclei were stained with Sytox (Invitrogen). For T cells and B cells, nuclei were stained with DAPI

(Invitrogen). Antibodies used were: Caveolin-1 (Santa Cruz Biotechnology, sc-894), monocyte marker Mac-1 (BD Pharmingen 553308), PMN marker Gr-1 (BD Pharmingen 553124), T cell marker CD3 (BD Pharmingen 555342), and B cell marker CD19 (BD Pharmingen 555413). Images were acquired using a Zeiss 510SML Laser Confocal Microscope as above.

Gelatin zymography - 8 % SDS-PAGE gels were prepared with 1 mg/ml of gelatin in the resolving gel. After electrophoresis under non-reducing conditions of aliquots of medium derived from equal numbers of cells, gels were incubated for 30 min in 2.5% Triton X-100, 15 min in water, and overnight at 37ºC in 50 mM Tris pH 8.0/ 5 mM $CaCl_2$. Gels were then stained 30 min with Coomassie Blue G (dissolve 0.63 g in 112 ml methanol, filter, mix with 112 ml water and 25 ml stock acetic acid) and destained with changes of 20 % methanol, 7 % acetic acid, in water. Gelatinase activity was visualized as clear bands on a uniform blue background.

Statistical Analyses - Immunoreactive bands were quantified by densitometry using ImageJ1.32 NIH software. For statistical analyses, raw densitometric data were processed and analyzed using Prism 3.0 (GraphPad Software Inc.) software. Protein expression levels were analyzed using Student's *t*-test (Figs. 1-3 and Supplemental Figs. 1, 2) and using one-way ANOVA followed by the Bonferroni post-test (Fig. 4). Multivariate ANOVA was used to analyze multiple variables (Figs. 5, 6). In all Figures, *** indicates $p < 0.001$, ** indicates $p < 0.01$, and * indicates $p < 0.05$.

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SUPPLEMENTAL TABLE 1

Under a protocol approved by the Institutional Review Board for Human Research, patients with systemic sclerosis (scleroderma, SSc) were recruited from the Scleroderma Clinic at the Medical University of South Carolina. All patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for scleroderma (1), except one patient with an undifferentiated connective tissue disease (UCTD) who had features of scleroderma. Demographic characteristics of the study population are summarized in Supplemental Table 1. Systemic sclerosis patients were classified as having either limited cutaneous (lcSSc) or diffuse cutaneous (dcSSc) systemic sclerosis according to the criteria proposed by LeRoy et al. (2). Disease duration was determined based on when the first non-Raynaud phenomenon symptoms were documented. The modified Rodnan skin thickness score was assessed in all patients at 17 body areas by clinical palpation and was graded on a scale of 0-3, with a maximum total score of 51 (3).

The following criteria were used to define visceral involvement:
Pulmonary – Demonstration of abnormalities on high resolution computed tomographic (HRCT) scan (ground glass changes and/or fibrosis), pulmonary hypertension based on a right heart catheterization, restrictive changes on pulmonary function testing, or reduced diffusing capacity for carbon monoxide.
GI – History of gastro-esophageal reflux disease based on either subjective and/or objective findings. Some patients had symptoms of reflux requiring treatment with a proton pump inhibitor and/or H2-antagonist. Others had abnormal motility documented by esophageal manometry or findings of esophagitis on upper endoscopy.
Cardiac – Evidence on echocardiogram of left ventricular diastolic dysfunction, a pericardial effusion, elevated peak right ventricular systolic pressure, right ventricular and/or right atrial dilatation. Conduction abnormalities on a 12 – lead EKG was also considered sufficient.
Renal – History of rapidly progressive renal failure.

Antinuclear antibodies (ANA) and anti-centromere antibodies were determined by immunofluorescent analysis on Hep-2 cell substrates. Anti-Scl-70 (topoisomerase I) antibodies and anti-RNA polymerase III antibodies were determined by enzyme immunoassay.

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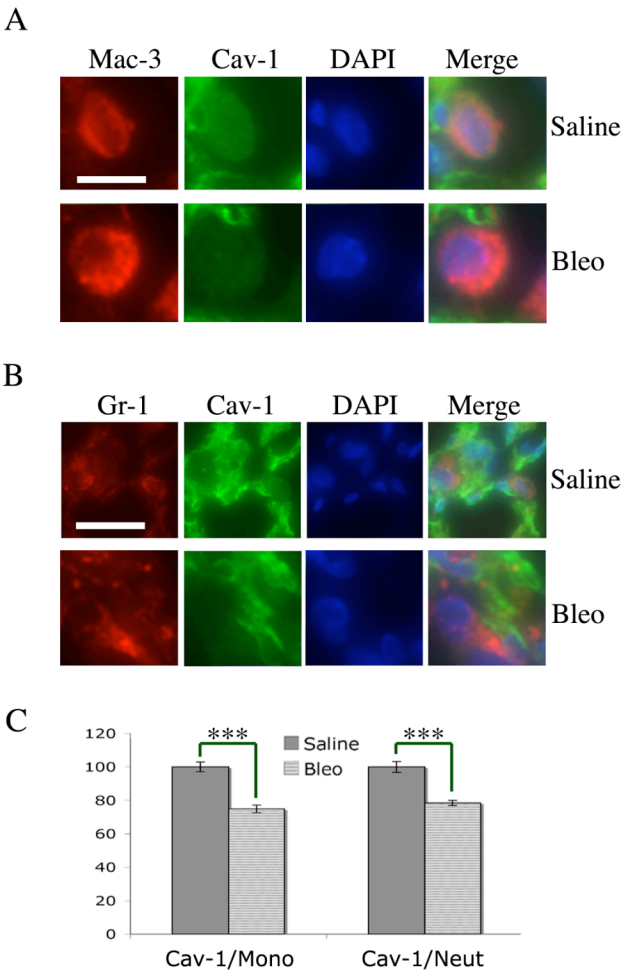
Supplemental Table 1: Clinical Features of SSc Patients

Gender and Race:	3 Caucasian Males 10 Caucasian Females 1 African-American Male 4 African-American Females	
Disease: Limited Cutaneous	6	
Diffuse Cutaneous	11	
UCTD	1	
Age: Mean ± SD (range), yr		51 ± 12.8 (22-73)
Disease duration: Mean ± SD (range), yr		7.6 ± 6.8 (1-28)
Pulmonary Involvement		18/18 (100 %)
Abnormal HRCT		15/18 (83 %)
FVC, Mean ± SD, % predicted		70.5±18.7
DLCO, Mean ± SD, % predicted		53.5±18.8
GI Involvement		18/18 (100 %)
Cardiac Involvement		11/18 (61 %)
Renal Involvement		1/18 (5 %)
Modified Rodnan skin score		10.3 ± 8.8
Medications (current)		
Cytoxan		0/18
Cellcept		5/18
Imuran		1/18
Prednisone		5/18
Sildenafil		2/18
Bosentan		1/18
Autoantibodies:ANA+		11/12
Scl-70+		4/14
RNA pol III+		1/2
Anti-centromere		0/12

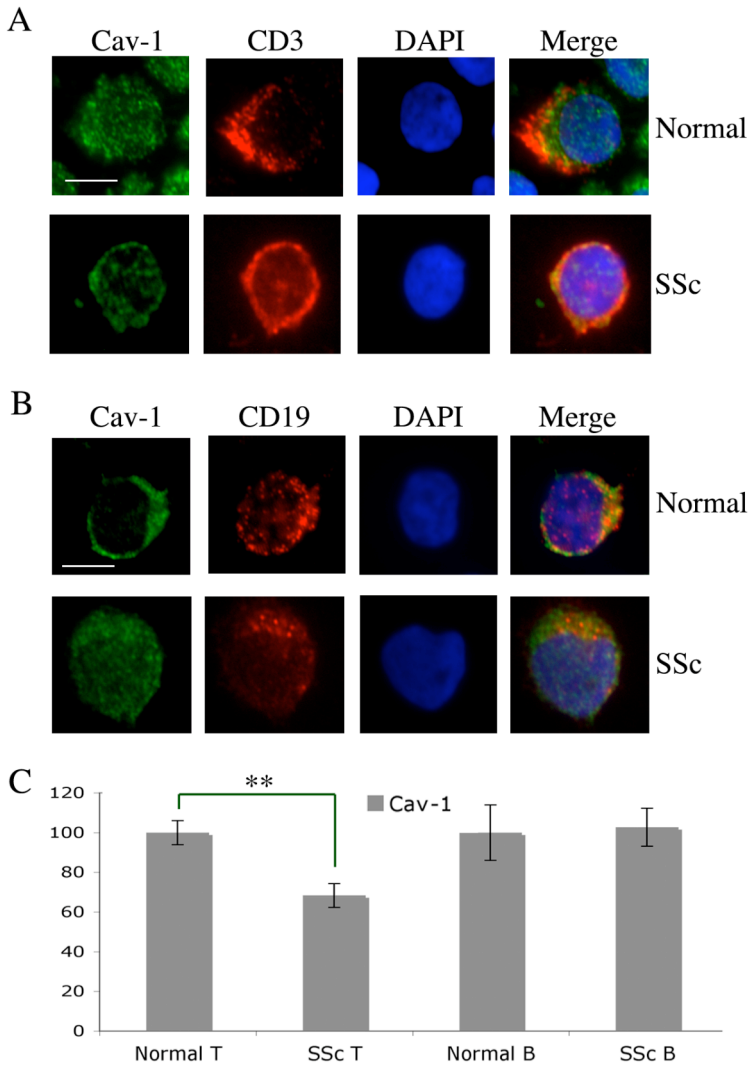
UCTD, undifferentiated connective tissue disease
HRCT, high resolution Computed Tomographic scan (ground glass and/or fibrosis)
FVC, forced vital capacity
DLCO, diffusing capacity for carbon monoxide

SUPPLEMENTAL FIGURE 1



Supplemental Figure 1. Bleomycin treatment inhibits caveolin-1 expression in lung tissue monocytes and neutrophils. Sections from saline-treated or bleomycin-treated mice were harvested 7 days after treatment and stained with DAPI, anti-caveolin-1, and with either Gr-1 or Mac-3. Representative images are shown of staining of Mac-3-positive **(A)** and Gr-1-positive **(B)** cells. Bars = 5 μm. Caveolin-1 staining intensity in randomly chosen Mac-3-positive monocytes (Cav/Mono) and Gr-1-positive neutrophils (Cav/Neut) from saline- and bleomycin-treated mice is quantified (average ± SEM) in **C**. In each case, the data were obtained from 20 to 60 cells from three saline-treated and six bleomycin-treated mice. Note that there is a modest, but statistically significant, decrease in caveolin-1 expression in both lung tissue monocytes and neutrophils in bleomycin-treated mice. *** $P < 0.001$.

SUPPLEMENTAL FIGURE 2



Supplemental Figure 2. Caveolin-1 expression in normal and scleroderma peripheral blood T cells and B cells. The expression of caveolin-1 in isolated T cells **(A)** and B cells **(B)** was evaluated by immunofluorescent microscopy. Bars = 5 µm. Caveolin-1 staining intensity in randomly chosen CD-3-positive cells (i.e. T cells) and CD-19-positive cells (i.e. B cells) from Normal and Scleroderma donors is quantified (average ± SEM) in **C**. In each case, the data were obtained from 20 to 60 cells from 4 donors. Note that there is a statistically significant decrease in caveolin-1 expression in scleroderma T cells, but not in scleroderma B cells. ** $P < 0.01$.