SUPPLEMENTARY MATERIAL AND METHODS

Study population

Human tissue samples of SSc lungs were obtained from archival and anonymised lung tissue samples removed as part of the patient's medical care or post-mortem examination (Department of Pathology of the University of Pittsburgh) or from SSc patients undergoing lung transplantation (Department of Surgery, Division of Thoracic Surgery, Medical University of Vienna, Austria). Downsized non-transplanted donor lungs (Division of Thoracic Surgery, Medical University of Vienna) served as healthy controls. The protocol and tissue usage was approved by the local authorities (Vienna: institutional ethics committee [976/2010]; Pittsburgh: institutional review board [PRO1110204]) and patient consent was obtained before lung transplantation. Scleroderma patients met American College of Rheumatology diagnostic criteria for systemic sclerosis or LeRoy and Medsger criteria for early systemic sclerosis [1, 2]. The patients' characteristics are listed in table S1.

Animal experiments

Female Fra-2 transgenic (TG) mice and wild-type (WT) littermates were maintained under specific pathogen free conditions in isolated ventilated cages with 12 hour light/dark cycles. All animal experiments met EU guidelines 2010/63/EU and were approved by the local authorities (Austrian Ministry of Education, Science and Culture). The characterisation of hemodynamic and lung function was performed in Fra-2 TG and WT mice at 20 weeks of age. 25 mg/kg anakinra (Kineret, Swedish Orphan Biovitrum, Stockhholm, Sweden) was given via daily intraperitoneal injection for a total of eight weeks as previously described [3]. Control groups received injections with an equal volume of sterile saline solution. Mice were sacrificed at 18-19 weeks of age. Anakinra treatment was performed in 2 independent experiments with five to seven mice per group.

Bronchoalveolar lavage fluid (BALF)

After sacrifice animals were lavaged with 1 ml PBS containing protease inhibitor cocktail (Roche) and 1mM EDTA and total cell counts were made.

Single cell lung tissue homogenates

Single cell lung tissue homogenates were performed as previously described [4]. In short, the lower right lobe was digested with 0.7 mg/ml Collagenase and 30 µg/ml DNAse for 40 minutes at 37°C. The tissue was passed through 100 µm cell strainer to obtain a single cell suspension.

Flow cytometry

BAL and single cell lung tissue homogenates were analysed using a LSRII flow cytometer and analysed with the FACSDiva software (BD Biosciences) as previously described [4]. Cells were initially gated on CD45 positivity and were identified as follows: neutrophils (CD11b+, CD11c-, Gr-1+), macrophages (CD11b low, CD11c+, Siglec-F+), dendritic cells (CD11b+, CD11c+, MHC-II high), T helper cells (CD3+, CD4+), cytotoxic T cells (CD3+, CD8+), B cells (CD19+), and eosinophils (CD11b+, CD11c-, Siglec F+). Antibody details are provided in table S2.

Immunohistochemistry and tissue staining

Human and murine lung samples were formalin-fixed, paraffin embedded and cut into 2.5 µm sections. Sections were deparaffinised in xylene followed by rehydration in decreasing concentrations of ethanol. Tissue was stained with Masson's trichrome or Sirius red for histological collagen analysis. For immunohistochemical analyses the antigen retrieval was done in sodium citrate pH6 or EDTA-Tris pH9. Stainings were performed using ZytoChem Plus AP-Fast Red Kit (Zymed Laboratories, USA) or ImmPress^{TM} Kit with NovaRed Substrate, (Vector Laboratories, USA) or Vector Vip Peroxidase (HRP) Substrate Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Negative controls were performed by omission of the first antibody. Antibodies used are listed in table S3. Images were digitalised using a VS120 slide scanning microscope (Olympus, Germany).

Quantification of vascular remodelling and histological scoring

Tissue sections were analysed using Visiopharm integrated software VIS (Visiopharm, Denmark). The degree of muscularisation was quantified on mouse lung tissue sections stained by double immunohistochemistry with endothelial marker von Willebrand factor (vWF) and α-smooth muscle actin (α-Sma) as described previously [5]. Per mouse, 145±80

(minimum: 36, maximum 373) vessels, ranging from 10 to 100 µm in size, were analysed. Deposition of collagen was analysed on one Sirius red stained lung section per mouse. To obtain values reflecting collagen deposition in the parenchyma only, bronchi and vessels with a diameter larger than 200 µm, including 50 µm surrounding, were excluded from the analysis.

Histological scoring was performed on hematoxylin-eosin and trichrome stained lung sections by a pathologist in a blinded manner. Vascular and parenchymal remodelling was determined giving scores from 0 (no remodelling) to 3 (severe remodelling). Further, inflammation in the perivascular, peribronchial and interstitial lung compartments was assessed with scores representing no (0), mild (1), moderate (2) and severe (3) inflammation.

Cell isolation and cell culture

Human primary pulmonary arterial smooth muscle cells (PASMCs) and parenchymal fibroblasts (PFs) were obtained from downsizing donor lungs. Experiments were performed with cells between passages 2-6. Cells were grown in full medium (DMEM-F12, 10% FCS, 1% glutamine and antibiotic/antimycotics, Gibco for PFs; VascuLife SMC Complete Kit; LifeLine Technology for PASMCs). Cells were starved for a minimum of 12 hours in basal medium without FCS prior to stimulation with 1 ng/mL of recombinant IL-1α (Peprotech, USA) or 10 ng/mL of recombinant IL-1β (Peprotech, USA). In inhibitor experiments, cells were pretreated with 50 µM U0126 (Sigma-Aldrich, Austria), or PS1145 (Tocris Bioscience, UK) for 1 hour before addition of IL-1 stimulus. Fra-2 was overexpressed by transfecting PFs with a CMV-hu-Fra-2 (pCDNA3.1) vector construct using Lipofectamine 3000 Reagent (Thermo Scientific, Austria) for 24 hours. The construct (CMV-hu-Fra-2 [pcDNA3]) was a kind gift from Latifa Bakiri, PhD (Spanish National Cancer Research Center Genes, Spain).

Proliferation

Proliferation of PASMCs and PFs was determined by [^{3}H] thymidine incorporation assay (BIOTREND Chemikalien, Germany). 5000 cells per well were seeded in a 96 well plate. The cells were starved in basal medium without FCS overnight prior to stimulation with IL-1α (1 ng/ml) or IL-1β (10 ng/ml). PDGF-BB (10 ng/mL, Sigma Aldrich) stimulated cells served as

positive control. [^{3}H] thymidine incorporation was measured after 24 or 48 hours. Results are expressed as the relative proliferation compared to untreated cells in basal media.

Western blotting

Proteins were isolated from mouse lung homogenate samples using RIPA buffer (Sigma). To obtain nuclear or cytoplasmic fractions, the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Austria) was used. Protein samples were separated by SDS-PAGE and transferred to PVDF membranes (GE Healthcare, UK). Membranes were incubated with the primary antibody overnight at 4°C and one hour at room temperature with the HRP-conjugated secondary antibody. Primary and secondary antibodies used in this study are listed in table S3. Membranes were incubated with ECL prime developing solution (GE Healthcare, UK) and signal detection was done using a ChemiDoc Touch Imaging System (Bio-Rad, USA).

RNA isolation and real-time RT-PCR

Total RNA, isolated from lung homogenates or cells using the peqGOLD Total RNA Kit (Peqlab, Germany), was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad, USA). The real-time RT-PCR reaction was run on a LightCycler 480 System (Roche Applied Science, Austria) using a QuantiFast SYBR Green PCR kit (Qiagen, Germany). Melting curve analysis and gel electrophoresis was performed to confirm the specific amplification of the expected PCR products. Hydroxymethylbilane synthase (HMBS) and beta-2-microglobulin (B2M) served as reference genes. The difference in threshold cycle (Ct) values was calculated as follows: ΔCt = meanCt reference genes – Ct target gene. $\Delta\Delta Ct = \Delta Ct - \Delta Ct^{(\text{untreated control})}$. Primer sequences are provided in table S4.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed using nuclear extracts (NE-PER Nuclear and Cytoplasmic Extraction Kit, Thermo Scientific) from Fra-2-overexpressing human PFs and from lung homogenates of Fra-2 TG mice and WT littermate controls. 3´biotin-labelled probes, corresponding to predicted AP-1 binding sites in the promoter regions of human or mouse IL-1α gene, were used and are depicted in table S5. DNA binding was assessed using the LightShift Chemiluminescent EMSA

Kit (Thermo Scientific, Austria), following manufacturer's protocol. Equal loading of nuclear extract was confirmed by Western blotting.

Enzyme-linked immunosorbent assay (ELISA)

Protein concentrations of IL-1α and IL-1β in plasma and bronchoalveolar lavage (BAL) fluid of Fra-2 TG mice and WT littermate controls were measured by ELISA according to the manufacturer's instruction (IL-1alpha/IL-1beta Mouse Uncoated ELISA Kit, Invitrogen/ThermoFisher Scientific, Austria).

Statistics

Statistical analysis was performed in GraphPad Prism 5 software (Graph Pad Software Inc., USA). Data are expressed as single data points with median, if not stated otherwise. Comparisons between two groups with equal variances were done with unpaired student's t-test. Two groups with significantly different variances were compared using the Mann Whitney test. Treatment effects of anakinra on WT and TG mice were analysed by 2-way ANOVA with Bonferroni's post-test. Multi-group comparisons of cell culture protein and expression data were done using a Kruskal-Wallis test with Dunn's post-test for multiple comparisons. All statistical tests used for a specific data set are indicated in the Figure Legends. p-values below 0.05 were considered as statistically significant.

SUPPLEMENTARY RESULTS

In the next step, we analysed which major inflammatory signalling pathways, such as mitogen-activated protein (MAP) kinases and inflammation-associated transcription factors, were affected by IL-1α and IL-1β in PASMCs and PFs. IL-1α and IL-1β induced a time-dependent phosphorylation of JNK, p38, Fra-2 and cJun in both PASMC and PF (Figure S3A). Fra-2 phosphorylation was evident by the appearance of higher molecular weight bands in the nuclear fraction. No activation of c-fos and AKT was observed (data not shown). Interestingly, PASMCs and PFs showed differential pathway regulation upon IL-1 stimulation: IL-1α and IL-1β induced phosphorylation of ERK in PASMC but not in PF, whereas treatment with IL-1α and IL-1β induced translocation of the p65 NF-κB subunit into the nucleus in PF but not in PASMC (Figure S2A). Using pharmacological inhibitors of JNK, ERK, p38 and NF-κB, it became evident that IL-1α- and IL-1β-induced COL1 downregulation in PASMCs and PFs was mediated by ERK and NF-κB, respectively (Figure S3B).

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Table S1: patients characteristics

Patient case	Gender	Age (years)	histologic description	mPAP (mmHg)	PAWP (mmHg)	FVC (% predicted)	FEV1 (% predicted)	DLCO (% predicted)
5	Male	59	UIP with non-necrotizing granulomas and arteriopathy	NA	NA	61	66	53
12	Female	56	UIP with NSIP areas	NA	NA	62	74	NA
13	Male	63	UIP with prominent NSIP areas associated with granulomas	22	14	57	64	54
28	Male	50	fibrointimal arterial thickening, desquamative interstitial pneumonia, generally preserved lung architecture	57	13	87	79,5	51

Table S2: Antibody details for flow cytometry

Antigen	Label	Company	Clone	Isotype	Dilution Factor
CD3	FITC	eBioscience	145-2C11	Hamster IgG	1:20
CD4	APC	Biolegend	GK1.5	Rat IgG2b, κ	1:100
CD8	PE	Biolegend	53-6.7	Rat IgG2a, κ	1:200
CD11b	V500	BD Bioscience	M1/70	Rat IgG2b, κ	1:50
CD11c	ef450	eBioscience	N418	Hamster IgG	1:50
CD19	AF700	Biolegend	6D5	Rat IgG2a, κ	1:100
CD24	PerCP Cy5.5	BD Bioscience	M1/69	Rat IgG2b, κ	1:500
CD25	APC-Cy7	Biolegend	PC61	Rat IgG1, λ	1:50
CD45	PerCP-Cy5.5	eBioscience	30-F11	Rat IgG2b, κ	1:200
CD45	FITC	Biolegend	30-F11	Rat IgG2b, κ	1:200
CD64	AF647	BD Bioscience	X54-5/7.1	Mouse IgG1, κ	1:20
gdTCR	BV421	Biolegend	GL3	Hamster IgG	1:50
Gr-1	PE-Cy7	Biolegend	RB6-8C5	Rat IgG2b, κ	1:800
MHC-II	APC-Cy7	Biolegend	M5/114.15.2	Rat IgG2b, κ	1:400
Siglec F	PE	BD Bioscience	E50-2440	Rat IgG2a, κ	1:20

Table S3: Antibodies details for immunohistochemistry and western blotting

Antibody	Catalogue #	Company	purpose	Dilution
vWF	A0082	DAKO	IHC - double staining	1:900
α-SMA	EB06450	Everest Biotech	IHC	1:100
			Western Blotting	1:1000
Fra-2 (H103)	sc-13017	Santa Cruz	IHC	1:200
			Western Blotting	1:1000
IL-1α (H-159)	sc7929	Santa Cruz	IHC	1:100
IL-1β (H-153)	sc7884	Santa Cruz	IHC	1:50
Collagen I	1310-01	Southern Biotech	IHC	1:800
			Western Blotting	1:1000
Relmα	ab39626	Abcam	IHC	1:200
α-tubulin	#2125S	Cell signaling	Western Blotting	1:5000
p-JNK	#4671S	Cell signaling	Western Blotting	1:1000
p-p38	#9211S	Cell signaling	Western Blotting	1:1000
p38	#9212S	Cell signaling	Western Blotting	1:1000
p-ERK1/2	#9101S	Cell signaling	Western Blotting	1:1000
ERK1/2	#9102S	Cell signaling	Western Blotting	1:1000
p-cJun	#3270S	Cell signaling	Western Blotting	1:1000
cJun	#9165S	Cell signaling	Western Blotting	1:1000
p65	#3987S	Cell signaling	Western Blotting	1:1000
Lamin	#2032S	Cell signaling	Western Blotting	1:1000

Table S4: Primers used in this study

Name	Species	Forward (5'-3')	Reverse (5'-3')
Il1a	Mouse	CGAAGACTACAGTTCTGCCATT	GACGTTTCAGAGGTTCTCAGAG
Il1b	Mouse	GCCACCTTTTGACAGTGATGAG	GACAGCCCAGGTCAAAGGTT
Col1a1	Mouse	AATGGCACGGCTGTGTGCGA	AACGGGTCCCCTTGGGCCTT
Col1a2	Mouse	TGTTGGCCCATCTGGTAAAGA	CAGGGAATCCGATGTTGCC
Col3a1	Mouse	GCCCTCCCGGGAATAACGGC	TGGCTCTCCCTTCGCACCGT
Acta2	Mouse	CAGCCAGTCGCTGTCAGGAACC	CCAGCGAAGCCGGCCTTACA
Il4	Mouse	ATGGATGTGCCAAACGTCCT	TGCAGCTCCATGAGAACACT
Il13	Mouse	GCCAAGATCTGTGTCTCTCCC	CCAGGTCCACACTCCATACC
I17	Mouse	AGGACGCGCAAACATGAGTC	GGACACGCTGAGCTTTGAGG
Il12p35	Mouse	GACCCTGTGCCTTGGTAGCATC	TGCTTCTCCCACAGGAGGTTTC
Ifng	Mouse	CAGCAACAGCAAGGCGAAAAAGG	TTTCCGCTTCCTGAGGCTGGAT
Retnla	Mouse	TGGCTTTGCCTGTGGATCTT	GCAGTGGTCCAGTCAACGAGTA
Chil3	Mouse	CCAGAAGCAATCCTGAAGACAC	GCACATCAGCTGGTAGGAAG
Igf1	Mouse	TCAGAAGTCCCCGTCCCTAT	TGGGAGGCTCCTCCTACATT
Hmbs	Mouse	GCCAGAGAAAAGTGCCGTGGG	TCCGGAGGCGGGTGTTGAGG
β2m	Mouse	CGGCCTGTATGCTATCCAGAAAACC	TGTGAGGCGGGTGGAACTGTG
COL1A1	Human	ACATGTTCAGCTTTGTGGACC	TGTACGCAGGTGATTGGTGG
ACTA2	Human	GCCTTGGTGTGTGACAATGG	ACCATCACCCCCTGATGTCT
HMBS	Human	TCGGAGCCATCTGCAAGCGG	GCCGGGTGTTGAGGTTTCCCC
β2M	Human	CCTGGAGGCTATCCAGCGTACTCC	TGTCGGATGGATGAAACCCAGACA

Table S5: EMSA probes used in this study

Gene	sequence	label
hulL1A_sense	GTTCTCTGTTGCAGAAGTCAAGATG	3'-biotinylated
hulL1A_antisense	CATCTTGACTTCTGCAACAGAGAAC	
mull1a_sense	CAGAGAAGCCTGACTCAGACTTAAGTC	3'-biotinylated
mull1a_antisense	GACTTAAGTCTGAGTCAGGCTTCTCTG	

Table S6: Histological scoring of Fra-2 TG mice with (TG+A) or without (TG) anakinra treatment.

0: no remodelling/inflammation; 1: mild remodelling/inflammation; 2: moderate remodelling/inflammation; 3: severe remodelling/inflammation; A: arteries;V: veins; Alv: alveolar;Ly: lymphocytes; H: histiocytes; Eo: eosinophils; N: neutrophils; PC: plasma cells; M: macrophages, Mono: monocytes; OP: organizing pneumonia; IMT: inflammatory myofibrobastic tumor; ns: not significant.

	vascular remodelling	**fibrosis**	**perivascular inflammation**	**peribronchial inflammation**	**interstitial inflammation**	**other comments**
TG01	3 A (intima + media)	1 (interstitial)	2/1 A/V (Ly, PC, Eo)/(Ly, Eo)	1 (Ly, Eo, H)	1 (Ly, Eo, H)	giant cells, AlvMp, Ly, N
TG02	0	0	1 A+V (Ly, Eo, PC, M, H)	1 (Ly, Eo, H)	0	--
TG03	0	0	0	0-1 (Ly, Eo)	0	--
TG04	3 A (intima + media)	1 (tumor-like)	2A+V (Ly, Eo, M, PC, H)	2 (Eo, Ly, PC)	0	OP, fibrosis like IMT
TG05	1 media	3 (subpleural)	2 A+V (Ly, Eo, PC)	1 (Ly, Eo, PC)	1 (Ly, Eo)	OP, in alveoli giant cells
TG06	0	0	0	0	0	0
TG07	0	3 (subpleural, tumor-like)	2 A+V (Ly, Eo, PC, H)	1 (Ly, Eo, PC)	0	0
TG08	0	3 (subpleural, just focal tumor-like nodule)	1 A+V (Ly, Eo, PC)	0	0	0

TG09	0	0	0	0	0	0
TG+A01	0	3 (subpleural, tumor-like)	2 A+V (Ly, PC, Eo)	3 destroyed bronchi	2 (Ly, PC, Eo, N)	giant cells, fat, Alv: Eos+++, M, N
TG+A02	0	1 (perivascular, septum)	1 A (Ly, PC)	1 (Ly, PC)	0	giant cells, OP, Ly in Alv
TG+A03	1 media	3 (tumor-like, subpleural, interstital, perivascular)	2 A+V (Ly, PC, Eo)	3 destroyed bronchi	3 (Eo, Ly, PC, N)	giant cells, fat, Alv: Eos+++, M, N
TG+A04	0	0	2 A+V (Ly, Eo, H)	2 (Ly, H, Eo)	1 (Ly)	0
TG+A05	0	1 (tumor-like)	1 A+V (Ly, Eo, PC; Mono)	1 (Ly, Eo, PC)	0	0
TG+A06	3 A (intima + media)	1 (tumor-like)	1 A+V (Ly, PC, Eo)	2 (Ly, Eo)	3 (Eo, Ly, PC, N)	Alv: M, N; OP, giant cells
TG+A07	2	0	1 A+V (Ly, Eo, PC)	1 (Ly, PC, Eo)	0	0
TG+A08	3 A (intima + media)	1 (perivascular)	2 A+V (Ly, PC, Eo)	1 (Ly, PC, Eo)	2 (Eo, Ly, N, PC)	OP, neutrophilic pneumonia, giant cells
TG+A09	3	1 (perivascular, tumor-like)	2 A+V (Ly, Eo, PC)	2 (Ly, Eo, PC)	1 (Eo, Ly, PC)	0
p-value	ns	ns	ns	** p=0,0090	* p=0,0209	