Multifaceted contribution of the TLR4-activated IRF5 transcription factor in systemic sclerosis

Ryosuke Saigusa[a], Yoshihide Asano[a,1], Takashi Taniguchi[a], Takashi Yamashita[a], Yohei Ichimura[a], Takehiro Takahashi[a], Tetsuo Toyama[a], Ayumi Yoshizaki[a], Koji Sugawara[b], Daisuke Tsuruta[b], Tadatsugu Taniguchi[c,d,1], and Shinichi Sato[a]

[a]Department of Dermatology, University of Tokyo Graduate School of Medicine, Bunkyo-ku, Tokyo 113-8655, Japan; [b]Department of Dermatology, Osaka City University Graduate School of Medicine, Abeno-ku, Osaka-shi, Osaka 545-8585, Japan; [c]Department of Molecular Immunology, Institute of Industrial Science, The University of Tokyo, Meguro-ku, Tokyo 153-8505, Japan; and [d]Max Planck-The University of Tokyo Center for Integrative Inflammology, Meguro-ku, Tokyo 153-8505, Japan

Contributed by Tadatsugu Taniguchi, October 28, 2015 (sent for review September 20, 2015; reviewed by Rudolf Grosschedl and Toshifumi Matsuyama)

Systemic sclerosis (SSc) is a multisystem autoimmune disorder with clinical manifestations resulting from tissue fibrosis and extensive vasculopathy. A potential disease susceptibility gene for SSc is IFN regulatory factor 5 (*IRF5*), whose SNP is associated with milder clinical manifestations; however, the underlying mechanisms of this association remain elusive. In this study we examined IRF5-deficient (*Irf5*$^{-/-}$) mice in the bleomycin-treated SSc murine model. We show that dermal and pulmonary fibrosis induced by bleomycin is attenuated in *Irf5*$^{-/-}$ mice. Interestingly, we find that multiple SSc-associated events, such as fibroblast activation, inflammatory cell infiltration, endothelial-to-mesenchymal transition, vascular destabilization, Th2/Th17 skewed immune polarization, and B-cell activation, are suppressed in these mice. We further provide evidence that IRF5, activated by Toll-like receptor 4 (TLR4), binds to the promoters of various key genes involved in SSc disease pathology. These observations are congruent with the high level of expression of IRF5, TLR4, and potential endogenous TLR4 ligands in SSc skin lesions. Our study sheds light on the TLR4-IRF5 pathway in the pathology of SSc with clinical implications of targeting the IRF5 pathways in the suppression of disease development.

interferon regulatory factor 5 | systemic sclerosis | fibrosis | vasculopathy | Toll-like receptor 4

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by immune abnormalities, vasculopathy, and extensive tissue fibrosis (1). Based on the results of etiological and genetic studies, the conventional wisdom is that SSc is caused by a complex interplay between genetic factors and environmental influences. For instance, the biggest risk factor for SSc is family history (2). On the other hand, concordance for SSc is around 5% in twins and is similar in monozygotic and dizygotic twins, whereas antinuclear antibodies are detected more frequently in the healthy monozygotic twin sibling than in the healthy dizygotic twin sibling of an SSc patient (3). In addition, most SSc susceptibility genes are HLA haplotypes and non-HLA immune-related genes that are shared by other collagen diseases (4). Therefore, genetic factors are likely associated with autoimmunity, increasing the susceptibility to autoimmune diseases including SSc, but additional environmental factors are required to induce clinically definite SSc in genetically predisposed individuals. Despite these etiological and genetic data, the entire process of the SSc development and pathogenesis remains elusive.

Therefore it is important to elucidate the molecular mechanism(s) underlying SSc pathogenesis. In this regard, much attention has been focused recently on the innate immune signaling via Toll-like receptors (TLRs) in various pathological conditions. For instance, fibroblasts and endothelial cells in SSc lesional skin highly express TLR4, originally identified as the receptor for bacterial LPS, and TLR4 signaling amplifies the sensitivity to TGF-β in dermal fibroblasts (5–7). It also was shown that dermal and lung fibrosis is attenuated in bleomycin (BLM)-treated TLR4-deficient mice (7). Endogenous potential TLR4 ligands are up-regulated in SSc lesional skin (5–7), and serum levels correlate with severe organ involvement and immunological abnormalities (8, 9). Therefore, the TLR4 signaling pathway is suspected to play a central role in the SSc pathogenesis.

Although how the TLR4 signaling pathway contributes to SSc pathogenesis remains enigmatic, it is interesting that several independent case-control and genome-wide association studies identify IFN regulatory factor 5 (*IRF5*), a member of the IFN regulatory factor (IRF) family, as an SSc susceptibility gene (10–15). IRFs were identified primarily in the research of the type I IFN system and have been shown to have functionally diverse roles in the regulation of the innate and adaptive immune responses (16). Reflecting such property of IRFs, SNPs of IRFs have been linked to the development of various immune and inflammatory disorders. IRF5 is of particular interest, being implicated in multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and SSc (14). Thus far an association of certain SNPs within the *IRF5* promoter with the risk and severity of SSc has been reported (10–15), but whether and how IRF5 is activated to contribute to disease development remains unknown.

Stimulation of TLRs triggers the activation of myeloid differentiation factor 88 (MyD88)-dependent and/or independent pathways (16). IRF5 is activated via the MyD88 pathway in dendritic cells and macrophages (17). TLR-activated IRF5 mediates the induction of genes IL-6, IL-12, and TNF-α (17). Hence, an intriguing possibility is that TLR4-mediated activation

Significance

The present study is, to our knowledge, the first demonstration of the molecular mechanisms underlying the association of IFN regulatory factor 5 (IRF5) expression with milder clinical manifestations of systemic sclerosis (SSc). It is speculated that endogenous ligands induce Toll-like receptor 4 signaling and promote IRF5 transcriptional regulation of its target gene promoters, which may be required for the development of SSc. Our present study supports this notion. Symptoms associated with SSc in humans were suppressed in mice deficient in the *Irf5* gene. As such, this study offers previously unidentified insight into the complexity of SSc pathology, giving impetus to further clinical studies for the treatment of the disease.

Author contributions: R.S., Y.A., Tadatsugu Taniguchi, and S.S. designed research; R.S., Takashi Taniguchi, T.Y., Y.I., T. Takahashi, T. Toyama, A.Y., K.S., and D.T. performed research; K.S. and D.T. contributed new reagents/analytic tools; R.S., Y.A., Takashi Taniguchi, T.Y., Y.I., T. Takahashi, T. Toyama, A.Y., K.S., D.T., Tadatsugu Taniguchi, and S.S. analyzed data; and R.S., Y.A., Tadatsugu Taniguchi, and S.S. wrote the paper.

Reviewers: R.G., Max Planck Institute of Immunobiology and Epigenetics; and T.M., Nagasaki University Graduate School of Biomedical Sciences.

The authors declare no conflict of interest.

[1]To whom correspondence may be addressed. Email: yasano-tky@umin.ac.jp or tada@m.u-tokyo.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1520997112/-/DCSupplemental.

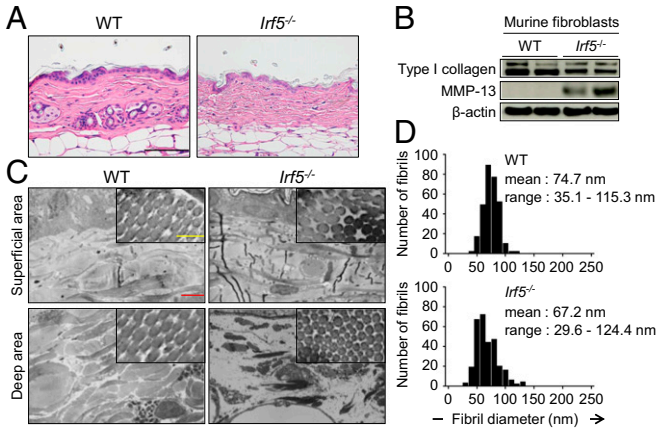

Fig. 1. Loss of *Irf5* impaired collagen metabolism and fibrillogenesis in vivo. (*A*) Representative skin image of WT and *Irf5*$^{-/-}$ mice. (Scale bar, 100 μm.) (*B*) Immunoblotting with cell lysates from WT and *Irf5*$^{-/-}$ murine dermal fibroblasts. (*C*) Ultrastructure of dermal collagen fibrils evaluated by electron microscopy. (Yellow scale bar, 200 nm; red scale bar, 2 μm.) (*D*) Frequency distribution profiles of fibril diameter in the dermis of WT and *Irf5*$^{-/-}$ mice (500 collagen fibrils per group).

of IRF5 is involved in SSc. We therefore studied the role of IRF5 in the regulation of genes associated with the susceptibility to and the severity of SSc using IRF5-deficient mice in the context of TLR4 signaling. We show that IRF5, activated by TLR4, binds to the promoters of various key genes involved in the disease symptoms. We discuss our findings in terms of the complexity of SSc and its clinical implications.

Results

Involvement of IRF5 in the Fibrosis- and Fibrillogenesis-Related Genes in Dermal Fibroblasts. First, to investigate the role of IRF5 in skin homeostasis, we examined by histology the skin of *Irf5*$^{-/-}$ mice (12 wk after birth) without BLM treatment. As shown in Fig. 1*A*, thinner collagen bundles were found in the dermis of *Irf5*$^{-/-}$ mice than in the dermis of WT littermate mice, but other skin structures in *Irf5*$^{-/-}$ mice looked normal. Consistent with this finding, collagen content decreased in the skin of *Irf5*$^{-/-}$ mice (Fig. S1*A*), and *Irf5*$^{-/-}$ dermal fibroblasts exhibited lower expression of type I collagen and higher expression of matrix metallopeptidase (MMP)-13 (Fig. 1*B* and Fig. S1*B*). Further analyses with electron microscopy delineated thinner collagen fibrils, especially in deep dermis (Fig. 1*C*), and higher variability in fibril diameter in *Irf5*$^{-/-}$ mice (Fig. 1*D*), suggesting an abnormality in collagen fibrillogenesis.

Interestingly, the ChIP assay revealed IRF5 binding to the promoters of the collagen, type 1, α1 (*Col1a1*), collagen, type 1, α2 (*Col1a2*), and *Mmp13* genes, indicating the potential involvement of IRF5 in the regulation of these genes (Fig. S1*C*). In addition, a notable decrease in the mRNAs for fibrillogenesis-related genes, namely a disintegrin and metalloprotease domain with thrombospondin type 1, motif 2 (*Adamts2*), lysyl oxidase (*Lox*), decorin (*Dcn*), and lumican (*Lum*), was observed in *Irf5*$^{-/-}$ dermal fibroblasts as compared with WT dermal fibroblasts (Fig. S1*D*). A similar mRNA expression pattern also was detected in the skin of *Irf5*$^{-/-}$ mice (Fig. S1*E*). Thus, IRF5, perhaps constantly but weakly activated in these mice, may influence extracellular matrix homeostasis by regulating fibrosis- and fibrillogenesis-associated gene expression in dermal fibroblasts (see below). It is worth noting that, except for the *Dcn* gene, these gene-expression profiles are contrary to those of SSc (18).

TLR4-Activated IRF5 Regulates *COL1A2* Gene Expression in Dermal Fibroblasts. In addition to murine dermal fibroblasts, we also detected IRF5 binding to the promoters for the *COL1A1*, *COL1A2*, and *MMP1* genes in human dermal fibroblasts (Fig. 2*A*). In the case of the *COL1A2* promoter, sequence-specific binding of IRF5 to the IFN-stimulated response element (ISRE) was confirmed by an oligonucleotide pull-down assay (Fig. 2*B*). We further analyzed the role of IRF5 in *COL1A2* gene expression in human dermal fibroblasts by a transient assay using a *COL1A2*-promoter-Luciferase gene and found that ectopic expression of IRF5 increased *COL1A2* promoter activity in a dose-dependent manner (Fig. 2*C*). Of note, LPS and TGF-β, known to induce the expression of type I collagen (5), further augmented the reporter gene expression (Fig. 2*C*), suggesting that TLR4 signaling regulates the transcriptional activity of IRF5. Indeed, IRF5 binding to the *COL1A2* promoter was enhanced significantly by simultaneous stimulation of LPS and TGF-β1 (Fig. 2*D*). Interestingly, the enhancement of IRF5 binding to the *COL1A2* promoter also was observed when the cells were stimulated by high-mobility group box 1 (HMGB1), which is also known to activate TLR4 in lieu of LPS (Fig. 2*E*) (19). Consistent with this notion, ChIP in *Tlr4*$^{-/-}$ murine dermal fibroblasts showed a remarkable decrease in IRF5 binding to the *Col1a2* promoter (Fig. 2*F*), but *Tlr4* deficiency did not affect the expression of IRF5 (Fig. 2*G*). Thus, these observations underscore the evidence that the TLR4–IRF5 axis induces *COL1A2* gene expression in dermal fibroblasts.

Attenuated Dermal and Pulmonary Fibrosis in BLM-Treated *Irf5*$^{-/-}$ Mice. The role of IRF5 in tissue fibrosis was investigated further in BLM-treated mice. In line with in vitro data, dermal thickness, collagen content, and the number of myofibroblasts were significantly decreased in BLM-treated *Irf5*$^{-/-}$ mice as compared with BLM-treated WT mice (Fig. 3*A* and Fig. S2 *A*–*C*). The expression profile of fibrosis- and fibrillogenesis-related genes in *Irf5*$^{-/-}$ dermal fibroblasts, which was confirmed at the protein level by immunostaining for MMP-13 (Fig. S2 *E* and *F*), was maintained in the skin of BLM-treated *Irf5*$^{-/-}$ mice (Fig. S2*D*). Also, lung histology revealed less fibrosis, less destruction of

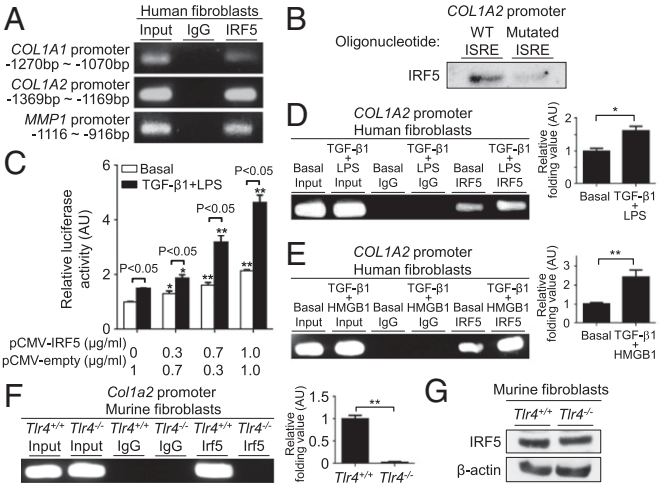

Fig. 2. TLR4-activated IRF5 induces the profibrotic phenotype in dermal fibroblasts. (*A*, *D*, and *E*) ChIP analysis with anti-IRF5 antibody in human dermal fibroblasts ($n = 4$). (*B*) Proteins pulled down by oligonucleotides including WT or mutated ISRE of the *COL1A2* promoter were subjected to immunoblotting with anti-IRF5 antibody. (*C*) Luciferase assay with the *COL1A2* promoter construct in human dermal fibroblasts ($n = 4$). Significant differences shown with asterisks are compared with the columns of the same color at the far left. (*F* and *G*) ChIP assay (*F*) and immunoblotting (*G*) with anti-IRF5 antibody in *Tlr4*$^{+/+}$ and *Tlr4*$^{-/-}$ murine dermal fibroblasts. In *C*–*E*, some cells were stimulated with TGF-β1 and LPS or HMGB1 for 24 h. In *D*–*F*, quantification by qRT-PCR is shown in the right panels ($n = 5$). $^{*}P < 0.05$ and $^{**}P < 0.01$ by two-tailed unpaired *t*-test. AU, arbitrary units.

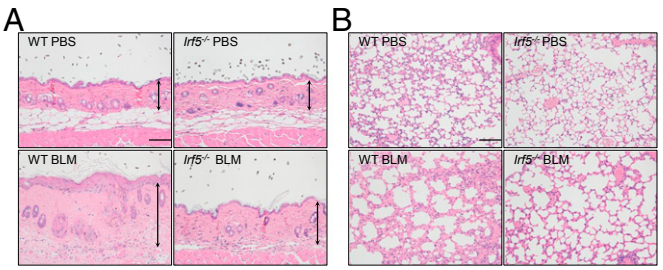

Fig. 3. Deletion of *Irf5* attenuates BLM-induced dermal and pulmonary fibrosis. Representative sections of skin (*A*) and lung (*B*) in WT and *Irf5*$^{-/-}$ mice injected with PBS or BLM. Vertical bars with arrows represent dermal thickness. (Horizontal scale bars, 100 μm.)

alveolar structures, and less inflammatory cell infiltration in BLM-treated *Irf5*$^{-/-}$ mice than in BLM-treated WT mice (Fig. 3*B* and Fig. S2*G*). Thus, *Irf5* deficiency suppresses pathological dermal and pulmonary fibrosis in BLM-treated mice.

Promotion of Th1 Immune Polarization and Attenuation of B-Cell Activation by the Absence of IRF5. We next asked whether the loss of IRF5 also influences immune cells in BLM-treated mice. As shown in Fig. 4*A*, immunostaining analysis revealed reduced infiltration of T cells, B cells, mast cells, and macrophages in the skin and lungs of BLM-treated *Irf5*$^{-/-}$ mice (see also Fig. S3*A*), suggesting that IRF5 is involved in the BLM-induced inflammatory responses.

Consistent with this observation, the expression of mRNAs for fibrosis-related cytokines and chemokines was different in the skin and lungs of BLM-treated WT and *Irf5*$^{-/-}$ mice; IFN-γ mRNA levels were increased significantly, but IL-4 and IL-6 mRNA levels were decreased in the absence of IRF5 (Fig. S3 *B* and *C*). These observations suggested a Th1-type immune polarization in BLM-treated *Irf5*$^{-/-}$ mice. This notion was confirmed by intracellular flow cytometry for cytokines and master transcription factors within lymphocytes of draining lymph nodes, showing an increase in Th1 cells but no change of Th2 and Th17 cells in BLM-treated *Irf5*$^{-/-}$ mice as compared with BLM-treated WT mice (Fig. 4 *B* and *C* and Fig. S3 *D* and *E*).

In this regard, we also measured by ChIP assay IRF5 binding to the T-box expressed in T cells (*Tbet*) promoter in CD4^{+} T cells. The results suggest that the absence of IRF5 may promote Th1 polarization through induction of the T-bet transcription factor; in other words, IRF5 may serve as a repressor of the *Tbet* gene (Fig. 4*D*). IRF5 is known to function as a positive regulator of various genes in immune cells (17), but a transcriptional repressive function has not been reported. To confirm this notion, we carried out a reporter gene-based assay in which activation of a *Tbet* promoter induces the expression of luciferase (Tbet-Luc) in HEK293T cells. When IRF5A, a constitutive active type isoform of IRF5 which lacks a nuclear export signal (20), was coexpressed with Tbet-Luc, luciferase activity was suppressed in a dose-dependent manner (Fig. 4*E*), indicating the repressive action of IRF5 on this promoter. In view of a previously published report that TLR4 signaling in T cells promotes an inflammatory response (21), one may envisage that the TLR4–IRF5 axis also is involved in the BLM-induced immune pathogenesis.

We also extended our study to the role of IRF5 in B cells. We observed that expression of CD19, a critical positive-response regulator (22), was expressed at lower levels in IRF5-deficient B cells (Fig. S3*F*). Furthermore, IRF5-deficient B cells produced lower amounts of IL-6 than did WT B cells in response to LPS and/or anti-CD40 antibody (Fig. S3*G*). Of note, the *Cd19* and *Il6* promoters also were bound by IRF5 (Fig. 4*F* and Fig. S3*H*), and the sequence-specific binding of IRF5 to the ISRE of the *Il6* promoter also was confirmed (Fig. S3*I*).

It is interesting that IRF5 binding to the *Tbet* and *Il6* promoters also was suppressed in *Tlr4*$^{-/-}$ CD4^{+} T cells and *Tlr4*$^{-/-}$ B cells, respectively (Fig. 4 *D* and *F*). Furthermore, enhanced IRF5 binding to the *Il6* promoter by HMGB1 also was observed in B cells (Fig. 4*F*). Overall, these results indicate that TLR4-activated IRF5 is directly involved in the regulation of Th1 cell differentiation and B-cell activation.

Regulation of Antifibrotic Property by IRF5 in Endothelial Cells. We next examined the vascular aspect of BLM-treated *Irf5*$^{-/-}$ mice by focusing on cell-adhesion molecules, vascular stability, and endothelial-to-mesenchymal transition (EndoMT). In the skin of BLM-treated mice, selectin P (*Selp*) and selectin E (*Sele*) mRNA levels were increased by the absence of IRF5, but intercellular adhesion molecule 1 (*Icam1*) mRNA levels were decreased (Fig. S4*A*). In the lung, the lack of IRF5 significantly reduced *Selp* and *Icam1* and especially glycosylation-dependent cell-adhesion molecule 1 (*Glycam1*) mRNA expression levels (Fig. S4*B*). Given that L-selectin, a ligand for GlyCAM-1, and ICAM-1 promote Th2 and Th17 cell infiltration, whereas P-selectin and

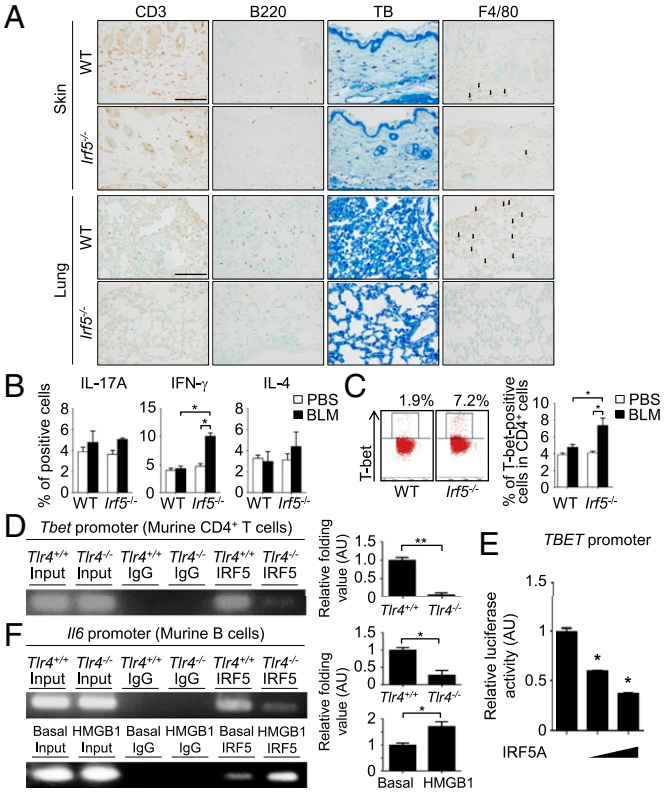

Fig. 4. Loss of *Irf5* suppresses the induction of profibrotic inflammatory responses in BLM-treated mice. (*A*) Immunohistochemistry for CD3, B220, and F4/80 and toluidine blue (TB) staining in the skin and lung of BLM-treated WT and *Irf5*$^{-/-}$ mice. (Scale bars, 100 μm.) Arrows indicate F4/80^{+} cells ($n = 5$). (*B* and *C*) Percentage of IL-17A–, IFN-γ–, and IL-4–producing CD4^{+} T cells (*B*) and T-bet^{+} CD4^{+} T cells (*C*) in draining lymph nodes of BLM-treated mice determined by intracellular staining ($n = 4$). Representative FACS plots of intracellular T-bet staining are shown in *C*. (*D* and *F*) ChIP assay with anti-IRF5 antibody in murine CD4^{+} T cells (*D*) and B cells (*F*) with or without homozygous *Tlr4* deletion. In some experiments, B cells were stimulated with 10 μg/mL of HMGB1 for 24 h. For each ChIP assay, quantification by qRT-PCR is shown in the panels at right ($n = 5$). (*E*) Luciferase assay using the *TBET* promoter constructs in HEK293T cells cotransfected with IRF5A expression vector ($n = 4$). *$P < 0.05$ and **$P < 0.01$ by two-tailed unpaired *t*-test.

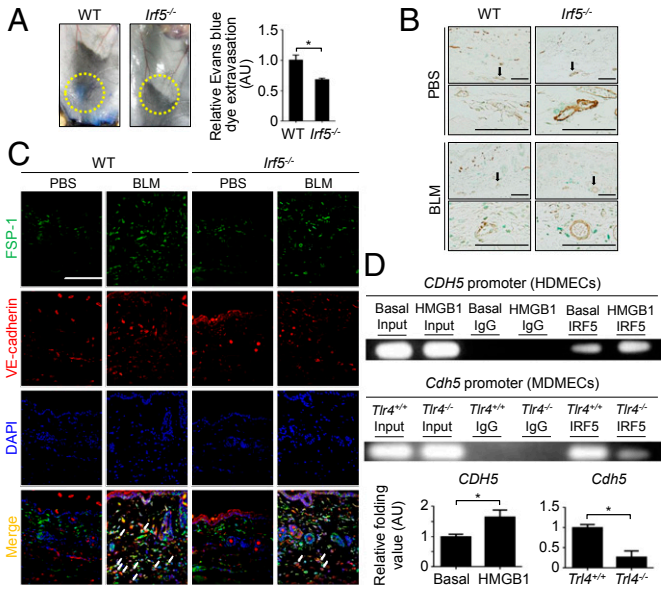

Fig. 5. *Irf5* deletion abrogates vascular destabilization and EndoMT induced by BLM. (*A*, *Left*) Evaluation for the extravasation of Evans blue dye injected into the caudal vein of BLM-treated mice. (*Right*) Quantification of Evans blue dye extravasation by formamide extraction ($n = 6$). (*B*) Immunohistochemistry for α-SMA in the skin of PBS- or BLM-treated mice. (Scale bars, 100 μm.) (*C*) Immunofluorescence staining for FSP1 (green), VE-cadherin (red), and DAPI (blue) in skin samples from each group. Arrows represent FSP1/VE-cadherin double-positive cells. (Scale bar, 100 μm.) ($n = 4$). (*D*, *Upper*) ChIP assay with anti-IRF5 antibody in human dermal microvascular endothelial cells with or without HMGB1 stimulation (10 μg/mL for 24 h) and in $Tlr4^{+/+}$ and $Tlr4^{-/-}$ murine dermal microvascular endothelial cells. (*Lower*) Quantification by qRT-PCR ($n = 5$). $^*P < 0.05$ by two-tailed unpaired *t*-test.

E-selectin facilitate Th1 cell infiltration, IRF5 may modulate the expression of cell-adhesion molecules, resulting in the promotion of Th1 cell infiltration in BLM-treated mice; this activity is consistent with T-helper cytokine profiles in the skin and lungs of BLM-treated mice (Fig. S3 *B* and *C*).

In the vascular permeability assay, the absence of IRF5 attenuated Evans blue dye extravasation induced by BLM (Fig. 5*A*), suggesting that IRF5 has a suppressive role in vascular stabilization. Because a mural cell phenotype contributes to vascular maturation, and α-smooth muscle actin (α-SMA) serves as a marker of mature vessels (23), α-SMA expression in dermal small vessels was evaluated by immunohistochemistry. The loss of IRF5 augments α-SMA expression, irrespective of injected agents (Fig. 5*B*). In agreement with this observation, dermal microvascular endothelial cells from $Irf5^{-/-}$ mice expressed PDGF-B and vascular endothelial (VE)-cadherin, which stabilize vasculature by acting on mural cells, at higher levels than cells from WT counterparts (Fig. S4*C*). Furthermore, IRF5 also bound the *Pdgfb* and cadherin-5 (*Cdh5*) promoters as measured by ChIP assay (Fig. S4*D*). Collectively, *Irf5* deficiency is likely to promote vascular stabilization by directly inducing the expression of PDGF-B and VE-cadherin.

Another critical event in the pathology of tissue fibrosis, EndoMT, was assessed further by double immunofluorescence for fibroblast secretory protein-1 (FSP1), a fibroblast marker, and VE-cadherin, an endothelial cell marker, revealing that *Irf5* deletion suppressed BLM-dependent induction of double-positive cells (Fig. 5*C* and Fig. S4*E*). Importantly, in human dermal microvascular endothelial cells (HDMECs), IRF5 was bound to the promoter of the snail family zinc finger 1 (*SNAIL1*) gene, encoding an important regulator of EndoMT (Fig. S4*F*). Therefore, in BLM-treated mice *Irf5* deficiency appears to inhibit EndoMT directly, contributing to the attenuation of tissue fibrosis.

In HDMECs, IRF5 was bound to the promoters of various genes, including the *ICAM1*, *PDGFB*, *CDH5*, and *SNAIL1* genes (Fig. S4*F*). When HDMECs were treated with HMGB1, the occupancy of the *CDH5* promoter by IRF5 was increased (Fig. 5*D*). Furthermore, $Tlr4^{-/-}$ murine dermal microvascular endothelial cells exhibited a marked reduction of IRF5 binding to the *Cdh5* promoter (Fig. 5*D*). These data indicate that IRF5 regulates the profibrotic/antifibrotic and proangiogenic/angiostatic phenotypes of endothelial cells, depending on TLR4 activation status. Overall, our results in toto suggest a multifaceted transcriptional regulation by IRF5, functioning as either a transcriptional activator or a repressor in this SSc model.

IRF5 Expression and Its Clinical Correlation in SSc Patients. Finally, we evaluated the association of IRF5 expression with clinical features in SSc patients. *IRF5* mRNA expression was elevated significantly in SSc lesional skin compared with healthy control skin (Fig. 6*A*), and this finding was confirmed at the protein level in dermal fibroblasts and endothelial cells by immunohistochemistry (Fig. 6 *B* and *C*). Notably, increased *IRF5* mRNA levels correlated significantly with an increase of the *COL1A1*, *COL1A2*, and connective tissue growth factor (*CTGF*) mRNA levels in SSc lesional skin (Fig. S5*A*). Furthermore, *IRF5* mRNA levels were elevated significantly in patients with diffuse cutaneous SSc (dcSSc) as compared with patients with limited cutaneous SSc (lcSSc) and in SSc patients with a current and past history of digital ulcers as compared with patients without such a history (Fig. S5*B*). Thus, in so far as we could determine in these SSc patients, these data are consistent with the murine model of SSc.

In in vitro studies we confirmed that *IRF5* mRNA levels were elevated in SSc dermal fibroblasts as compared with normal dermal fibroblasts (Fig. S5*C*) and correlated positively with *COL1A2* mRNA levels in normal and SSc dermal fibroblasts (Fig. S5*D*). Importantly, in normal human fibroblasts IRF5 expression was increased by LPS and/or TGF-β stimulation at the mRNA and protein levels (Fig. S5*E*). Therefore IRF5 itself may be up-regulated in response to profibrotic stimuli in SSc dermal fibroblasts.

The SNP rs4728142 is located in the *IRF5* promoter, and its minor allele A is seen in 9% of Japanese population (24). Supporting the relationship between the rs4728142 A allele and less serious clinical presentation (24), *IRF5* mRNA levels in the

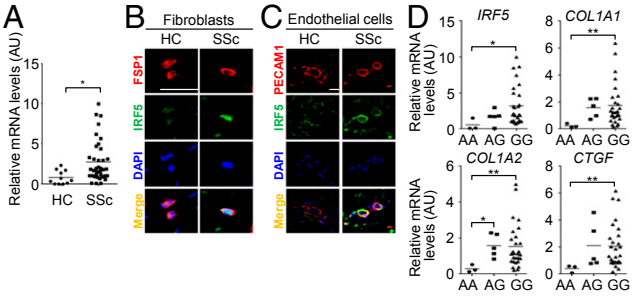

Fig. 6. IRF5 expression and its association with the profibrotic condition in SSc. (*A*) *IRF5* mRNA expression in bulk skin from healthy controls (HC) and SSc patients. (*B*) Immunofluorescence staining for FSP1 (red), IRF5 (green), and DAPI (blue) in skin samples from HC and SSc subjects ($n = 4$). (Scale bar, 20 μm.) (*C*) Immunofluorescence staining for platelet/endothelial cell adhesion molecule 1 (PECAM1) (red), IRF5 (green), and DAPI (blue) in skin samples from HC and SSc subjects. (Scale bar, 20 μm.) (*D*) The comparison of relative mRNA levels of the *IRF5*, *COL1A1*, *COL1A2*, and *CTGF* genes in bulk skin from SSc patients with the AA genotype, AG genotype, and GG genotype. $^*P < 0.05$ and $^{**}P < 0.01$ by two-tailed unpaired *t*-test.

lesional skin were observed to be significantly lower in SSc patients with the AA genotype than in those with the GG genotypes (Fig. 6*D*). Importantly, *COL1A1*, *COL1A2*, and *CTGF* mRNA levels were decreased significantly in SSc patients with the AA genotype as compared with those with the GG genotype (Fig. 6*D*). Furthermore, this allele tended to be related to milder interstitial lung disease with higher percent diffusing capacity for carbon monoxide (%DLco) (Table S1). Therefore, the rs4728142 A allele leads to a milder clinical presentation of SSc, possibly correlated with inhibited activation of fibroblasts.

Discussion

In this study we provide the first evidence, to our knowledge, of a critical role of IRF5 in the mouse model of SSc. Reflecting its critical role as a regulator of the immune system, IRF5 has been identified as a susceptibility gene in various diseases related to immune abnormalities, including SSc (14). Our study reveals that IRF5 is involved in many aspects of pathological processes (e.g., fibrotic and vascular aspects and immune cell abnormalities), as either a transcriptional activator or a repressor. Supporting this idea, IRF5 was observed to bind the promoters of various target genes in fibroblasts, endothelial cells, and lymphocytes. Of note, a direct involvement of IRF5 in immune polarization of $CD4^+$ T cells and B-cell activation was suggested also. Most importantly, loss of *Irf5* attenuated tissue fibrosis, vascular activation, and inflammation in BLM-treated mice. To the best of our knowledge, this is the first report disclosing the diverse effects of IRF5 on all three cardinal pathological features of SSc.

Innate immune signaling-mediated fibroblast activation was recently reported in SSc and its animal models. In SSc, serum levels and dermal expression levels of endogenous TLR4 ligands, such as HMGB1, hyaluronan, and fibronectin extra domain, are elevated (5, 6, 8, 9). In in vitro experiments, TLR4 signaling induced by these candidate ligands results in fibroblast activation (5, 6). TLR4 signaling also augments TGF-β signaling and, indeed, TLR4 blockade suppresses basal and TGF-β–dependent activation of fibroblasts (5, 6). Importantly, in *Tlr4*-deficient mice BLM-induced dermal fibrosis is attenuated significantly despite the elevation of endogenous TLR4 ligands (7). Taken together, TLR4 signaling appears to be involved in pathological dermal fibrosis by directly activating dermal fibroblasts. As described in this study, $Irf5^{-/-}$ dermal fibroblasts exhibited an antifibrotic phenotype under normal physiological conditions. In addition, ectopic expression of IRF5 enhanced *COL1A2* promoter activity, especially by the stimulatory signals via TGF-β1 and TLR4, both of which also increase IRF5 expression in human dermal fibroblasts. Most importantly, TLR4 activation augmented IRF5's occupancy on the *COL1A2* promoter in human dermal fibroblasts, whereas IRF5 binding to the *Col1a2* promoter was reduced remarkably in $Tlr4^{-/-}$ murine dermal fibroblasts. Therefore, the amplification of TGF-β stimulation by TLR4 signaling may be exerted, at least partially, through IRF5 activation in dermal fibroblasts. Given that the skin phenotype of $Irf5^{-/-}$ mice was largely contrary to that of human SSc at the histological and ultrastructural levels and that IRF5 was up-regulated in SSc dermal fibroblasts, we conclude IRF5 plays a pivotal role in tissue fibrosis in SSc. This notion also is supported by the significant positive correlation of *IRF5* mRNA levels with *COL1A1*, *COL1A2*, and *CTGF* mRNA levels in SSc lesional skin.

SSc vasculopathy is marked by specific structural and functional abnormalities (1). The structural changes of SSc vasculature are largely attributable to vascular instability closely related to the altered phenotype of mural cells. In SSc lesional skin, α-SMA expression is decreased in mural cells, reflecting the weak interaction of endothelial cells with mural cells and the activation of proangiogenic signaling pathways (23). This feature was reproducible in BLM-treated mice, but in $Irf5^{-/-}$ mice a high α-SMA expression was maintained in the skin vasculature even after BLM injection, indicating that IRF5 is a key regulator of the balance between angiostatic and proangiogenic conditions. Consistent with this notion, IRF5 bound the promoters of the *CDH5* and *PDGFB* genes encoding key molecules—VE-cadherin and PDGF-B, respectively—regulating vascular stabilization and angiogenesis. Because of its involvement in mechanisms connecting vascular activation and tissue fibrosis, EndoMT is a critical pathological event in human and murine models of SSc (25, 26). Because the loss of *Irf5* decreased the number of double-positive cells for FSP1 and VE-cadherin in the perivascular region of BLM-treated skin, IRF5 is implicated as a critical regulator of EndoMT, as also was confirmed by IRF5 binding to the *SNAIL1* promoter. Importantly, IRF5 occupancy on the *CDH5* promoter was enhanced by TLR4 activation. Together with the evidence that TLR4 and its endogenous ligands have been implicated in angiogenesis (27, 28), the present data provide a previously unidentified insight into the role of IRF5 in TLR4-mediated angiogenesis and suggest that the TLR4–IRF5 axis potentially regulates vascular features of SSc.

The regulation of $CD4^+$ T-cell infiltration by cell adhesion molecules has attracted much attention in the context of pathological tissue fibrosis, including SSc and its animal models (25, 29). Studies with mice deficient for cell adhesion molecules have revealed that ICAM-1 and GlyCAM-1 promote tissue fibrosis along with the infiltration of Th2/Th17 cells, macrophages, and mast cells, whereas P-selectin and E-selectin suppress tissue fibrosis accompanying Th1 cell infiltration in response to BLM (29). In lesional skin of early dcSSc, the expression levels of the profibrotic cell adhesion molecules ICAM-1 and GlyCAM-1 are increased relative to that of the antifibrotic cell adhesion molecules E-selectin and P-selectin (25). Therefore our observation that the deletion of *Irf5* reduced the relative expression of profibrotic cell adhesion molecules to antifibrotic molecules, in turn leading to the decreased infiltration of macrophages and mast cells and a Th1-predominant cytokine balance, indicates a critical role for IRF5 in the induction of profibrotic inflammation. In addition, we also demonstrated that Th1-skewed immune polarization in BLM-treated $Irf5^{-/-}$ mice is linked to IRF5 binding to the *Tbet* promoter, suggesting that IRF5 directly suppresses Th1 cell differentiation. Given that IRF5's occupancy of the *Tbet* promoter was decreased in $Tlr4^{-/-}$ $CD4^+$ T cells, the activation of TLR4–IRF5 axis promotes a Th2/Th17-predominant inflammatory condition through the direct suppression of Th1 cell differentiation, which may be characteristic of SSc (1).

B-cell activation also is an important part of immune abnormalities in SSc (22). In addition to fibroblasts, endothelial cells, and T cells, deletion of *Irf5* induced the phenotypical alteration of B cells, such as down-regulation of CD19 and decreased IL-6 release, a state contrary to SSc B cells. Furthermore, IRF5 bound the *Cd19* and *Il6* promoters in murine B cells, and occupancy of the *Il6* promoter was decreased in $Tlr4^{-/-}$ B cells and increased in $Tlr4^{+/+}$ B cells upon TLR4 activation. Therefore, the TLR4–IRF5 axis directly regulates B-cell activation. Given that *Il6* mRNA levels were decreased markedly in the skin of BLM-treated $Irf5^{-/-}$ mice and that IL-6 is a classic inflammatory cytokine produced by various cells, we surmise that IRF5 regulates IL-6 production in other cell types. Because IL-6 enhances collagen synthesis by inducing myofibroblastic differentiation, promotes Th2 and Th17 differentiation, and inhibits Th1 polarization (30, 31), the loss of IL-6 production largely contributes to the attenuation of tissue fibrosis in BLM-treated $Irf5^{-/-}$ mice.

Based on data in mice, it is assumed that up-regulated expression of IRF5 is involved in the development of three cardinal features of SSc. Supporting this idea, IRF5 expression was increased in dermal fibroblasts and vascular cells in the lesional skin of SSc patients. Furthermore, *IRF5* mRNA levels correlated positively with *COL1A1*, *COL1A2*, and *CTGF* mRNA levels in

SSc dermal fibroblasts. Moreover, as is consistent with the linking of rs4728142 A allele to a milder clinical phenotype resulting from the loss of IRF5 induction, SSc dermal fibroblasts with the AA genotype expressed *COL1A1*, *COL1A2*, and *CTGF* mRNA at lower levels than SSc dermal fibroblasts with the GG genotype. Reflecting the role of IRF5 in SSc vasculopathy, SSc patients with a current and past history of digital ulcers had higher *IRF5* mRNA levels than patients with no history of digital ulcers. Given that SSc progresses via a complex interaction between fibroblasts, endothelial cells, and immune cells with SSc-specific phenotypes, the attenuation of SSc phenotypes in each of those cells resulting from IRF5 down-regulation appears to result in milder clinical symptoms of this disease. To our knowledge, these are the first data providing a molecular basis for an association of the rs4728142 A allele with a milder SSc phenotype.

In summary, we provide evidence that IRF5 is involved in the three cardinal pathological processes of SSc through the direct transcriptional regulation of target genes in various cell types. In particular, it is interesting that IRF5 could serve as a potent repressor for the *Tbet* gene, because IRF5 acts primarily as a potent inducer of various immune-related and inflammatory genes (17). Also, contrary to our previous findings on serum MMP-13 levels and endothelial VE-cadherin expression in SSc (23, 32), *Irf5* deficiency induced *Mmp13* gene expression in dermal fibroblasts and *Cdh5* gene expression in endothelial cells, indicating a role for IRF5 as a transcriptional repressor in various types of cells. In this regard, it may be worth recalling that such a bifunctional action has been shown for IRF3 (20). Obviously, further work is required to elucidate the underlying mechanisms by which IRF5 exerts a repressive function. Importantly, our study revealed the contribution of innate immune signaling via the TLR4–IRF5 axis to the induction of SSc phenotypes in various cell types, offering clues for further understanding of the roles of environmental factors and endogenous ligands in the development and persistence of disease. Finally, our study may provide impetus for new drug development by targeting IRF5 in the treatment of SSc.

Methods

All experimental procedures were approved by the ethical committee of University of Tokyo Graduate School of Medicine. Written informed consent was obtained from all patients and healthy individuals. The study was performed according to the Declaration of Helsinki. All animal studies and procedures were approved by the Committee on Animal Experimentation of University of Tokyo Graduate School of Medicine. Generation of BLM-treated mice, histological assessment, the hydroxyproline assay, transmission electron microscopy, immunoblotting, RNA isolation, quantitative RT-PCR (qRT-PCR), ChIP, the oligonucleotide pull-down assay, the reporter assay, flow cytometric analysis, the vascular permeability assay, immunofluorescence, cell culture, plasmid information, and the SNP genotyping assay are described in *SI Methods*. Primer sequences for qRT-PCR and ChIP are summarized in Tables S2 and S3, respectively.

Mice. C57BL/6 female mice (6 to 8 wk old) were used in this study. *Irf5*$^{-/-}$ mice were described previously (17). *Tlr4*$^{-/-}$ mice and tight skin mice were purchased from The Jackson Laboratory.

Patients. cDNA and genomic DNA were prepared from forearm skin samples of 36 SSc patients [33 females; median age (25–75 percentile): 57 y; range 41.0–67.0 y] and 11 healthy donors (8 females; median age, 50 y; range 38.0–69.0 y). Fibroblasts were obtained by skin biopsy from eight sex- and site-matched SSc patients and eight healthy donors of closely matched age.

Statistical Analysis. Statistical analysis was carried out with one-way ANOVA with Bonferroni post hoc tests for multiple group comparisons and the two-tailed unpaired *t*-test for two group comparisons. For comparing two group values that did not follow Gaussian distribution, the two-tailed Mann–Whitney *u* test was used. $P < 0.05$ was considered statistically significant. Within-group distributions are expressed as mean ± SEM.

ACKNOWLEDGMENTS. This study was supported by grants from the Ministry of Health, Labor, and Welfare of Japan.

1. Asano Y, Sato S (2015) Vasculopathy in scleroderma. *Semin Immunopathol* 37(5):489–500.
2. Arnett FC, et al. (2001) Familial occurrence frequencies and relative risks for systemic sclerosis (scleroderma) in three United States cohorts. *Arthritis Rheum* 44(6):1359–1362.
3. Feghali-Bostwick C, Medsger TA, Jr, Wright TM (2003) Analysis of systemic sclerosis in twins reveals low concordance for disease and high concordance for the presence of antinuclear antibodies. *Arthritis Rheum* 48(7):1956–1963.
4. Broen JC, Radstake TR, Rossato M (2014) The role of genetics and epigenetics in the pathogenesis of systemic sclerosis. *Nat Rev Rheumatol* 10(11):671–681.
5. Bhattacharyya S, et al. (2013) Toll-like receptor 4 signaling augments transforming growth factor-β responses: A novel mechanism for maintaining and amplifying fibrosis in scleroderma. *Am J Pathol* 182(1):192–205.
6. Bhattacharyya S, et al. (2014) FibronectinEDA promotes chronic cutaneous fibrosis through Toll-like receptor signaling. *Sci Transl Med* 6(232):232ra50.
7. Takahashi T, et al. (2015) Amelioration of tissue fibrosis by toll-like receptor 4 knockout in murine models of systemic sclerosis. *Arthritis Rheumatol* 67(1):254–265.
8. Yoshizaki A, et al. (2009) Clinical significance of serum HMGB-1 and sRAGE levels in systemic sclerosis: Association with disease severity. *J Clin Immunol* 29(2):180–189.
9. Yoshizaki A, et al. (2008) Clinical significance of serum hyaluronan levels in systemic sclerosis: Association with disease severity. *J Rheumatol* 35(9):1825–1829.
10. Dieudé P, et al. (2009) Association between the IRF5 rs2004640 functional polymorphism and systemic sclerosis: A new perspective for pulmonary fibrosis. *Arthritis Rheum* 60(1):225–233.
11. Ito I, et al. (2009) Association of a functional polymorphism in the IRF5 region with systemic sclerosis in a Japanese population. *Arthritis Rheum* 60(6):1845–1850.
12. Dieude P, et al. (2010) Phenotype-haplotype correlation of IRF5 in systemic sclerosis: Role of 2 haplotypes in disease severity. *J Rheumatol* 37(5):987–992.
13. Radstake TR, et al.; Spanish Scleroderma Group (2010) Genome-wide association study of systemic sclerosis identifies CD247 as a new susceptibility locus. *Nat Genet* 42(5):426–429.
14. Tang L, Chen B, Ma B, Nie S (2014) Association between IRF5 polymorphisms and autoimmune diseases: A meta-analysis. *Genet Mol Res* 13(2):4473–4485.
15. Allanore Y, et al. (2011) Genome-wide scan identifies TNIP1, PSORS1C1, and RHOB as novel risk loci for systemic sclerosis. *PLoS Genet* 7(7):e1002091.
16. Honda K, Taniguchi T (2006) IRFs: Master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol* 6(9):644–658.
17. Takaoka A, et al. (2005) Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* 434(7030):243–249.
18. Noda S, et al. (2014) Simultaneous downregulation of KLF5 and Fli1 is a key feature underlying systemic sclerosis. *Nat Commun* 5:5797.
19. Yang H, Antoine DJ, Andersson U, Tracey KJ (2013) The many faces of HMGB1: Molecular structure-functional activity in inflammation, apoptosis, and chemotaxis. *J Leukoc Biol* 93(6):865–873.
20. Negishi H, et al. (2012) Cross-interference of RLR and TLR signaling pathways modulates antibacterial T cell responses. *Nat Immunol* 13(7):659–666.
21. Reynolds JM, Martinez GJ, Chung Y, Dong C (2012) Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. *Proc Natl Acad Sci USA* 109(32):13064–13069.
22. Sato S, Fujimoto M, Hasegawa M, Takehara K, Tedder TF (2004) Altered B lymphocyte function induces systemic autoimmunity in systemic sclerosis. *Mol Immunol* 41(12):1123–1133.
23. Asano Y, et al. (2010) Endothelial Fli1 deficiency impairs vascular homeostasis: A role in scleroderma vasculopathy. *Am J Pathol* 176(4):1983–1998.
24. Sharif R, et al. (2012) IRF5 polymorphism predicts prognosis in patients with systemic sclerosis. *Ann Rheum Dis* 71(7):1197–1202.
25. Taniguchi T, et al. (2015) Fibrosis, vascular activation, and immune abnormalities resembling systemic sclerosis in bleomycin-treated Fli-1-haploinsufficient mice. *Arthritis Rheumatol* 67(2):517–526.
26. Jimenez SA (2013) Role of endothelial to mesenchymal transition in the pathogenesis of the vascular alterations in systemic sclerosis. *ISRN Rheumatol* 2013:835948.
27. Murad S (2014) Toll-like receptor 4 in inflammation and angiogenesis: A double-edged sword. *Front Immunol* 5:313.
28. Yang S, Xu L, Yang T, Wang F (2014) High-mobility group box-1 and its role in angiogenesis. *J Leukoc Biol* 95(4):563–574.
29. Yoshizaki A, et al. (2010) Cell adhesion molecules regulate fibrotic process via Th1/Th2/Th17 cell balance in a bleomycin-induced scleroderma model. *J Immunol* 185(4):2502–2515.
30. O'Reilly S, Cant R, Ciechomska M, van Laar JM (2013) Interleukin-6: A new therapeutic target in systemic sclerosis? *Clin Transl Immunology* 2(4):e4.
31. Rincón M, Anguita J, Nakamura T, Fikrig E, Flavell RA (1997) Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. *J Exp Med* 185(3):461–469.
32. Asano Y, et al. (2006) Clinical significance of serum levels of matrix metalloproteinase-13 in patients with systemic sclerosis. *Rheumatology (Oxford)* 45(3):303–307.