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Induction of Inflammation and Fibrosis by Semaphorin 4A in Systemic Sclerosis

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Objective. To analyze the potential role of semaphorin 4A (Sema4A) in inflammatory and fibrotic processes involved in the pathology of systemic sclerosis (SSc).

Methods. Sema4A levels in the plasma of healthy controls (n = 11) and SSc patients (n = 20) were determined by enzyme-linked immunosorbent assay (ELISA). The expression of Sema4A and its receptors in monocytes and CD4+ T cells from healthy controls and SSc patients (n = 6–7 per group) was determined by ELISA and flow cytometry. Th17 cytokine production by CD4+ T cells (n = 5–7) was analyzed by ELISA and flow cytometry. The production of inflammatory mediators and extracellular matrix (ECM) components by dermal fibroblast cells (n = 6) was analyzed by quantitative polymerase chain reaction, ELISA, Western blotting, confocal microscopy, and ECM deposition assay.

Results. Plasma levels of Sema4A, and Sema4A expression by circulating monocytes and CD4+ T cells, were significantly higher in SSc patients than in healthy controls ($P < 0.05$). Inflammatory mediators significantly up-regulated the secretion of Sema4A by monocytes and CD4+ T cells from SSc patients ($P < 0.05$ versus unstimulated SSc cells). Functional assays showed that Sema4A significantly enhanced the expression of Th17 cytokines induced by CD3/CD28 in total CD4+ T cells as well in different CD4+ T cell subsets ($P < 0.05$ versus unstimulated SSc cells). Finally, Sema4A induced a profibrotic phenotype in dermal fibroblasts from both healthy controls and SSc patients, which was abrogated by blocking or silencing the expression of Sema4A receptors.

Conclusion. Our findings indicate that Sema4A plays direct and dual roles in promoting inflammation and fibrosis, 2 main features of SSc, suggesting that Sema4A might be a novel therapeutic target in SSc.

INTRODUCTION

Systemic sclerosis (SSc) is a severe autoimmune inflammatory disease of unknown etiology with high morbidity and mortality rates, characterized by activation of the immune system, vascular abnormalities, and fibrosis. The resultant skin thickening and stiffness and loss of internal organ function leads to profound disability and premature death (1,2). Fibrosis is marked by the excessive deposition of extracellular matrix (ECM) proteins, as well as increased numbers of fibroblasts expressing the contractile protein α-smooth muscle actin (α-SMA) (3,4). Accumulating evidence has also shown that immune responses are deregulated in SSc patients, contributing to pathology (5,6). One consequence of this immune deregulation is the alteration of T cell homeostasis, with an elevated frequency of Th17 cells in SSc patient peripheral blood and skin (7–11). Interleukin-17 (IL-17) is a cytokine involved in many pathologic features contributing to SSc pathology, including proinflammatory cytokine secretion, monocyte recruitment, and granulocyte–macrophage colony-stimulating factor production (12–14).

The semaphorin family is a large group of proteins initially described as axonal guidance molecules, but now appreciated for their roles in other physiologic and pathologic processes,

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including the regulation of immune responses, angiogenesis, cell migration, and tissue invasion (15,16). Semaphorin 4A (Sema4A) is a transmembrane protein that can also be cleaved and released into circulation. Both transmembrane and soluble Sema4A bind to multiple receptors, the best characterized of which are plexin B2, plexin D1, and neuropilin 1 (NRP-1) (17,18). Sema4A is a key molecule in the regulation of T cell homeostasis, activation, and Th1/2/17 differentiation (18–20). Sema4A deficiency or inhibition reduces disease severity in murine models of multiple sclerosis (MS) and autoimmune myocarditis, but enhances the severity of experimental asthma due to impaired Th1/Th17 differentiation and skewing towards a Th2 polarization (19,21–23). Reciprocally, serum levels of Sema4A are increased in MS patients and positively associated with Th17 skewing (23). Thus, Sema4A may play a suppressive role in Th2-driven disease while driving Th1- and Th17-dependent diseases. Sema4A might also play a direct role in fibrosis, inducing collagen contraction by SSc patient lung fibroblasts (24). In this study, we examined whether Sema4A signaling might serve to connect altered Th17 behavior with fibrotic processes in SSc.

MATERIALS AND METHODS

Patients. Blood from patients and sex- and age-matched healthy controls was obtained from the University Medical Center Utrecht and Maasstad Hospital Rotterdam. All subjects provided written informed consent approved by the local institutional medical ethics review boards prior to inclusion in this study. Samples and clinical information were treated anonymously immediately after collection. Patients fulfilled the American College of Rheumatology/European League Against Rheumatism 2013 classification criteria for SSc (25), and the demographic and clinical characteristics of the patients are detailed in Supplementary Tables 1–3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40915/abstract.

Cell isolation. Peripheral blood mononuclear cells (PBMCs) from healthy controls and SSc patients were isolated by Ficoll gradient (GE Healthcare). Cells were processed for further isolation using magnetic beads and an AutoMACS Pro Separator for monocytes and CD4+ T cells, according to the manufacturer's instructions (Miltenyi Biotec). Purity was routinely >95% for CD4+ T cells and >90% for monocytes, as assessed by flow cytometry. Total CD4+ T cells were stained with allophycocyanin (APC)–eFluor 780–conjugated anti-CD4 (eBioscience), BV-510–conjugated anti-CD27 (BioLegend), phycoerythrin (PE)–conjugated anti-CD25, Alexa Fluor 647–conjugated anti-CD127, and PE–Cy7–conjugated anti-CD45RO (BD Biosciences) antibodies. Cell sorting was performed on a BD FACSAria III cell sorter (BD Bioscience) to obtain pure populations of naive CD4+CD25–CD27+CD45RO–, effector memory CD4+CD25–CD27–CD45RO+, and central memory CD4+CD25–CD27+CD45RO+ T cells (>99% purity).

Flow cytometric analysis. PBMCs were stained with Fixable Viability Dye (for dead cell exclusion) (eBioscience), antibodies to PE-Cy7–conjugated CD19 (Beckman Coulter), APC-conjugated CD1c, APC–eFluor780–conjugated CD4, PerCP–Cy5.5–conjugated CD303, BV785-conjugated CD14, Alexa Fluor 700–conjugated CD3 (BioLegend), BV605-conjugated HLA–DR, BV711-conjugated CD141, V500-conjugated CD8, PE–CF594–conjugated CD56 (BD Biosciences), fluorescein isothiocyanate (FITC)–conjugated CD16 (BioConnect), and PE-conjugated Sema4A and its isotype control (R&D Systems). For intracellular staining, PBMCs were first fixed and permeabilized using a FoxP3/Transcription Factor Staining Buffer set (eBioscience). Cells were acquired on an LSRFortessa (BD Biosciences).

CD4+ T cells were stained with Fixable Viability Dye and antibodies to PE-conjugated NRP-1 (Miltenyi Biotec), FITC-conjugated plexin D1, APC-conjugated plexin B2, and their respective isotype controls (R&D Systems). Alternatively, cells were fixed and permeabilized using a FoxP3/Transcription Factor Staining Buffer set, and stained with FITC-conjugated IL-17A, APC-conjugated IL-22, PerCP–Cy5.5–conjugated interferon-γ (IFNγ) (all from eBioscience), BV711-conjugated IL-4, PE-conjugated IL-21, and BV421-conjugated tumor necrosis factor (TNF; all from BD Biosciences). All data were acquired on an LSRFortessa (BD Biosciences). After excluding debris, doublets, and dead cells, cell populations were analyzed using FlowJo software (Tree Star). (See Supplementary Table 4, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40915/abstract, for gating strategy). All flow cytometry data are presented as the percentage of positive cells or the change in median fluorescence intensity (ΔMFI), where ΔMFI = MFI of positive staining − MFI of isotype staining.

Monocyte stimulation. Monocytes were cultured for 30 minutes in RPMI GlutaMax (ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS; BioWest) and 10,000 IU penicillin–streptomycin (ThermoFisher Scientific), and then left unstimulated or stimulated with lipopolysaccharide (LPS; 100 ng/ml), R848 (1 μg/ml), high molecular weight poly(I-C) (5 μg/ml) (all from InvivoGen), or CXCL4 (5 μg/ml; PeproTech) for 24 hours. Cells were lysed for messenger RNA (mRNA) expression analysis and cell-free tissue culture supernatants were harvested for cytokine analysis.

CD4+ T cell stimulation. CD4+ total, naive, central memory, or effector memory T cells were activated with Dynabeads Human T-Activator CD3/CD28 (ThermoFisher Scientific) at a bead-to-cell ratio of 1:5 in the absence or presence of recombinant human Sema4A (200 ng/ml; R&D Systems) for 2–7 days. Alternatively, CD4+ T cells were preincubated for 1 hour with neutralizing antibodies to anti–plexin D1 or NRP-1 (R&D Systems) before cell activation and stimulation with Sema4A, as described above, for 5 days. For intracellular cytokine staining,

phorbol 12-myristate 13-acetate (1 μg/ml), ionomycin (50 ng/ml) (both from Sigma-Aldrich) and BD GolgiStop (BD Biosciences) were added for the final 4 hours of stimulation. For proliferation analysis, CD4+ T cells were labeled with CellTrace Violet (1.5 μ*M*; ThermoFisher Scientific) prior to culture.

CD4+ T cell transfection. CD4+ T cells were transfected by electroporation using a Neon Transfection System (ThermoFisher Scientific). CD4+ T cells were activated with Dynabeads Human T-Activator CD3/CD28 at a bead-to-cell ratio of 1:5 for 48 hours. Before transfection, Dynabeads were removed, and activated CD4+ T cells were transfected with plexin B2–specific or scrambled (Sc) nontargeting small interfering RNAs (siRNAs) (50 n*M*; Thermo Scientific) in RPMI GlutaMax containing 10% FBS in the presence of Dynabeads (bead-to-cell ratio of 1:5) for 24 hours, and experiments were performed 4 days after transfection. The efficiency of the transfection was >60% for both healthy control and SSc CD4+ T cells (Supplementary Figures 1A and B, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40915/abstract).

Dermal fibroblast culture and stimulation. SSc dermal fibroblasts (n = 6) were isolated from 3–4-mm skin biopsy sections obtained from a clinically affected area. Healthy control dermal fibroblasts (n = 6) were obtained from skin biopsy sections as resected material after cosmetic surgery. Dermal fibroblast isolation was performed using a whole skin dissociation kit (Miltenyi Biotec) following the manufacturer's instructions, and fibroblasts were routinely maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FBS and 10,000 IU penicillin–streptomycin. Cells were used for experiments between passages 3 and 5, and stimulations were performed after overnight starvation in medium containing 0.1% FBS. Fibroblasts were left unstimulated or preincubated for 1 hour with neutralizing antibodies to anti–plexin D1 or NRP-1 and afterward left unstimulated or stimulated with Sema4A (200 ng/ml) for 24–72 hours. Alternatively, conditioned medium from CD4+ T cells was preincubated for 1 hour at 37°C in the presence of a neutralizing anti–IL-17A antibody (secukinumab 100 ng/ml; kindly provided by Dr. Erik Lubberts, Erasmus Medical Center, Rotterdam, The Netherlands) or its isotype control (IgG1κ; eBioscience) and applied to fibroblasts for 24 hours.

Dermal fibroblast transfection. Dermal fibroblasts were transfected using Dharmafect 1 (Thermo Scientific). Plexin B2–specific or Sc nontargeting siRNAs (50 n*M*; Thermo Scientific) were mixed with Dharmafect 1 prior to transfection for 24 hours. Experiments were performed 48–72 hours after transfection. The efficiency of the silencing was >60% (Supplementary Figures 1C and D).

Cytokine measurement. Sema4A (Biomatik), IL-6, IL-8 (Sanquin), and IL-17A (eBioscience) were measured by enzyme-linked immunosorbent assay in cell-free supernatants and plasma from healthy controls and SSc patients, according to the manufacturer's instructions.

Immunoblotting. Dermal fibroblasts were lysed in Laemmli buffer, and protein content was quantified with a BCA Protein Assay Kit (Pierce). Equivalent amounts of total protein lysate were subjected to electrophoresis on NuPAGE 4–12% Bis-Tris protein gels (ThermoFisher Scientific) and proteins were transferred to PVDF membranes (Millipore). Membranes were incubated overnight at 4°C with primary antibodies specific for α-SMA (Sigma), vimentin, histone 3 (Cell Signaling Technology), type III collagen (Millipore), type VI collagen, and plexin B2 (Abcam). Membranes were then washed and incubated in Tris buffered saline–Tween containing horseradish peroxidase–conjugated secondary antibody. Protein was detected with Lumi-Light Plus Western blotting substrate (Roche Diagnostics) using a ChemiDoc MP System (Bio-Rad). Densitometric analysis was performed with Image J software. Relative protein expression was normalized to histone H3 expression.

Reverse transcriptase–polymerase chain reaction (PCR) and quantitative PCR. RNA from monocytes, CD4+ T cells, and dermal fibroblasts was isolated using an RNeasy kit and RNase-Free DNase set (Qiagen). Total RNA was reverse-transcribed using an iScript cDNA Synthesis kit (Bio-Rad). Duplicate PCR reactions were performed using SYBR Green (Applied Biosystems) with a StepOnePlus Real-Time PCR system (Applied Biosystems). Complementary DNA was amplified using specific primers (Invitrogen) (see Supplementary Table 5, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40915/abstract). Relative levels of gene expression were normalized to the housekeeping genes *B2M* (for monocytes and CD4+ T cells) and *GAPDH* (for fibroblasts). The relative quantity of mRNA was calculated using the formula $2^{-\Delta Ct} \times 1{,}000$.

Confocal microscopy. Nunc Lab-Tek II chamber slides (ThermoFisher Scientific) were precoated with 0.001% poly-L-lysine (Sigma-Aldrich), washed with phosphate buffered saline (PBS), and air-dried. Dermal fibroblasts were seeded in DMEM containing 10% FBS for 24 hours and then incubated overnight in DMEM containing 0.1% FBS. Cells were then stimulated with Sema4A (200 ng/ml) in DMEM containing 2% FBS and 50 μg/ml of L-ascorbic acid, and refreshed 48 hours later. After 72 hours, cells were fixed with 4% paraformaldehyde, washed with PBS–1% bovine serum albumin (BSA), and blocked in 5% normal donkey serum/1% BSA. Cells were incubated with primary antibodies specific for type I collagen (SouthernBiotech), type VI collagen (Abcam), and fibronectin (R&D Systems) for 1 hour at

room temperature, washed, and incubated with secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 594, and Alexa Fluor 647 (ThermoFisher Scientific) for 30 minutes at room temperature. Finally, cells were incubated with DAPI (Sigma-Aldrich Chemie) and slides mounted with Mowiol (Sigma-Aldrich Chemie). Imaging data were acquired on a Zen2009 LSM 710 confocal microscope (Zeiss).

Extracellular matrix deposition assay. Black/clear flat-bottomed imaging plates (96 wells each; Corning) were coated with 0.2% gelatin solution for 1 hour at 37°C, washed with Dulbecco's PBS containing 1 m*M* $Ca2^+$ and 1 m*M* $Mg2^+$ (DPBS+), and incubated with 1% glutaraldehyde for 30 minutes at room temperature. Plates were washed again with DPBS+ and incubated for 30 minutes at room temperature with 1*M* ethanolamine. After washing, dermal fibroblasts were seeded, incubated for 24 hours in complete medium, and starved overnight in DMEM containing 0.1% FBS. Cells were then stimulated with Sema4A (200 ng/ml) in DMEM containing 2% FBS and 50 μg/ml of L-ascorbic acid and refreshed 48 hours later. After 72 hours, cells were lysed with 0.5% volume/volume Triton X-100 containing 20 m*M* NH_4OH in PBS, and plates were kept at 4°C overnight. Cellular debris was removed, and wells were fixed in ice-cold 100% methanol. After washing, the ECM was blocked at room temperature with 1% normal donkey serum (Jackson ImmunoResearch) and incubated with primary antibodies specific for type I collagen, type IV collagen, and fibronectin. Afterward, wells were washed, and secondary antibodies (IRDye 800CW and IRDye 680RD; Li-Cor) were added for 1 hour at room temperature. Plates were measured using an Odyssey Sa Infrared Imaging System (Li-Cor Biotechnology).

Statistical analysis. Correlations were analyzed by Spearman's correlation analysis using SPSS software, version 25. Statistical analysis was performed using Windows GraphPad Prism 6 software. Potential differences between experimental groups were analyzed by nonparametric test, Wilcoxon's test, Mann-Whitney test, Kruskal-Wallis test, or Friedman's test, as appropriate. *P* values less than 0.05 were considered significant.

RESULTS

Elevated expression of Sema4A in plasma and immune cells from SSc patients. Plasma levels of Sema4A were significantly higher in SSc patients than in healthy controls and, interestingly, positively correlated with the severity of skin thickening (r = 0.529, *P* = 0.016) (Figures 1A and B). Since Sema4A is a transmembrane protein that can be cleaved and released into the circulation (23), we analyzed the intracellular expression of Sema4A in monocytes, dendritic cells, and B and T cells from healthy control and SSc patient peripheral blood, to determine if circulating white blood cells might contribute to the increased Sema4A expression observed in SSc patients. Sema4A expression was significantly higher in monocytes from SSc patients compared to healthy controls, as was the percentage of monocytes expressing Sema4A (Figures 1C and D and Supplementary Figure 2A, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40915/abstract). We observed similar results in CD4+ T cells, but expression of Sema4A in T cells was much lower than that in monocytes. We did not find differences in Sema4A expression in the other cell subsets analyzed (Supplementary Figures 2B and C). We also analyzed the cellular surface expression of Sema4A and found that the percentage of Sema4A-positive monocytes was higher in SSc patients than in healthy controls. However, CD4+ T cells did not express Sema4A on the cell surface and no differences in expression were found in the other cell populations (Supplementary Figures 2D and E).

Next, we analyzed whether Sema4A expression was regulated in monocytes by different inflammatory mediators involved in the pathogenesis of SSc, namely Toll-like receptor (TLR) agonists and CXCL4 (5,26). LPS and R848 did not modulate the expression of Sema4A (data not shown), but poly(I-C) and CXCL4 significantly induced the secretion of Sema4A by monocytes from SSc patients (Figure 1E). CD3/CD28-induced CD4+ T cell activation also induced Sema4A secretion in both healthy controls and SSc patients, albeit at lower levels compared to monocytes (Figure 1F). Importantly, and consistent with the results of intracellular staining, both basal and stimulated secretion of Sema4A were significantly higher in monocytes and CD4+ T cells from SSc patients than those from healthy controls. Taken together, these data demonstrate that Sema4A expression is deregulated in SSc patient monocytes and, to a lesser extent, CD4+ T cells.

Increased frequency of CD4+ T cells expressing plexin B2 in SSc patients. Since Sema4A plays a key role in the homeostasis and activation of CD4+ T cells (19,20), we determined the expression of the best-characterized Sema4A receptors in healthy control and SSc patient CD4+ T cells. NRP-1 was not expressed by CD4+ T cells (data not shown) and plexin D1 expression was similar between healthy controls and SSc patients. However, the percentage of CD4+ T cells expressing plexin B2 protein was significantly higher in SSc patients (Figure 1G). CD4+ T cells comprise different cell subsets (naive, effector, effector memory, and central memory CD4+ T cells) and, consistent with previous observations (27), frequencies of naive and central memory subsets were significantly altered in SSc patients (Supplementary Figures 3A and B, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40915/abstract). Therefore, we analyzed the expression of Sema4A receptors in these cell populations. Again, NRP-1 was not expressed in any population (data not shown) and we did not find differences in the expression of plexin D1. However, we observed a higher frequency

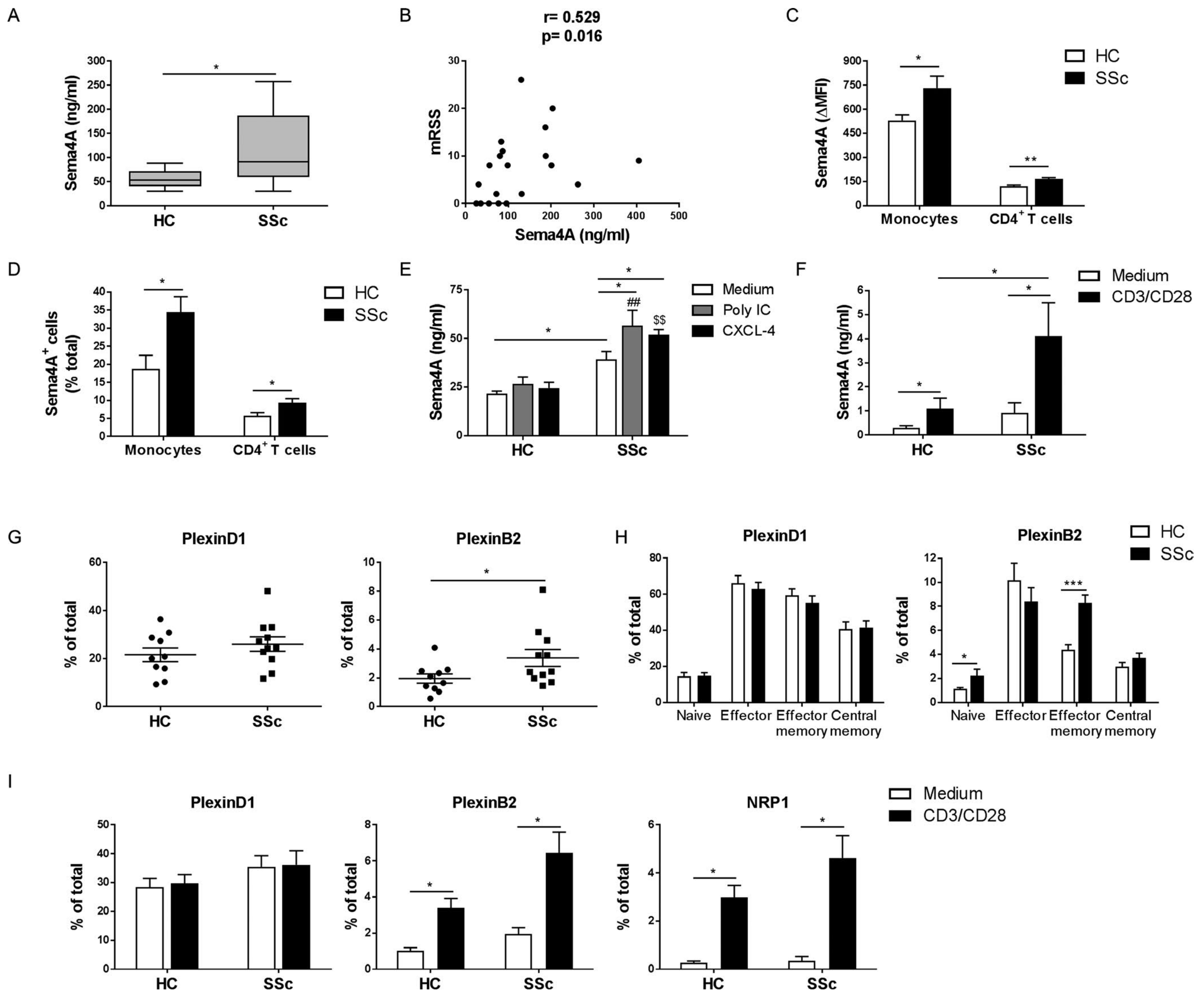

Figure 1. Semaphorin 4A (Sema4A) is elevated in plasma, monocytes, and CD4+ T cells from patients with systemic sclerosis (SSc) and is induced by inflammatory stimuli. **A**, Sema4A levels in plasma from healthy controls (HC; n = 11) and SSc patients (n = 20). Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. **B**, Correlation between Sema4A plasma levels and the modified Rodnan skin thickness score (MRSS). **C** and **D**, Intracellular Sema4A expression in monocytes and CD4+ T cells from healthy controls (n = 6) and SSc patients (n = 6). Data are shown as the change in median fluorescence intensity (ΔMFI) (**C**) or the percentage of positive cells (**D**). **E**, Expression of Sema4A protein by monocytes from healthy controls and SSc patients (n = 6 per group). Cells were stimulated with poly(I-C) or CXCL4 for 48 hours. **F**, Expression of Sema4A protein by CD4+ T cells from healthy controls and SSc patients (n = 6 per group). Cells were stimulated with CD3/CD28 Dynabeads for 5 days. **G**, Surface expression of plexin D1 and plexin B2 in CD4+ T cells from healthy controls and SSc patients. Circles represent individual subjects; horizontal lines and error bars show the mean ± SEM. **H**, Surface expression of plexin D1 and plexin B2 in CD4+ T cell subsets from healthy controls and SSc patients (n = 7 per group). **I**, Surface expression of plexin D1, plexin B2, and neuropilin 1 (NRP-1) in total CD4+ T cells from healthy controls and SSc patients (n = 7 per group). Cells were stimulated with CD3/CD28 Dynabeads. In **C–F**, **H**, and **I**, bars show the mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ for the indicated comparisons. ## = $P < 0.05$ versus healthy control samples stimulated with poly(I-C); $$ = $P < 0.05$ versus healthy control samples stimulated with CXCL4.

of plexin B2–positive cells in the naive and effector memory CD4+ T cell populations (Figure 1H).

We next determined the effect of CD4+ T cell activation on the expression of the Sema4A receptors. CD3/CD28 stimulation did not affect the expression of plexin D1, but significantly increased the percentage of cells expressing plexin B2 and NRP-1 (Figures 1I and Supplementary Figures 3C and D). Additionally, we found a trend toward a higher frequency of plexin B2–positive and NRP-1–positive cells in SSc patients compared to healthy controls, although the differences were not significant. The induction of plexin B2 and NRP-1 was not specific to any of the CD4+ T cell subsets (Supplementary Figure 3E).

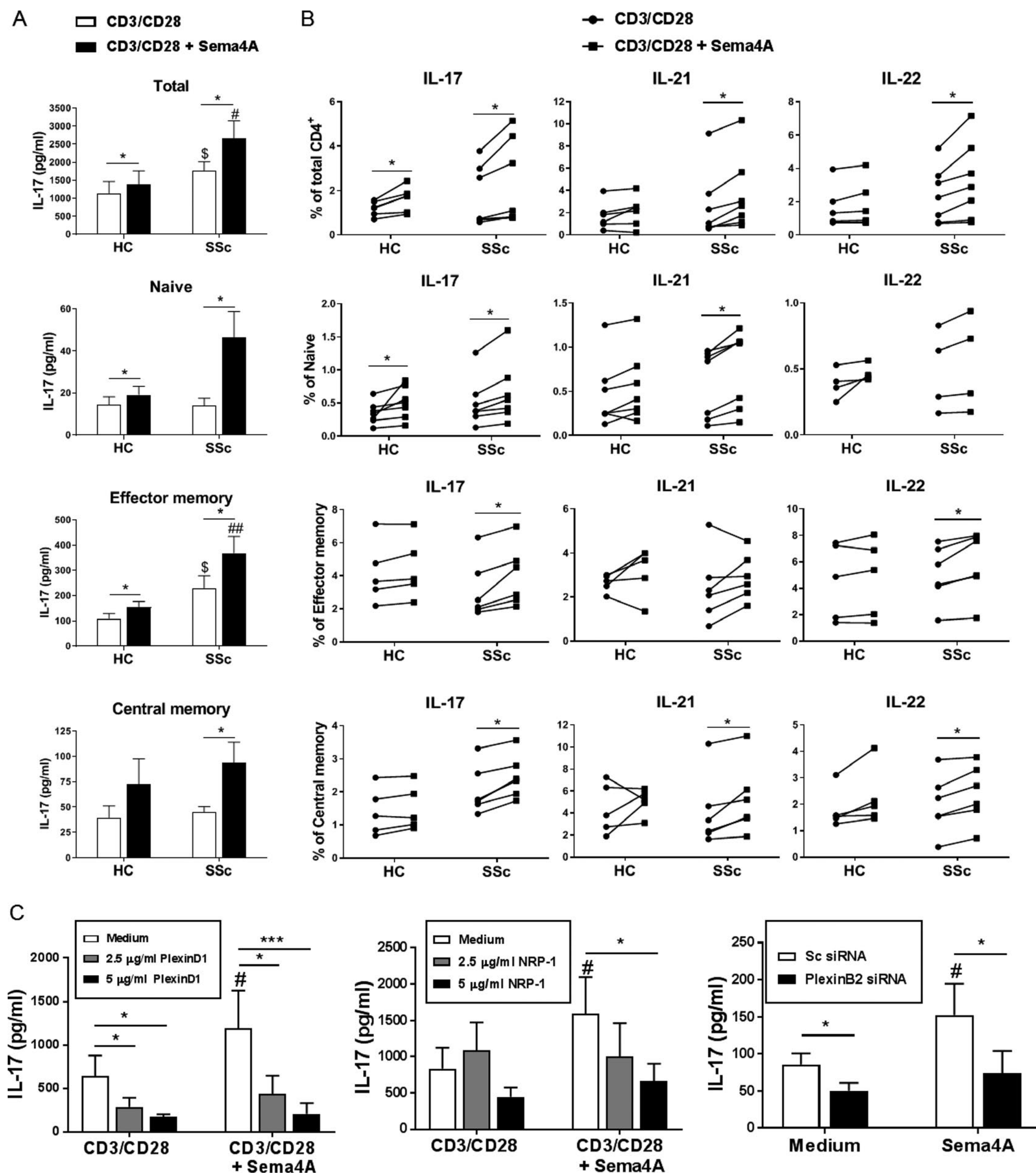

Figure 2. Semaphorin 4A (Sema4A)–induced Th17 cytokine production in CD4+ T cells. **A** and **B**, Interleukin-17 (IL-17) secretion (**A**) and intracellular levels of IL-17, IL-21, and IL-22 (**B**) in total CD4+ T cells and different CD4+ T cell subsets from healthy controls (HCs) and patients with systemic sclerosis (SSc). Cells were activated with CD3/CD28 Dynabeads in the absence or presence of Sema4A for 5 days (for total CD4+ T cells), 7 days (for naive CD4+ T cells), or 2 days (for effector memory and central memory CD4+ T cells). **C**, Secretion of IL-17 by total CD4+ T cells from SSc patients following 5 days of activation with CD3/CD28 Dynabeads in the absence or presence of Sema4A, which had previously been incubated for 1 hour with increasing concentrations of blocking anti–plexin D1 or anti–neuropilin 1 (anti–NRP-1) antibodies or after plexin B2 silencing. In **A** and **C**, bars show the mean ± SEM of 5–7 independent experiments. In **B**, symbols represent individual subjects. * = $P < 0.05$; *** = $P < 0.001$ for the indicated comparisons. $ = $P < 0.05$ versus CD3/CD28-activated cells from healthy controls; # = $P < 0.05$; ## = $P < 0.01$, versus CD3/CD28-activated, Sema4A-treated cells from healthy controls in **A** and versus medium or scrambled (Sc) small interfering RNA (siRNA) in **C**.

Sema4A enhances production of Th17 cytokines by CD4+ T cells. Mouse studies have shown that Sema4A is involved in Th17 skewing (21–23). We therefore analyzed the functional consequences of Sema4A ligation on the human production of Th17 cytokines. Sema4A did not affect cell viability or proliferation in response to CD3/CD28 stimulation of CD4+ T cells from either healthy controls or SSc patients (Supplementary Figures 4A and B, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40915/abstract). However, in both healthy controls and SSc patients, Sema4A enhanced IL-17 secretion induced by CD3/CD28 stimulation, as well the frequency of IL-17–positive, IL-21–positive,

and IL-22–positive cells (Figures 2A and B and Supplementary Figure 4C). In contrast, Sema4A did not regulate the expression of Th1 and Th2 cytokines (Supplementary Figure 5, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40915/abstract). Effects of Sema4A on T cell cytokine production were not due to differences in healthy control and SSc patient T cell subset frequencies, since Sema4A enhanced the secretion of the production of IL-17, IL-21, and IL-22 induced by CD3/CD28 in naive, effector memory, and central memory T cell subsets from healthy controls and SSc patients (Figures 2A and B), while not affecting the production of Th1 and Th2 cytokines (Supplementary Figure 5). Importantly, the production of IL-17 was significantly higher in total, naive, and effector memory CD4+ T cells from SSc patients than in those from healthy controls.

We next determined the effect of blocking the expression of Sema4A receptors on Sema4A-induced production of Th17 cytokines in total CD4+ T cells from healthy controls and SSc patients. The blocking of plexin D1 and NRP-1 with specific antibodies, and the silencing of plexin B2 expression with siRNA, significantly reduced Sema4A-induced secretion of IL-17, as well Sema4A-enhanced frequency of IL-17–positive, IL-21–positive, and IL-22–positive cells in both healthy controls and SSc patients (Figure 2C and Supplementary Figures 6 and 7, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40915/abstract). Notably, this reduction was also observed in the absence of exogenous Sema4A. Since antibody blocking and cell transfection did not affect CD4+ T cell viability or proliferation (data not shown), this effect is likely due to the endogenous secretion of Sema4A by CD4+ T cells (Figure 1F).

Sema4A directs fibroblast activation via IL-17 production by CD4+ T cells. Several studies have implicated IL-17 in SSc inflammatory and fibrotic processes (7–11). To determine if the enhanced IL-17 production induced by Sema4A was sufficient to induce biologic responses, we stimulated dermal fibroblasts from healthy controls with the conditioned medium of CD4+ T cells, previously preincubated with an anti–IL-17 antibody or its isotype control, and analyzed fibroblast expression of mRNA for ECM components and inflammatory mediators. Compared to the conditioned medium of unstimulated CD4+ T cells, the conditioned medium of CD4+ T cells stimulated with CD3/CD28 alone or in combination with Sema4A did not modulate the expression of mRNA for *COL1A1*, *COL3A1*, or *FN1* (Figure 3A). However, the conditioned medium of activated CD4+ T cells significantly induced expression of mRNA for *IL6*, *IL8*, *PTGS2*, *CCL2*, *CCL20*, and *CXCL2*, and the secretion of IL-6 and IL-8 proteins, compared to the conditioned medium of unstimulated cells. Importantly, the

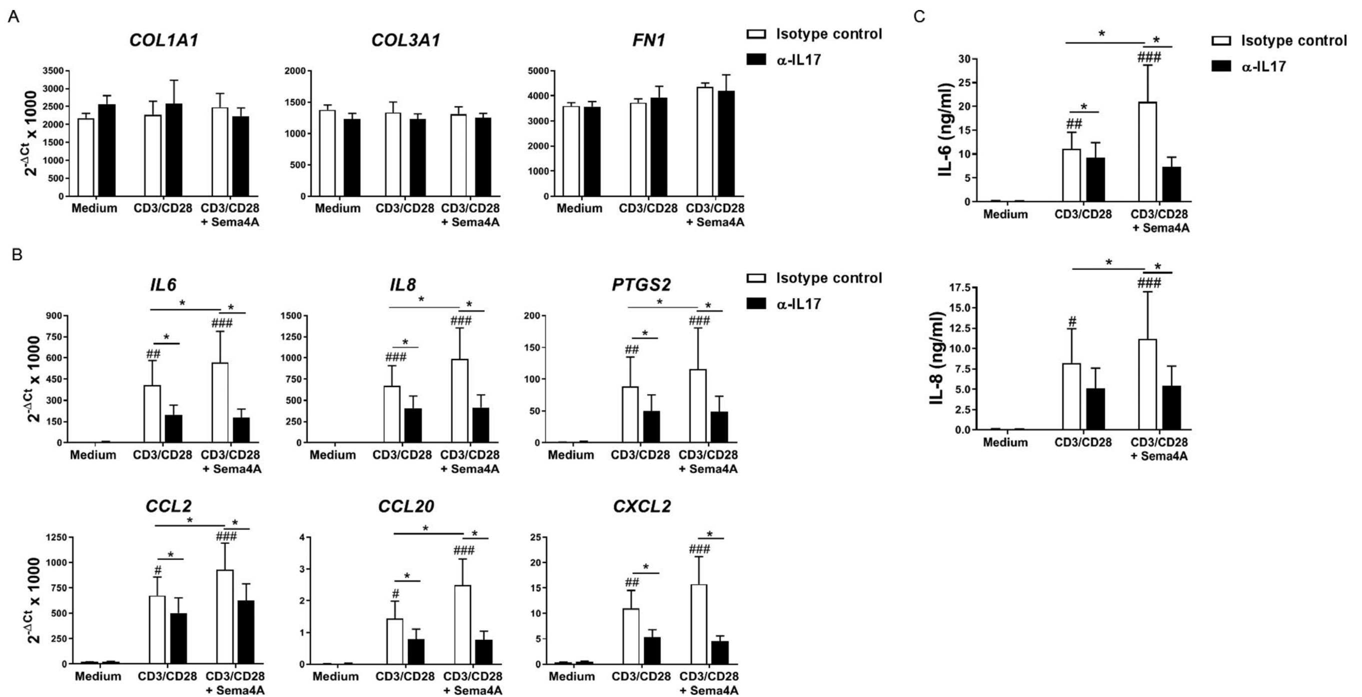

Figure 3. Semaphorin 4A (Sema4A) orchestrates fibroblast activation via interleukin-17 (IL-17) production by CD4+ T cells. **A** and **B**, Expression of mRNA for extracellular matrix components (**A**) and inflammatory mediators (**B**) in skin fibroblasts from healthy controls. Cells were incubated for 24 hours with conditioned medium of activated CD4+ T cells in the absence or presence of Sema4A, which had previously been incubated for 1 hour with an anti–IL-17 antibody or its isotype control. **C**, Expression of IL-6 and IL-8 protein (ng/ml) by skin fibroblasts from healthy controls. Cells were incubated for 24 hours with conditioned medium of activated CD4+ T cells in the absence or presence of Sema4A, which had previously been incubated for 1 hour with an anti–IL-17 antibody or its isotype control. Bars show the mean ± SEM of 7 independent experiments. * = $P < 0.05$ for the indicated comparisons. # = $P < 0.05$; ## = $P < 0.01$; ### = $P < 0.001$ versus medium.

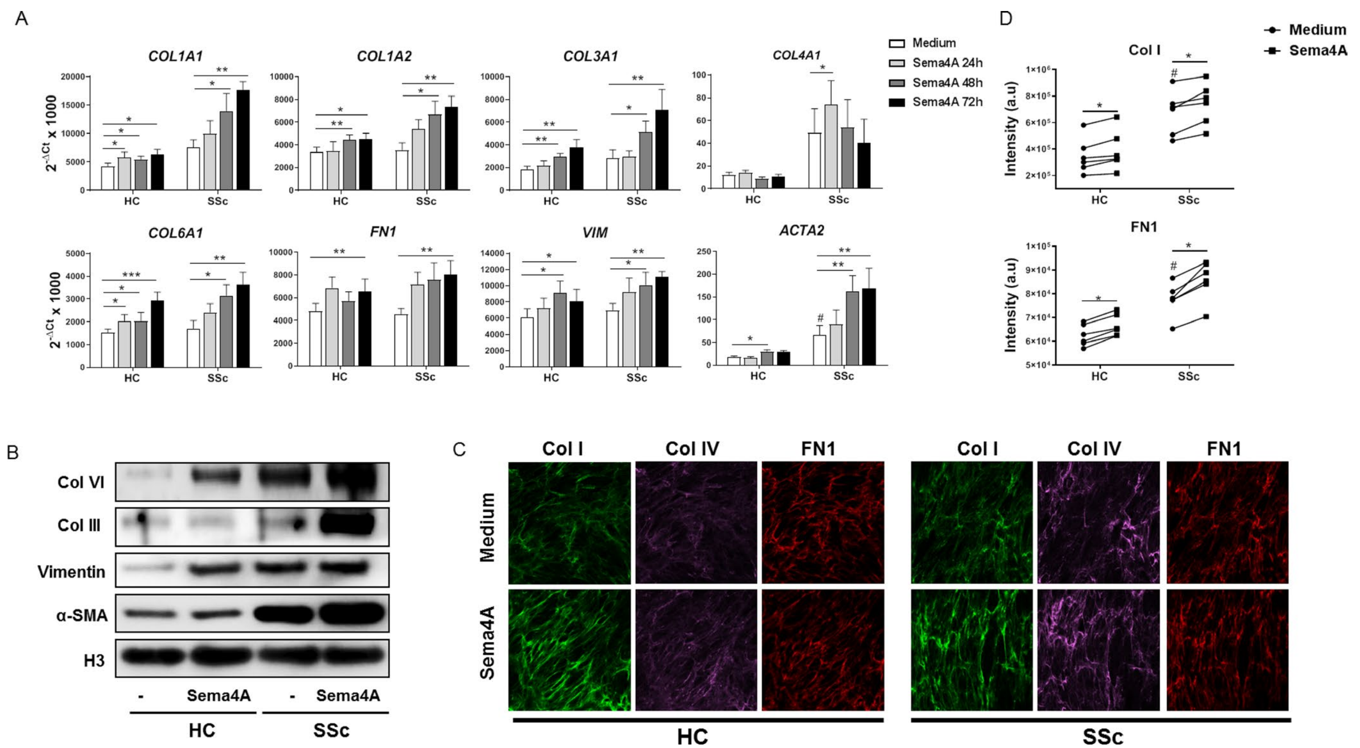

Figure 4. Semaphorin 4A (Sema4A)–induced expression and deposition of extracellular matrix (ECM) components. **A**, Expression of mRNA for ECM components by skin fibroblasts from healthy controls (HCs) and patients with systemic sclerosis (SSc). Cells were stimulated with Sema4A (200 ng/ml) for 24, 48, or 72 hours. Bars show the mean ± SEM of 6 independent experiments. **B**, Representative immunoblots showing expression of type VI collagen (Col VI), type III collagen, vimentin, and α-smooth muscle actin (α-SMA) protein in skin fibroblasts from healthy controls and SSc patients. Cells were stimulated with Sema4A for 72 hours. **C** and **D**, Type I collagen, type IV collagen, and fibronectin 1 (FN1) production (**C**) and deposition (**D**) by skin fibroblasts from healthy controls and SSc patients. Cells were stimulated with Sema4A for 7 days. Images in **C** are representative of 4 independent experiments. Original magnification × 20. Symbols in **D** represent individual subjects. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ for the indicated comparisons. # = $P < 0.05$ versus unstimulated healthy control fibroblasts.

expression of these mediators was significantly enhanced by the conditioned medium of CD4+ T cells activated in the presence of Sema4A and was significantly inhibited when supernatants were preincubated with anti–IL-17 antibody (Figures 3B and C). Taken together, these results suggest that Sema4A-induced IL-17 expression by T cells is partially responsible for dermal fibroblast expression of inflammatory mediators.

Sema4A induces a profibrotic phenotype in dermal fibroblasts. Since Sema4A can modulate collagen contraction by lung fibroblasts (24), we analyzed the potential profibrotic effect of Sema4A on skin fibroblasts. Sema4A up-regulated, in a time-dependent manner, the expression of mRNA for various ECM components involved in fibrotic processes, such as *COL1A1*, *COL1A2*, *COL3A1*, *COL4A1*, *COL6A1, VIM*, *FN1,* and *ACTA2*, the gene that encodes the myofibroblast marker α-SMA, in fibroblasts from both healthy controls and SSc patients (Figure 4A). Protein analysis confirmed these results, as Sema4A induced the expression of type III collagen, type VI collagen, vimentin, and α-SMA (Figure 4B). Sema4A also induced the production of type I collagen, type IV collagen, and fibronectin by both healthy control and SSc dermal fibroblasts (Figure 4C). ECM deposition analysis confirmed these findings and also demonstrated that the deposition of type I collagen and fibronectin was significantly higher in SSc fibroblasts than healthy control fibroblasts (Figure 4D).

Finally, we analyzed the effect of the inhibition of Sema4A signaling on the production of ECM components by SSc fibroblasts. Neutralization of NRP-1 did not affect the expression of mRNA for these components in Sema4A-stimulated fibroblasts (Supplementary Figure 8, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40915/abstract). However, plexin D1 neutralization or plexin B2 silencing significantly suppressed expression of mRNA for *COL1A1*, *COL1A2*, *COL3A1*, *COL6A1, VIM*, *FN1,* and *ACTA2* (Figures 5A and D), and the expression of type III collagen, type VI collagen, vimentin, and α-SMA protein in SSc patient fibroblasts (Figures 5B, C, E, and F). The lack of effect of NRP-1 was not due to differences in the expression levels of plexin D1, plexin B2, and NRP-1, as all 3 receptors were detected in dermal fibroblasts from both healthy controls and SSc patients. We did not find differences in the expression of mRNA for these receptors between healthy con-

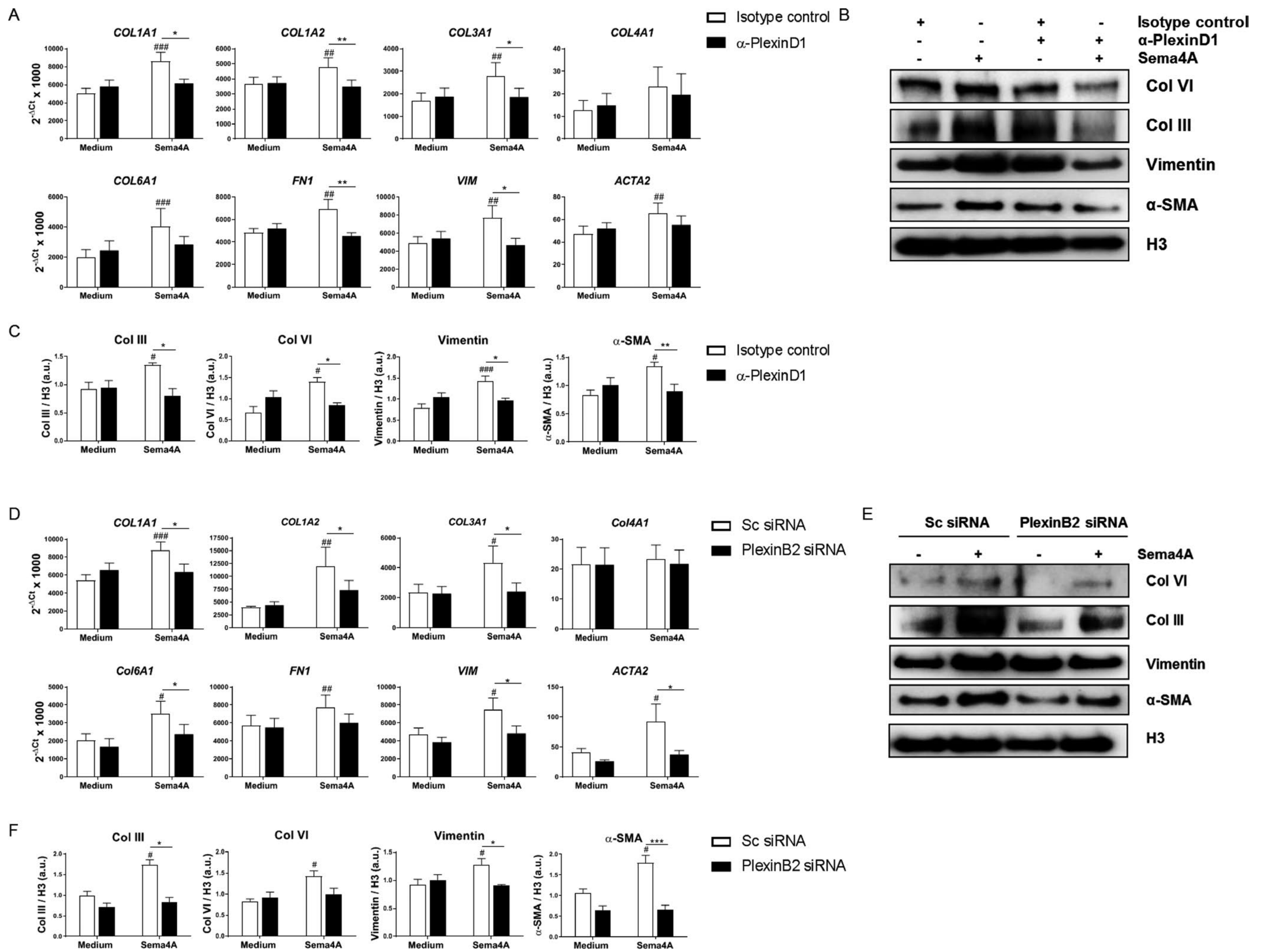

Figure 5. Plexin D1 blocking and plexin B2 silencing abrogate semaphorin 4A (Sema4A)–induced expression of extracellular matrix (ECM) components. **A** and **B**, Expression of mRNA for ECM components (**A**) and representative immunoblots showing expression of type VI collagen (Col VI), type III collagen, vimentin, and α-smooth muscle actin (α-SMA) protein (**B**) by skin fibroblasts from patients with systemic sclerosis (SSc). Cells were stimulated with Sema4A for 72 hours after 1 hour of preincubation with blocking anti–plexin D1 antibody or its isotype control. **C**, Densitometric analysis of type III collagen, type VI collagen, vimentin, and α-SMA protein expression. Data were normalized to histone H3 expression. **D** and **E**, Expression of mRNA for ECM components (**D**) and representative immunoblots showing expression of type VI collagen, type III collagen, vimentin, and α-SMA protein (**E**) by skin fibroblasts from SSc patients. Cells were stimulated with Sema4A for 72 hours after plexin B2 silencing. **F**, Densitometric analysis of type III collagen, type VI collagen, vimentin, and α-SMA protein expression. Data were normalized to histone H3 expression. In **A**, **C**, **D**, and **F**, bars show the mean ± SEM of 5–6 independent experiments. In **B** and **E**, results are representative of 5–6 independent experiments. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ for the indicated comparisons. # = $P < 0.05$; ## = $P < 0.01$; ### = $P < 0.001$, versus isotype control or unstimulated scrambled (Sc) small interfering RNA (siRNA)–transfected cells.

trols and SSc patients, but Sema4A up-regulated the expression of mRNA for *PLXND1*, *PLXNB2,* and *NRP1* in fibroblasts from both healthy controls and SSc patients (Supplementary Figure 9, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40915/abstract), suggesting that Sema4A might further enhance its profibrotic effect via the up-regulation of its receptors. Taken together, these data demonstrate that Sema4A directly induces profibrotic gene expression in healthy control and SSc dermal fibroblasts, dependent on signaling mediated by plexin D1 and plexin B2.

DISCUSSION

Herein, we demonstrated that Sema4A plays an essential role in inflammation and fibrosis, 2 main aspects of SSc pathology. Sema4A induces inflammation in an IL-17–dependent manner and directly induces a profibrotic phenotype in dermal fibroblasts (Figure 6). Abnormal activation of monocytes and T cells contributes to the ongoing inflammation observed in SSc patients (1,5,6,28,29). We found that plasma levels of Sema4A were elevated in SSc patients, similar to other autoimmune diseases such as rheumatoid arthritis and MS (23,30), and identified periph-

eral blood monocytes and CD4+ T cells as potential sources of Sema4A in SSc patients. Importantly, poly(I-C), a TLR-3 agonist, (31) and CXCL4, both important to the pathology of SSc (26), induced Sema4A secretion by circulating monocytes. CD3/CD28-mediated activation of CD4+ T cells also induced the secretion of Sema4A, as well as the surface expression of its receptors plexin B2 and NRP-1. Taken together, these results demonstrate that regulated expression of Sema4A and its receptors is disrupted in circulating SSc patient immune cells.

Previous mouse studies have demonstrated that Sema4A mediates Th17 skewing, and elevated serum levels of Sema4A in MS patients are positively associated with Th17 skewing (21–23). In this study we directly demonstrated a requisite role for Sema4A in the production of Th17, but not Th1 or Th2 cytokines, by human CD4+ T cells. In contrast with our observation, Sema4A was recently reported to induce proliferation and Th2 polarization in human CD4+ T cells (32). These differences may be attributed to the different recombinant Sema4A protein used, the different manner of CD4+ T cell activation and proliferation, and the use of CD4+ T cells from adult blood buffy coats. The effect of Sema4A on Th17 cytokine production was not considered in that study and therefore cannot be directly compared with our results. The effect of Sema4A on Th17 cytokine production was not restricted to a specific CD4+ T cell population, as we observed a similar induction of Th17 cytokines in naive, central memory, and effector memory populations. Importantly, Sema4A-induced IL-17 secretion was significantly higher in SSc patients, likely due to elevated expression of the receptors plexin B2 and NRP-1 by resting and activated SSc patient CD4+ T cells.

Inhibition of Sema4A signaling, through the blocking of the receptors NRP-1 and plexin D1, or the silencing of plexin B2, drastically reduced Th17 cytokine secretion. Notably, this effect was also observed in the absence of exogenous Sema4A, suggesting that CD4+ T cell production of Sema4A induces the expression of Th17 cytokines in an autocrine/paracrine manner. Sema4A can bind to different receptors in a cell type–dependent manner. Plexin D1 is the Sema4A receptor in macrophages, while Sema4A binds to plexin B2 in CD8+ T cells and to NRP-1 in Treg cells (33–35). Our results show that all 3 of these receptors are involved in Sema4A signaling in CD4+ T cells, although the highest inhibition of Sema4A-induced Th17 cytokine production was observed after the blocking of plexin D1. This finding might be explained simply by the fact that the percentage of CD4+ T cells expressing plexin D1 is much higher than those expressing plexin B2 and NRP-1, but we cannot rule out the potential differential blocking efficiency of antibodies used, residual expression of plexin B2 following gene silencing, or involvement of immunoglobulin-like transcript 4, a recently identified Sema4A receptor in CD4+ T cells (32).

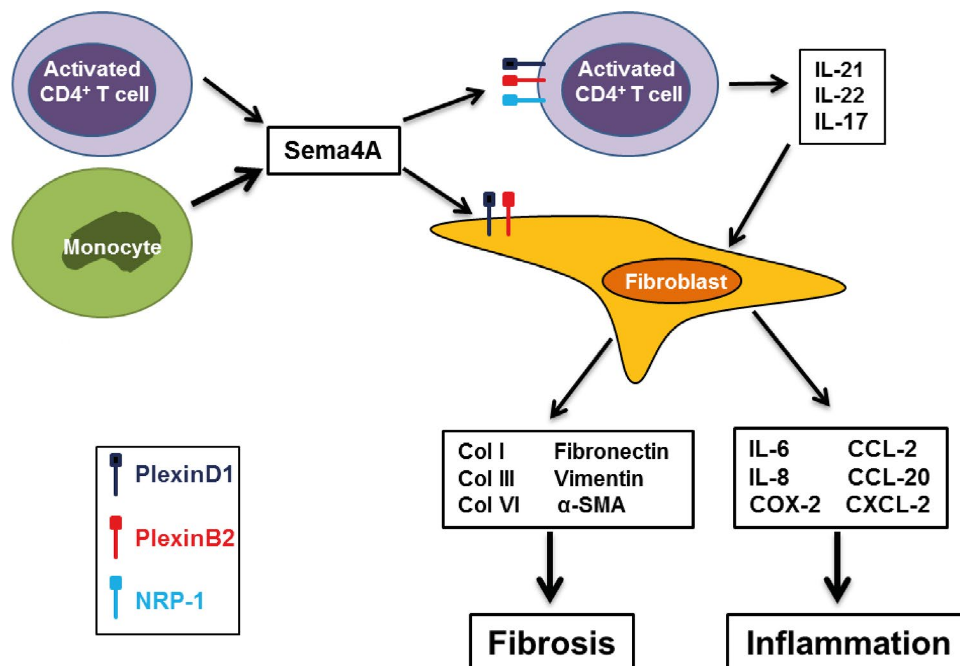

Figure 6. Schematic overview of the inflammatory and fibrotic roles of semaphorin 4A (Sema4A) in the pathogenesis of systemic sclerosis (SSc). Sema4A is elevated in the plasma of SSc patients, due to increased production by monocytes and, to a lesser extent, CD4+ T cells. In CD4+ T cells, Sema4A enhances the production of Th17 cytokines induced by CD3/CD28, and secreted interleukin-17 (IL-17) induces the production of inflammatory mediators and chemokines in dermal fibroblasts. Sema4A also plays a direct role in fibrosis by inducing the production of extracellular matrix components and the expression of the myofibroblast marker α-smooth muscle actin (α-SMA) in dermal fibroblasts. NRP-1 = neuropilin 1; Col I = type I collagen; COX-2 = cyclooxygenase 2.

To date, the role of IL-17 in SSc fibrotic processes has been uncertain, as mouse studies have shown that IL-17 plays a profibrotic role that was not observed in isolated human fibroblasts (36–39). In this study, we observed that IL-17 failed to modulate fibroblast expression of the ECM components examined, confirming that IL-17 does not induce a fibrotic phenotype in SSc fibroblasts. Instead, IL-17 can enhance dermal fibroblast expression of inflammatory mediators that are elevated in SSc patients and play an important role in disease pathology, including IL-6, IL-8, cyclooxygenase 2 (COX-2), CCL2, CCL20, and CXCL2 (1,40,41). Neutralization of IL-17 did not completely abrogate fibroblast production of inflammatory mediators in response to conditioned T cell supernatants, suggesting that other T cell cytokines are also involved in fibroblast activation. In this regard, IL-22 enhances TNF-induced expression of chemokines by healthy control and SSc dermal fibroblasts (42,43). Also, other mediators not regulated by Sema4A, such as TNF and IFNγ, are strong activators of dermal fibroblasts (43–45). However, this effect was not due to the Sema4A present in the supernatants, as the blocking of plexin D1 did not influence the expression of inflammatory mediators (Supplementary Figure 10, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40915/abstract).

Our results suggest that Sema4A plays a role in SSc pathology, through the induction of IL-17 by CD4+ T cells, which promote fibroblast expression of inflammatory mediators. Fibroblasts are also key contributors to fibrosis in SSc, and their activation in affected tissue leads to their differentiation into α-SMA–expressing myofibroblasts and excessive deposition of ECM components (4,46–48). In the present study, we found that Sema4A induced the expression of ECM components and α-SMA by healthy control and SSc patient dermal fibroblasts, effects mediated by the receptors plexin D1 and plexin B2. In conclusion, in this study we have identified Sema4A as a key mediator of Th17 production and fibrosis, and blocking Sema4A signaling might suppress both pathologic processes in SSc, a complex and heterogeneous disease for which currently available therapies can only treat organ manifestations and no antifibrotic drugs have yet to be approved (49,50).

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. García 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. Carvalheiro, Affandi, Marut, Reedquist, Radstake, García.

Acquisition of data. Carvalheiro, Affandi, Malvar-Fernández, Dullemond, Cossu, Ottria, Mertens, Giovannone, Bonte-Mineur, Kok, García.

Analysis and interpretation of data. Carvalheiro, Affandi, Malvar-Fernández, García.

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