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T Cells Expressing Allograft Inflammatory Factor 1 Display Increased Chemotaxis and Induce a Profibrotic Phenotype in Normal Fibroblasts In Vitro

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Objective. **Allograft inflammatory factor 1 (AIF-1) was first identified in rat cardiac allografts undergoing chronic rejection. The vasculopathy of chronic allograft rejection is strikingly similar to that seen in patients with systemic sclerosis (SSc). We previously demonstrated AIF-1 expression in inflammatory cells infiltrating skin and lungs from SSc patients, but its role in SSc pathogenesis is unknown. The present study was undertaken to investigate the effects of AIF-1 on T cell migration and production of cytokines capable of modulating normal dermal fibroblast functions.**

Methods. **Stably transfected Jurkat T cells expressing 2 AIF-1 splicing variants were prepared, and their migration toward fibroblast monolayers assayed in Transwell cultures. Cytokine production was assessed by real-time polymerase chain reaction (PCR) and multiplex enzyme-linked immunosorbent assay. Fibroblast gene expression was quantified by real-time PCR, and collagen production by Western blot analysis of culture media.**

Results. **AIF-1 significantly increased Jurkat T cell migration toward fibroblast monolayers. Expression of AIF-1 isoform 2 in Jurkat T cells up-regulated their production of interleukin-4 (IL-4) and IL-17. Conditioned media from AIF-1–expressing clones stimulated synthesis of types I and III collagen and expression of IL-6, transforming growth factor β, endothelin receptor, and α-smooth muscle actin by normal dermal fibroblasts.**

Conclusion. **These results suggest that AIF-1 may participate in the early pathogenesis of SSc by promoting tissue T cell infiltration and production of cytokines capable of inducing the expression of a fibrotic phenotype in normal fibroblasts.**

Systemic sclerosis (SSc) is a disease of unknown origin characterized by excessive deposition of collagen and other connective tissue macromolecules in skin and multiple internal organs, prominent and often severe alterations in the microvasculature, and humoral and cellular immunologic abnormalities. The most important clinical manifestations of SSc are caused by the exaggerated production of collagen and other connective tissue components by fibroblasts and other mesenchymal cells (1–4), resulting in severe and progressive fibrosis of the affected organs (5,6). There is strong evidence that chronic tissue inflammation plays a role in this process (7–9). Prominent mononuclear cell infiltration comprising predominantly macrophages and oligoclonal, activated CD4+ T cells is apparent in early SSc lesions (9–12). Indeed, the presence of several cytokines and growth factors of inflammatory cell origin in the affected tissues in SSc has been described (13,14).

Numerous studies examining the interplay between T cells and fibroblasts in the development of tissue fibrosis and fibroproliferative vasculopathy suggest that activated T cells can trigger fibroblast activation both by direct contact and by paracrine action of secreted cytokines (7,14–20). On the other hand, chemokines secreted by activated fibroblasts can induce chemotaxis of inflammatory cells, contributing to the amplification of the pathogenetic process

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Table 1. Primers used in cloning and in real-time PCR studies*

Gene	Forward primer	Reverse primer
AIF-1 isoform 1	ATGGAGTTTGACCTTAATGGAAATGGC	TCAGGGCAACTCAGAGATAGCTTT
AIF-1 isoform 2	ATGGAGTTTGACCTTAATGGAAATGGC	TCACATTTTTAGGATGGCAGATCTCTTG
AIF-1 isoform 3	ATGAGCCAAACCAGGGATTTACAG	TCAGGGCAACTCAGAGATAGCTTT
β-actin	TTGCCGACAGGATGCAGAA	GCCGATCCACACGGAGTACTT
IL-4	TGCTGCCTCCAAGAACACAA	TGTAGAACTGCCGGAGCACA
IL-6	TCCTTCTCCACAAGCGCC	AAGGCAGCAGGCAACACC
IL-10	AGGCTACGGCGCTGTCAT	GGCATTCTTCACCTGCTCCA
IL-17	CCTCACCTTGGAATCTCCACC	GCACTTTGCCTCCCAGATCA
TGFβ	CGAGCCTGAGGCCGACTA	AGATTTCGTTGTGGGTTTCCA
TNFα	CTGGCCCAGGCAGTCAGAT	GGGTTTGCTACAACATGGGCTA
IFNγ	TTCAGATGTAGCGGATAATGGAAC	TTCTGTCACTCTCCTCTTTCCA
CCR2	GCCACAAGCTGAACAGAGAAAGT	GAACGAGATGTGGACAGCATGT
CXCR4	GGAGGGGATCAGTATATACA	GAAGATGATGGAGTAGATGG
ET-R	CTGCTGCACATCGTCATTGAC	AGCTCCAAATGGCCAGTCCT
α-SMA	TGTATGTGGCTATCCAGGCG	AGAGTCCAGCACGATGCCAG
COL1A1	CCTCAAGGGCTCCAACGAG	TCAATCACTGTCTTGCCCCA
COL3A1	TTTGGCACAACAGGAAGCTG	GGACTGACCAAGATGGGAACAT

* PCR = polymerase chain reaction; AIF-1 = allograft inflammatory factor 1; IL-4 = interleukin-4; TGFβ = transforming growth factor β; TNFα = tumor necrosis factor α; IFNγ = interferon-γ; ET-R = endothelin receptor; α-SMA = α-smooth muscle actin.

(19,21,22). Therefore, a generally accepted hypothesis regarding the pathogenesis of tissue fibrosis in SSc is that activated T cells specifically infiltrate target organs and, once in the tissue, they release cytokines and growth factors, which in turn initiate and/or perpetuate the fibrotic process as well as the endothelial and vascular alterations (8,9,14,23). Immunohistochemistry and gene expression studies have identified numerous cytokines, growth factors, and other polypeptides that are selectively expressed in affected tissue in SSc and are therefore believed to participate in its pathogenesis (21,22,24–26). Allograft inflammatory factor 1 (AIF-1) is one of the most recently identified candidates (27,28).

AIF-1 is an intracellular protein, encoded by a phylogenetically conserved gene which was first identified in rat cardiac allografts undergoing chronic rejection (29,30). AIF-1 thus appears to play a very important role in the vasculopathy of transplant rejection, a process that bears striking resemblance to the vasculopathy of SSc.

Our group has demonstrated increased expression of AIF-1 in both skin and lungs of patients with SSc, particularly in endothelial cells, infiltrating T lymphocytes, and tissue macrophages (27,28), suggesting that the protein may have a role in SSc pathogenesis. Findings of recent genetic association and microarray gene expression studies have provided strong support for this notion (31–33); however, the putative mechanisms that may be involved are not known. In this study we examined whether the induced expression of AIF-1 in T cells could increase their migration toward dermal fibroblasts cultured in monolayers, and whether soluble factors produced by AIF-1–expressing T cells could result in increased collagen production by normal fibroblasts and expression of molecular markers reflecting a profibrotic phenotype in vitro.

MATERIALS AND METHODS

Cell lines. Buffy coat preparations were obtained from the blood bank at Thomas Jefferson University Hospital, in accordance with Industry Review Board–approved protocols. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation with Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) and processed for RNA extraction. I-32 Jurkat T cells (a kind gift from Dr. B. Perussia, Thomas Jefferson University) were cultured in T75 flasks and maintained at 6×10^5 cells/ml in RPMI 1640 supplemented with 5% fetal bovine serum and glutamine. Fibroblasts cultured from normal human dermis were obtained from the Scleroderma Center Tissue Bank, Thomas Jefferson University, and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, antibiotics, and glutamine (complete medium) until confluent. Fibroblasts were used at passages 4–10. For real-time polymerase chain reaction (PCR) experiments, fibroblasts were harvested with trypsin–EDTA, washed in phosphate buffered saline, and then processed for RNA extraction using an RNeasy kit according to the protocol

recommended by the manufacturer (Qiagen, Valencia, CA), including a genomic DNA digestion step.

AIF-1 isoform cloning. Total RNA was extracted from PBMCs from normal donors using an RNeasy kit. Total RNA (2 μg) was reverse transcribed using a Superscript II reverse transcriptase kit (Invitrogen, San Diego, CA) and amplified with primers designed to amplify the full-length complementary DNA of each AIF-1 isoform (Table 1). PCR products were electrophoresed on 2% agarose gels, and bands of expected length were eluted, purified, and sequenced to verify their specificity. PCR products were cloned into pCMV expression vector, according to the instructions of the manufacturer (Invitrogen). Clones were screened by PCR for insertion and orientation of the insert and resequenced for accuracy. Plasmids corresponding to AIF-1 isoforms 2 and 3 were verified by sequencing and were used for further studies. (The sequences were submitted and published on the NCBI database [Del Galdo F, Artlett C, and Jiménez SA: accession nos. EF070983 and EF070982]).

Stable transfectants. I-32 Jurkat T cells were screened for AIF-1 expression by real-time PCR, using isoform-specific primers as previously described (28). Four hundred thousand Jurkat T cells at a concentration of 10^6/ml were electroporated with 10 μg of pCMV plasmids carrying either AIF-1 isoform 2 or AIF-1 isoform 3 or with the empty vector, using a BTX ECM-630 electroporator (Genetronic, San Diego, CA) for 20 msec at 200V and 940 μF, in 1-mm–diameter cuvettes. Cells were selected by culturing for 9 weeks in G-418 medium (Sigma, St. Louis, MO) at concentrations of 900 μg/ml for the first week and 500 μg/ml thereafter. Bulk cultures were subcloned by limiting dilution, and high AIF-1 isoform–expressing clones were screened by real-time PCR as previously described (28), selected, and pooled for further studies.

Migration experiments. Migration assays were performed using 6-well Transwell plates with an 8-μm–pore-size polycarbonate filter (Costar, Cambridge, MA), as previously described (34). Briefly, normal dermal fibroblasts were placed in the lower chamber and grown to confluence in complete medium. Stably transfected I-32 Jurkat T cells (2×10^5) were loaded in the upper chamber. Transwell plates were then incubated for 3 hours or 6 hours at 37°C in a 5% CO_2 humidified atmosphere. Lower chambers were photographed using a Zeiss (Wetzlar, Germany) Axiovert 25 microscope equipped with a Spotcam digital camera. Image files were acquired with Spot advanced software and stored as jpeg files. Cell migration rates were determined by counting the Jurkat T cells that had migrated through the filter into the lower chamber in 6 random high-power fields (hpf) (200×). Experiments were performed in triplicate, examining the migration of 3 stably transfected pools of Jurkat T cells toward 3 different normal fibroblast cell lines. Migration inhibition experiments using a specific anti–monocyte chemotactic protein 1 (anti–MCP-1) antibody (BioLegend, San Diego, CA) were performed using 5 μg of antibody/10^6 cells as described (35). Cells migrating into the lower chamber were counted after 3 hours and 6 hours.

Real-time PCR. AIF-1 isoform and cytokine expression was verified and quantified using SYBR Green real-time PCR, as previously described (28). Primers for β-actin, interleukin-4 (IL-4), IL-6, IL-10, IL-17, interferon-γ (IFNγ), transforming growth factor β (TGFβ), tumor necrosis factor α (TNFα), CCR2, CCR4, endothelin receptor (ET-R), α-smooth muscle actin (α-SMA), and types I and III collagen were designed using Primer Express software (Applied Biosystems, Foster City, CA) and validated for specificity (Table 1). The differences in the number of messenger RNA (mRNA) copies in each PCR were corrected for human β-actin endogenous control transcript levels; levels in control experiments were set at 100, and all other values expressed as multiples thereof.

Enzyme-linked immunosorbent assay. SearchLight proteome array analyses (Pierce Biotechnology, Woburn, MA) were conducted to measure the levels of IL-4, IL-6, IL-17, and TGFβ in supernatants from AIF-1–expressing clones, as previously described (36–38). Briefly, samples were diluted 1:5, 1:50, or 1:1,000 and then incubated for 1 hour on the array plates, which had been prespotted with capture antibodies specific for each protein. Plates were decanted and washed 3 times before addition of a cocktail of biotinylated detection antibodies to each well. After incubation with detection antibodies for 30 minutes, plates were washed 3 times and incubated for 30 minutes with streptavidin–horseradish peroxidase. Plates were again washed, and SuperSignal Femto chemiluminescent substrate was added. The plates were immediately imaged using the SearchLight imaging system, and data were analyzed using ArrayVision software.

Stimulation of cultured fibroblasts with supernatants from AIF-1 isoform–expressing Jurkat T cells. Equal numbers of stably transfected Jurkat T cells were collected during their log phase of growth. Cells were centrifuged for 10 minutes at 1,500 revolutions per minute. Spun cells were processed for RNA extraction. The supernatants (AIF-1–conditioned medium) were filtered in a 0.22-μm filter, aliquoted, and stored at –20°C until used. Normal fibroblasts from adult forearm skin were cultured in complete medium in 6-well plates until confluence. Following preincubation for 24 hours with 40 μg/ml ascorbic acid (Sigma), fibroblasts were incubated for 24 hours in complete medium containing supernatants from each clone (at a 1:2 dilution). Fibroblasts were then harvested and processed for RNA extraction as described above. Type I collagen and type III collagen present in the culture media were quantified by Western blotting using specific anti-human type I or type III collagen polyclonal antibodies (Rockland, Gilbertsville, PA). For inhibition experiments, IL-4 neutralizing antibodies (BioLegend) were used as previously described (39). Briefly, conditioned supernatants (dilution 1:2) from AIF-1 isoform 2–expressing Jurkat T cells were preincubated for 1 hour at 37°C with or without anti–IL-4 antibodies at a 5-μg/ml final concentration and then used for stimulation experiments.

Statistical analysis. The statistical significance of the data was assessed by Student's 2-tailed *t*-test. *P* values less than 0.05 were considered significant. The raw number of transfected Jurkat T cells migrating toward the fibroblast monolayer in cell migration experiments was analyzed by repeated-measures analysis of variance (ANOVA). Additionally, single

conditions were subjected to Bonferroni adjustment for multiple comparisons.

RESULTS

Preparation of AIF-1 isoform expression vectors and stable transfection of Jurkat T cells. Several T cell lines were screened by real-time PCR to compare their AIF-1 isoform expression levels with those of PBMCs. All immortalized cell lines screened showed higher expression of AIF-1 isoform 1 compared with PBMCs (data not shown). The Jurkat T cell line I-32 displayed expression levels of AIF-1 isoforms 2 and 3 comparable with those of normal unstimulated PBMCs (Figure 1A) and was therefore selected for transfection. Transfection with empty vector did not affect AIF-1 isoform expression. Clones expressing high levels of AIF-1 isoforms 2 and 3 were identified by real-time PCR, selected, and pooled according to isoform specificity expression, and were used for further experiments (Figure 1B).

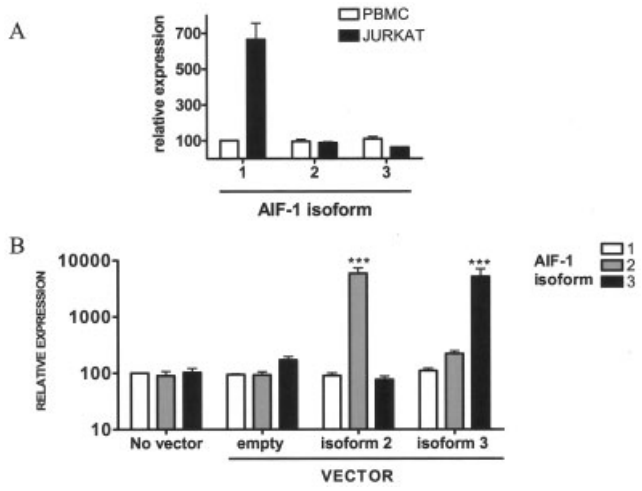

Figure 1. A, Similar levels of expression of allograft inflammatory factor 1 (AIF-1) isoforms 2 and 3 by Jurkat T cells and peripheral blood mononuclear cells (PBMCs). PBMCs and I-32 Jurkat T cells were processed for RNA extraction as described in Materials and Methods, and expression of AIF-1 isoforms was measured by real-time polymerase chain reaction (PCR). The mean expression level of each AIF-1 isoform in PBMCs from 3 different donors was arbitrarily set at 100, and Jurkat cell expression levels were expressed as multiples thereof. All real-time PCRs were performed in triplicate. Values are the mean and SD and are representative of 3 different experiments. **B,** Selective expression of AIF-1 isoform 2 or AIF-1 isoform 3 by stable transfectants, as measured by real-time PCR. Levels in nontransfected Jurkat cells were arbitrarily set at 100, and the other values expressed as multiples thereof. PCRs were performed in triplicate. Values are the mean and SD and are representative of 3 different experiments. *** = $P < 0.001$ versus expression levels of the same isoform in experiments with empty vector.

AIF-1 isoform 2 or 3 expression increases T lymphocyte migration toward fibroblast monolayers in vitro. Jurkat T cell lines stably transfected with an empty vector or with the vector expressing either isoform 2 or isoform 3 were assayed in migration experiments. The fibroblast monolayer itself induced Jurkat T cell migration, with a mean ± SD of 23.5 ± 4.27 cells/hpf migrating toward the fibroblast monolayer during the first 6 hours, whereas only 10.3 ± 2.02 cells/hpf migrated toward medium alone ($P = 0.008$). The migration of Jurkat T cells transfected with the empty vector was not different than the migration of nontransfected cells (Figure 2A). In contrast, Jurkat T cells expressing either AIF-1 isoform 2 or AIF-1 isoform 3 displayed a marked increase in migration toward the fibroblast monolayer (Figure 2B).

Quantitation of the effects of AIF-1 isoform expression on T cell migration showed that at the 3-hour time point, 15 ± 2.5 empty vector–transfected Jurkat T cells/hpf (mean ± SD) migrated toward the fibroblast monolayer, versus 51 ± 5.1 AIF-1 isoform 2–expressing cells/hpf and 41 ± 4.0 AIF-1 isoform 3–expressing cells/hpf (Figure 2C). At the 6-hour time point, 24 ± 5.9 empty vector–transfected Jurkat cells/hpf migrated toward the fibroblast monolayer, compared with 121 ± 15.2/hpf and 59 ± 15.8/hpf for AIF-1 isoform 2– and AIF-1 isoform 3–expressing cells, respectively (Figure 2C). The differences were highly significant when analyzed by repeated-measures ANOVA ($P \leq 0.0001$). At the 3-hour time point, AIF-1 isoform 2– and AIF-1 isoform 3–expressing cells displayed, respectively, 2.1-fold and 1.7-fold increased migration compared with empty vector–transfected cells, and at 6 hours, the increases were 7.0-fold and 3.83-fold, respectively ($P <$ 0.05 for all) (Figure 2D).

Up-regulation of CCR2 receptor expression is not responsible for AIF-1–mediated enhancement of T cell migration. To investigate the mechanisms of AIF-1–induced enhancement of T cell migration, the expression of chemokine receptor genes for CCR2 and CXCR4, functional receptors present on the cell surface of Jurkat T cells (21,39), was studied. CXCR4 gene expression was not affected by AIF-1 isoform 2 expression, whereas it was down-regulated by AIF-1 isoform 3 expression, compared with empty vector–transfected

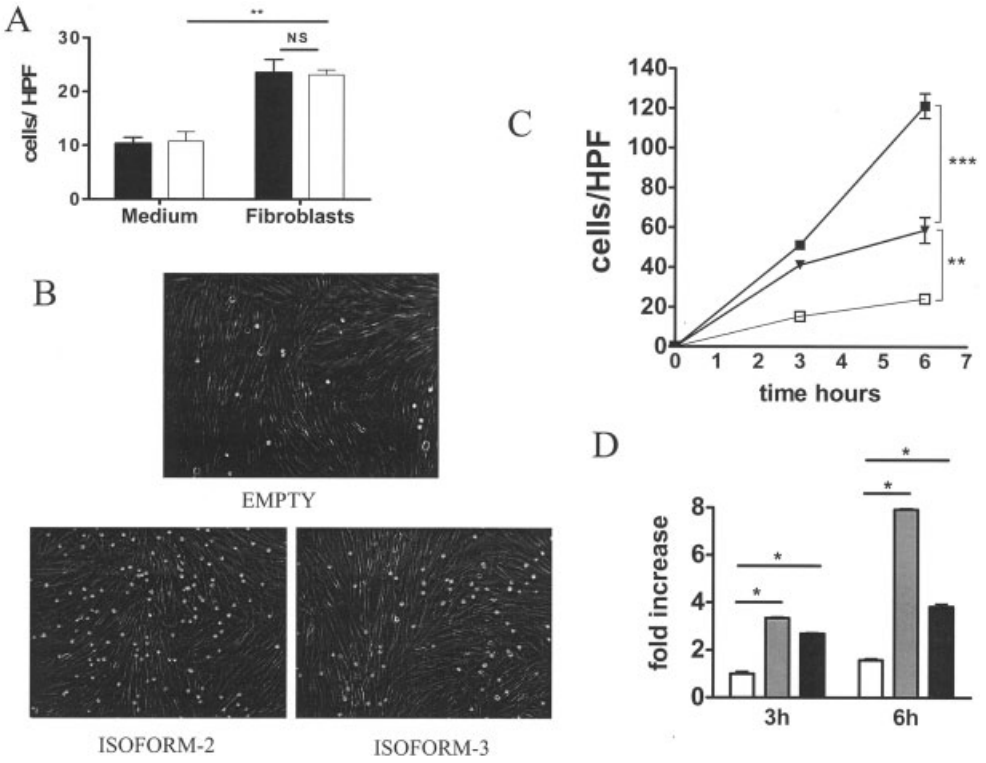

Figure 2. Allograft inflammatory factor 1 (AIF-1) increases Jurkat T cell migration toward a fibroblast monolayer. **A,** Induction of Jurkat T cell migration toward fibroblast monolayers. Migration toward medium alone or toward a fibroblast monolayer in nontransfected Jurkat T cells (solid bars) and empty vector stable transfectants (open bars) was measured as described in Materials and Methods. Following 6-hour incubation, 6 random high-power fields (hpf) (200×) were photographed, and cells counted. Values are the mean and SD. ** = $P = 0.008$. NS = not significant. **B,** Findings in Transwell lower chambers following 6-hour incubation. Representative hpf photographs of empty vector–transfected cells, AIF-1 isoform 2–transfected cells, and AIF-1 isoform 3–transfected cells are shown. Note the spindle-shaped fibroblast monolayer in the lower chamber. Jurkat cells are visible by phase-contrast microscopy as shining dots (original magnification × 200). **C,** Number of stably transfected Jurkat cells migrating into the lower chamber following 3 hours and 6 hours of incubation. Open squares = empty vector–transfected cells; solid squares = AIF-1 isoform 2–expressing cells; triangles = AIF-1 isoform 3–expressing cells. Values are the mean ± SD from 6 hpf and are representative of 3 different experiments. $P \leq 0.0001$ for all comparisons by repeated-measures analysis of variance. For comparisons among single groups after Bonferroni correction, ** = $P < 0.01$; *** = $P < 0.001$. **D,** Increase in the number of AIF-1 isoform 2– and AIF isoform 3–expressing cells (shaded bars and solid bars, respectively) migrating toward the fibroblast monolayer, compared with cells stably transfected with empty vector (open bars). The number of empty vector–transfected cells migrating toward the fibroblast monolayer at 3 hours was arbitrarily set at 1, and the mean and SD fold increase compared with this value (in 6 different hpf) was calculated. * = $P < 0.05$.

cells (Figure 3A). In contrast, CCR2 expression was strongly up-regulated in AIF-1 isoform 3–expressing cells (2-fold increase), whereas isoform 2–expressing cells displayed only 20% higher levels of CCR2 mRNA compared with empty vector–transfected controls (Figure 3A). To investigate whether the up-regulation of CCR2 mRNA was responsible for the observed increase in the migration of the transfected cells, inhibition experiments using MCP-1 neutralizing antibodies were performed as previously described (40). Blocking of MCP-1–CCR2 interaction did not affect the rate of migration of AIF-1 isoform 3–expressing cells toward normal fibroblast monolayers. Representative images from the Transwell lower chamber following 6-hour incubation are shown in Figure 3B, and the quantitative assessment of migrating T cells is shown in Figure 3C.

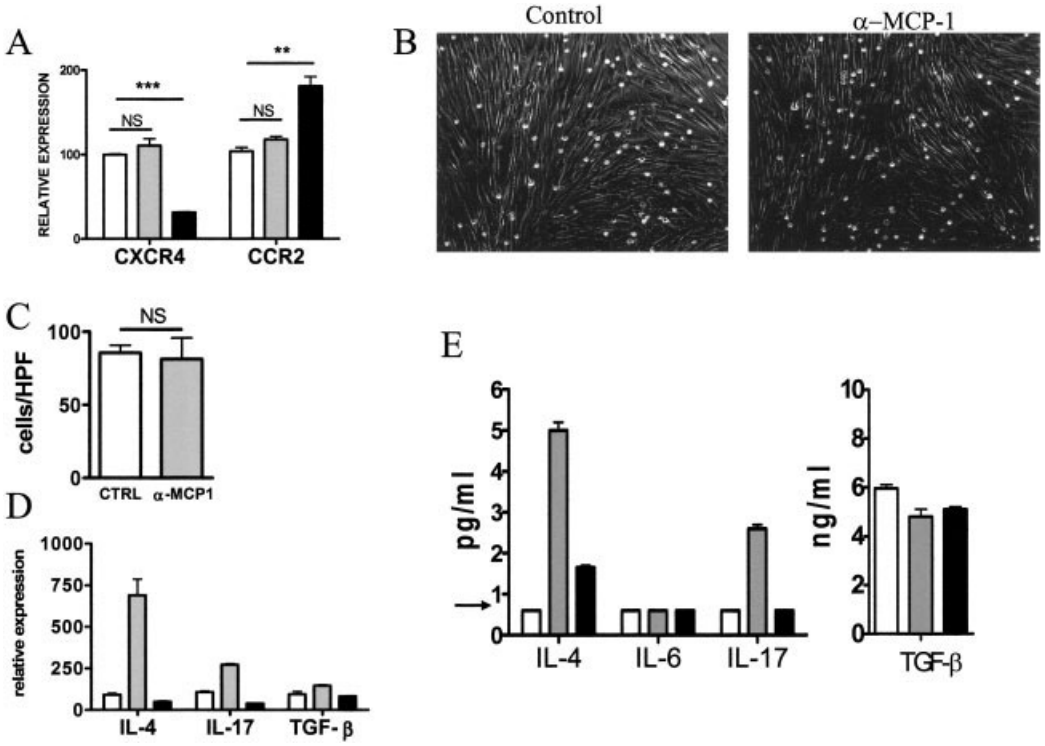

Figure 3. A, Chemokine receptor gene expression in allograft inflammatory factor 1 (AIF-1) transfectants. CCR2 and CXCR4 gene expression levels were measured by real-time polymerase chain reaction (PCR). Values are the mean and SD. ** = $P < 0.05$; *** = $P < 0.001$. NS = not significant. **B** and **C,** Lack of effect of blocking the monocyte chemotactic protein 1 (MCP-1)–CCR2 interaction on AIF-1 transfectant migration. AIF-1 isoform 3–expressing cells that were not preincubated (control [CTRL]) or were preincubated with neutralizing anti–MCP-1 antibody were assayed for migration toward a fibroblast monolayer. Qualitative results are shown in **B** (photomicrographs representative of 6 different high-power fields [hpf]), and quantitative results (mean and SD from 6 different hpf) in **C. D,** Effects of AIF-1 isoforms on cytokine gene expression by Jurkat T cells. Interleukin-4 (IL-4), IL-17, and transforming growth factor β (TGFβ) gene expression was measured by real-time PCR. PCRs were performed in triplicate. Values are the mean and SD from 3 different experiments. **E,** Levels of IL-4, IL-6, IL-17, and TGFβ in supernatants from the same experiments shown in **D,** measured by enzyme-linked immunosorbent assay. Reactions were conducted on 2 different supernatants, each in duplicate and in 3 different dilutions as described in Materials and Methods. Values are the mean and SD. Arrow shows the threshold of detection. In **A** and **C–E,** open bars = empty vector transfectants, shaded bars = AIF-1 isoform 2–expressing cells, and solid bars = AIF isoform 3–expressing cells. In **A** and **D,** gene expression levels in empty vector transfectants were arbitrarily set at 100, and values in AIF-1 isoform 2– and AIF isoform 3–expressing cells were expressed as multiples thereof.

AIF-1 isoform 2 and AIF-1 isoform 3 up-regulation of cytokine production by Jurkat T cells. Stably transfected Jurkat T cells were analyzed for IL-4, IL-6, IL-10, IL-17, TGFβ, IFNγ, and TNFα mRNA expression. AIF-1 isoform 2–expressing cells displayed a 700% increase in IL-4 expression and a 300% increase in IL-17 expression, whereas TGFβ mRNA expression was only modestly up-regulated (40%). In contrast, AIF-1 isoform 3–expressing cells exhibited a slight down-regulation of IL-4 and IL-17 expression and did not show any significant change in TGFβ expression (Figure 3D). IL-6, IL-10, TNFα, and IFNγ levels in AIF-1 isoform 2– or isoform 3–expressing cells were similar to those in empty vector–transfected cells (data not shown).

Multiplex enzyme-linked immunosorbent assay of the actual concentrations of IL-4, IL-6, IL-17, and TGFβ in the supernatants from the transfected cells showed that supernatants from AIF-1 isoform 2–expressing cells had higher levels of IL-4 and IL-17

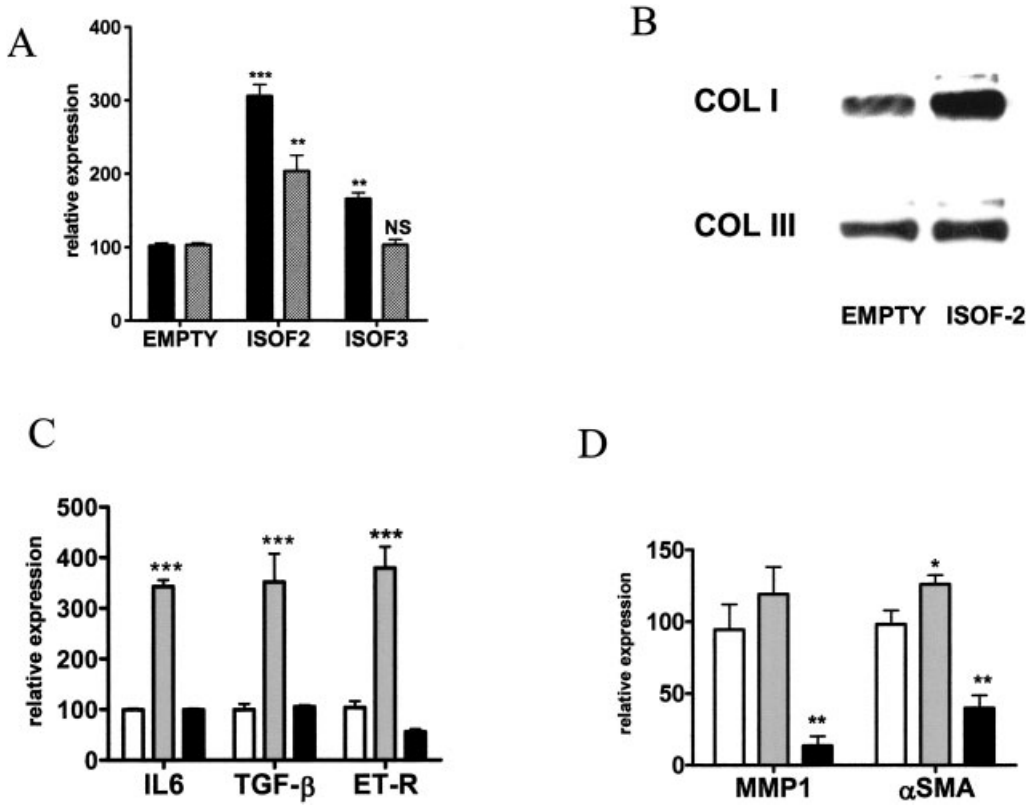

Figure 4. Conversion of normal fibroblasts to fibroblasts with a profibrotic phenotype induced by conditioned media from AIF-1–expressing Jurkat T cells. **A,** Fibroblast mRNA levels measured by real-time PCR following 24-hour incubation with conditioned medium from Jurkat T cells expressing either empty vector, AIF-1 isoform 2 (ISOF2), or AIF-1 isoform 3, as described in Materials and Methods. Levels of mRNA for COL1A1 (solid bars) and COL3A1 (shaded bars) in fibroblasts stimulated with conditioned medium from empty vector–transfected cells were arbitrarily set at 100, and the other values expressed as multiples thereof. Values are the mean and SD from 3 different PCRs and are representative of 3 different experiments. **B,** Western blot analysis of type I collagen (COL I) and type III collagen in fibroblast culture media following incubation with conditioned media from empty vector–transfected cells or AIF-1 isoform 2–expressing cells. **C,** Fibroblast expression of IL-6, TGFβ, and endothelin receptor (ET-R) measured by real-time PCR following 24-hour stimulation with conditioned media from either empty vector–, AIF-1 isoform 2–, or AIF-1 isoform 3–transfected Jurkat T cells. Values are the mean and SD from 3 different PCRs and are representative of 3 different experiments. **D,** Fibroblast expression of matrix metalloproteinase 1 (MMP-1) and α-smooth muscle actin (α-SMA) measured by real-time PCR as described in **C.** Values are the mean and SD from 3 different PCRs and are representative of 3 different experiments. In **C** and **D,** open bars = empty vector transfectants, shaded bars = AIF-1 isoform 2–expressing cells, and solid bars = AIF isoform 3–expressing cells; gene expression levels in empty vector transfectants were arbitrarily set at 100, and values in AIF-1 isoform 2– and AIF isoform 3–expressing cells were expressed as multiples thereof. $* = P < 0.05$; $** = P < 0.005$; $*** = P < 0.001$, versus empty vector. See Figure 3 for other definitions.

(5.0 pg/ml and 2.6 pg/ml, respectively) compared with either empty vector– or isoform 3–expressing cells, in which both were undetectable. In contrast, IL-6 was undetectable in all 3 supernatants, and TGFβ protein levels were essentially unchanged (6.1 ng/ml in the empty vector–transfected supernatants, and 4.5 and 5.0 ng/ml in the isoform 2– and isoform 3–transfected supernatants, respectively) (Figure 3E).

Conditioned media from AIF-1 isoform 2– or AIF-1 isoform 3–overexpressing Jurkat T cells increases collagen production by normal fibroblasts in vitro. We next examined whether secreted products from AIF-1

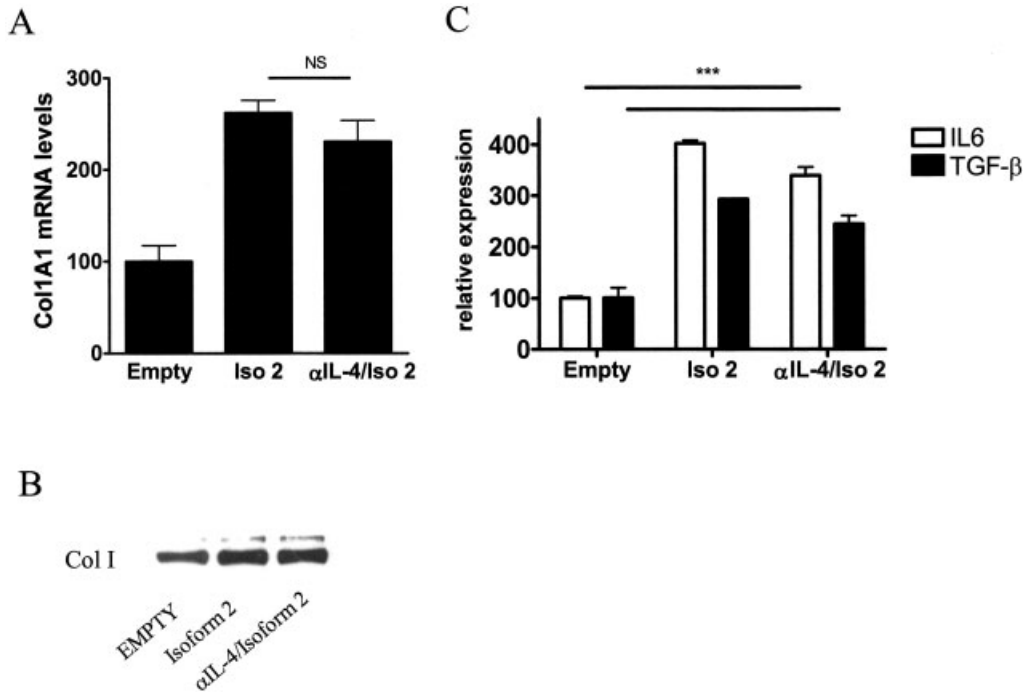

Figure 5. Confirmation that induction of fibroblast activation by AIF-1 isoform 2 (Iso 2)–conditioned media is independent of IL-4 secretion. **A,** Real-time PCR analysis of COL1A1 mRNA levels in fibroblasts following 24-hour stimulation with conditioned media from AIF-1 isoform 2 transfectants with or without preincubation with neutralizing anti–IL-4 antibody. COL1A1 mRNA levels in fibroblasts stimulated with conditioned media from AIF-1 isoform 2–expressing cells were up-regulated by 260% without anti–IL-4 preincubation and by 236% with anti–IL-4 preincubation. Values are the mean and SD from 3 different PCRs and are representative of 3 different experiments. **B,** Western blot analysis of type I collagen (Col I) in tissue culture media from the same experiments shown in **A. C**, Real-time PCR analysis of IL-6 and TGFβ mRNA levels assessed as described in **A.** Values are the mean and SD from 3 different PCRs and are representative of 3 different experiments. *** = $P <$ 0.001. In **A** and **C,** gene expression levels in empty vector transfectants were arbitrarily set at 100, and values in AIF-1 isoform 2–expressing cells were expressed as multiples thereof. See Figure 3 for other definitions.

isoform 2– or isoform 3–expressing T cells would induce a change in the phenotype of normal fibroblasts to one resembling that expressed by SSc fibroblasts. The results showed that AIF-1 isoform 2–conditioned media caused an average increase of ~300 % in COL1A1 mRNA expression and of ~200% in COL3A1 mRNA expression, whereas AIF-1 isoform 3 increased COL1A1 expression by ~66% and did not affect COL3A1 mRNA expression (Figure 4A). Western blotting studies revealed that these mRNA results were reflected at the level of collagen production (Figure 4B).

Induction of IL-6, TGFβ, ET-R, and α-SMA expression in normal fibroblasts by conditioned media from AIF-1 isoform 2–overexpressing Jurkat T cells. Normal fibroblast cell lines cultured with conditioned media from either AIF-1 isoform 2– or AIF-1 isoform 3–transfected Jurkat T cells or with conditioned media from Jurkat T cells transfected with empty vector were analyzed for expression of genes indicative of a profibrotic phenotype. AIF-1 isoform 2–conditioned media strongly up-regulated the expression of IL-6 and TGFβ genes (342% and 353 %, respectively). The expression of ET-R and α-SMA was also increased (400% and 130%, respectively) (Figures 4C and D). In contrast, matrix metalloproteinase 1 (MMP-1) levels were not significantly changed (Figure 4D). AIF-1 isoform 3–conditioned media did not up-regulate either IL-6, TGFβ, or ET-R mRNA levels, whereas both α-SMA and MMP-1 mRNA levels were down regulated (by 61% and 80%, respectively) (Figure 4D).

AIF-1 isoform 2–conditioned media induce fibroblast activation independently of IL-4 secretion. To analyze whether the fibroblast activation described above was mediated by the observed increase in IL-4

production (Figures 3D and E), IL-4 inhibition experiments were performed. Real-time PCR analysis showed that the stimulatory effect of AIF-1 isoform 2–conditioned media on COL1A1 mRNA levels was not affected by the presence of neutralizing IL-4 antibodies (Figure 5A). This was confirmed at the collagen production level by Western blot analysis of fibroblast culture supernatants (Figure 5B). We next investigated whether IL-4 inhibition abrogated the observed up-regulation of IL-6 and TGFβ in normal fibroblasts following culture with AIF-1 isoform 2–conditioned media. Neutralizing IL-4 antibodies failed to inhibit the potent up-regulation of IL-6 or TGFβ mRNA levels (290% and 240%, respectively) caused by AIF-1 isoform 2 supernatants compared with media from empty vector–transfected cells ($P < 0.001$ for both) (Figure 5C).

DISCUSSION

We recently demonstrated that T cells infiltrating the skin and lungs of SSc patients, as well as mononuclear cells within the neointima of pulmonary arteries of patients with SSc-related pulmonary hypertension, express high levels of AIF-1 (28). In this study we investigated whether AIF-1 expression in T cells could affect their ability to migrate toward fibroblast monolayers and/or induce their production of profibrogenic cytokines and growth factors. Due to well-known technical limitations inherent in the establishment of stable transfectants of normal human PBMCs, which include the length of the selection procedures, the limited survival of primary PBMCs in vitro in the absence of cytokine stimulation, and their genetic heterogeneity, we chose to study immortalized T cell lines, which are a homogeneous and virtually unlimited source of functional T cells. We used the CD4+ Jurkat T cell line I-32, which exhibited AIF-1 isoform 2 and AIF-1 isoform 3 expression similar to that of normal unstimulated PBMCs.

Analysis of the role of the various AIF-1 isoforms in Jurkat T cell migration toward a fibroblast monolayer showed that, consistent with previous findings by Kelemen and Autieri (41), AIF-1 isoform 3 promoted T cell migration. More importantly, AIF-1 isoform 2 promoted an even higher rate of migration. This observation is of substantial relevance, since AIF-1 isoform 2 is the splicing variant that we previously found to be selectively up-regulated by TGFβ (28), and harbors a single-nucleotide polymorphism that is highly expressed in SSc (31,32).

T cells migrate toward various stimuli through signals transduced mainly by chemokine receptors. MCP-1 and stromal cell–derived factor 1 are 2 chemokines known to be up-regulated in SSc lesions and are produced by fibroblasts; therefore, they are believed to mediate the tissue infiltration by inflammatory cells in SSc (21,22). To investigate whether AIF-1–induced enhancement of T cell migration involves MCP-1, we performed inhibition studies using specific antibodies. The results showed that MCP-1 blockage did not have any effect on T cell migration.

After observing that AIF-1 was able to affect T cell migration, we hypothesized that AIF-1 expression could play a critical role in other aspects of T cell activation as well, such as cytokine production. Indeed, we found that AIF-1 isoform 2 up-regulated the expression of IL-4 and IL-17 mRNA and increased the production of both cytokines. In contrast, levels of IFNγ and TNFα mRNA were not increased by either AIF-1 isoform 2 or AIF-1 isoform 3.

Since previous studies indicated that in vivo AIF-1–expressing T cells are in close proximity to activated skin fibroblasts (28), we next examined the effect of conditioned media from AIF-1–expressing Jurkat T cells on normal dermal fibroblast function. We performed coculture experiments under conditions that avoided the cell–cell contact of functional Jurkat T cells with HLA-mismatched fibroblasts that could potentially produce a T cell activation driven by allogenic diversity. We observed a robust and consistent increase in the production of types I and III collagen following 24 hours of stimulation with conditioned media from AIF-1 isoform 2–expressing T cells. This effect, evident at the protein level, was accompanied by a substantial increase in COL1A1 and COL3A1 mRNA levels and by a marked increase in levels of mRNA for IL-6, TGFβ, ET-R, and α-SMA, all considered to be markers of fibroblast activation (3,42–44).

To further investigate whether the profibrotic activation of normal fibroblasts demonstrated in vitro following stimulation with conditioned media from AIF-1–expressing cells was directly caused by the observed increase in IL-4 production, neutralization experiments were performed. The results (Figure 5) demonstrated that inhibition of IL-4 with specific antibodies did not have any significant effect either on collagen production or on cytokine mRNA levels in normal human dermal fibroblasts. Therefore, the observed profibrotic effect of AIF-1 isoform 2–expressing cells is not directly caused by IL-4. Further studies are in progress to identify the

cytokine(s) or growth factor(s) responsible for the observed effects on fibroblast function.

In conclusion, we have shown in this study that AIF-1 can induce T cell migration toward normal dermal fibroblasts, indicating that AIF-1 expression by T cells can promote their tissue infiltration. Furthermore, we have demonstrated that AIF-1 expression by T cells can induce activation of normal fibroblasts, increasing their collagen production and the expression of several markers of fibrosis including IL-6, TGFβ, α-SMA, and ET-R, while at the same time causing a reduction in their MMP-1 expression. Further understanding of the mechanisms by which AIF-1 can promote increased T cell migration and cytokine production, and identification of the cytokines produced by AIF-1–expressing cells that are responsible for the potent profibrotic effect on normal fibroblasts observed herein, will shed light on the interplay between T cells and fibroblasts in the early phase of SSc pathogenesis and on the role of AIF-1 in this process.

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AUTHOR CONTRIBUTIONS

Dr. Jiménez 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 design. Del Galdo, Jiménez.
Acquisition of data. Del Galdo.
Analysis and interpretation of data. Del Galdo, Jiménez.
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