

## LEGENDS TO SUPPLEMENTARY FIGURES

### **Supplementary Figure 1: MD2-TLR4 inhibitor blocks LPS-induced inflammation in vivo.**

C57/BL6 mice pretreated with T5342126 (1 mg/kg) received i.p. ultrapure LPS (10 mg/kg) or PBS followed 4 h later by i.p. luminol (100 mg/kg) and imaged by Bioluminescence imaging (BLI). Representative images.

### **Supplementary Figure 2: MD2-TLR4 inhibitor protects DBA/2J mice from skin fibrosis.**

DBA/2J mice received daily s.c. injections of PBS or bleomycin for 14 days. Treatment with vehicle or T5342126 (1 mg/kg/d) was started concurrently with bleomycin, or on day 14. Mice were sacrificed at day 22 (concurrent treatment) or day 28 (post treatment) and skin was harvested for analysis. **Left panel**, Masson's Trichrome stain. Representative images. Bar = 100  $\mu$ m. **Right panel**, dermal thickness (means  $\pm$  s.d. of five determinations/hpf from five mice/group). \*p < 0.05. **B**. Real-time qPCR. Results, normalized with GAPDH, represent the means  $\pm$  s.d. of triplicate determinations from four mice/group; \*p < 0.05.

### **Supplementary Figure 3: MD2-TLR4 inhibitor attenuates inflammatory response:**

C57/BL6 mice received daily s.c. bleomycin or PBS, together with T5342126 (1 mg/kg/d) i.p. started concurrently for 5 days. Mice were sacrificed at day 7 and skin was harvested for analysis. Immunofluorescence using antibodies to CD3 (red) and CD68 (green) or DAPI (purple). Representative images. Scale bar= 50  $\mu$ m. Quantification of immunopositive cells (means  $\pm$  SD from 3 hpf/mouse; 3 mice per group). \*p < 0.05.

**Supplementary Figure 4: MD2-TLR4 inhibitor reduces skin collagen gene expression in Tsk1/+ mice**

Six weeks-old Tsk1/+ mice treated with T5342126 (i.p.) daily were sacrificed at 12 weeks of age, and back skin was harvested. Real-time qPCR. Results, normalized with GAPDH, represent the means  $\pm$  s.d. of triplicate determinations from at least four mice/group; \*p < 0.05.

**Supplementary Figure 5: MD2-TLR4 inhibitor reduces TLR4 expression on macrophages and myofibroblasts**

C57/BL6 mice received daily s.c. injections of PBS or bleomycin alone, or together with T5342126 or vehicle started on day 0 or day 15. Mice were sacrificed at day 22 and skin was harvested for analysis. **Upper panels**, skin sections double immunolabelled with antibodies to TLR4 (green) and F4/80 (red) or TLR4 (green) and  $\alpha$ SMA (red) or DAPI (blue). Representative double-label immunofluorescence images. Scale bars, 25  $\mu$ m. **Lower panels**, quantitation of TLR4/F4/80 or TLR4/ $\alpha$ SMA double-positive cells (percent). Values are the mean  $\pm$ SD (3 hpf per mouse). \*p < 0.05.

**Supplementary Figure 6: MD2-TLR4 inhibitor reduces macrophage and myofibroblast TLR4 expression**

C57/BL6 mice received alternate-day i.p. injections of vehicle or chlorhexidine gluconate (CG) alone, or together with daily i.p. T5342126 started concurrently with, or one week following initiation of, CG. Mice were sacrificed at day 22, and parietal peritoneal membranes were harvested for analysis. Double-label immunofluorescence of peritoneal membranes double

immunolabelled with antibodies to TLR4 (green) and F4/80 (red) and TLR4 (green) and  $\alpha$ SMA (red) or DAPI (blue). **Upper panels**, representative images Scale bars, 25  $\mu$ m. **Lower panels**, quantitation of double-positive cells. Results (percentage) shown as means  $\pm$ SD (3 hpf per mouse). \* $p < 0.05$ .

**Supplementary Figure 7. MD2-TLR4 inhibitor attenuates DAMP-induced inflammatory and fibrotic responses in human skin fibroblasts and human skin explants.** **A.** Confluent human skin fibroblast cultures were incubated for with Fn<sup>EDA</sup> (10  $\mu$ g/ml) in media together with T5342126 (5 and 10  $\mu$ M) or vehicle, and harvested after 72 h. Real-time qPCR. Results, normalized with GAPDH, represent the means  $\pm$  s.d. of triplicate determinations. **B.** Normal human skin explants were injected every other day with tenascin-C (2  $\mu$ g/ml) together vehicle or T5342126 (10  $\mu$ M) for up to 14 days. At indicated intervals, tissues were harvested for real-time qPCR. Results, normalized with GAPDH, are means  $\pm$  s.d. of triplicate determinations from three explants/group; \* $p < 0.05$ .

**Supplementary Figure 8. TLR4 is specifically ablated in fibroblasts.**

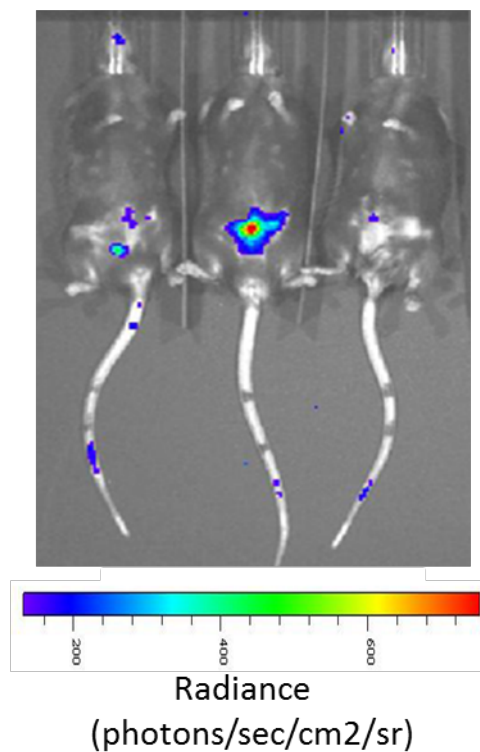
**A.** Schematic showing the generation of mice with fibroblast-specific inducible TLR4 ablation (TLR4<sup>fibcko</sup>) using COL1A2-CRE(ER)T and *TLR4*<sup>fl/fl</sup> mice. **B.** Timeline of tamoxifen and bleomycin treatments. **C.** Mice were sacrificed on day 7 following the last injection of tamoxifen or corn oil. Skin fibroblasts and bone-marrow-derived macrophages (M $\phi$ ) were obtained from two mice in each group and whole cell lysates subjected to Western analysis. Representative immunoblots.

**Supplementary Figure 9. TLR4-response gene signature scores in SSc skin biopsies:  
validation cohort.**

Expression of the experimentally derived TLR4-responsive gene signature (GSE79621) in an independent SSc validation cohort (GSE32413). Heatmap shows hierarchical clustering of the dataset generating diffuse and inflammatory intrinsic subsets. Clustering was performed using 112 probes representing 102 unique genes at an FDR of 5% with at least 1.5-fold change from in vitro experiment with TLR4 overexpression. Color coding indicate intrinsic subset designations as shown. Pearson's correlation of the TLR4-responsive gene signature in each biopsy specimen is shown below the heatmap.

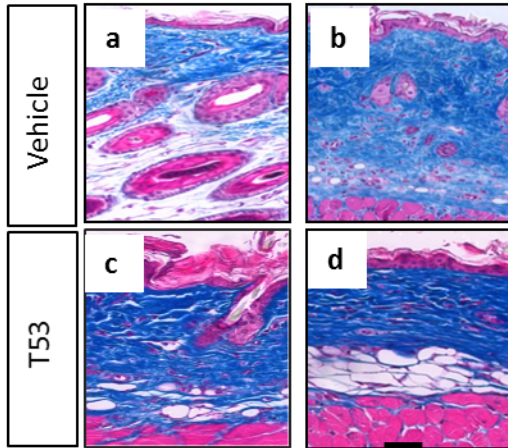
### Supplementary Fig. 1

LPS (mg/kg)	-	10	10
T53 (mg/kg)	-	-	1

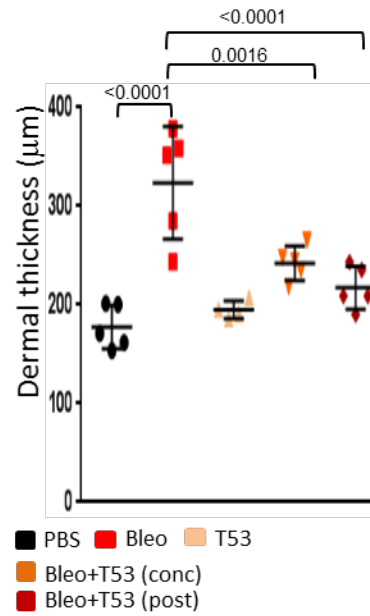


Supplementary Fig. 2

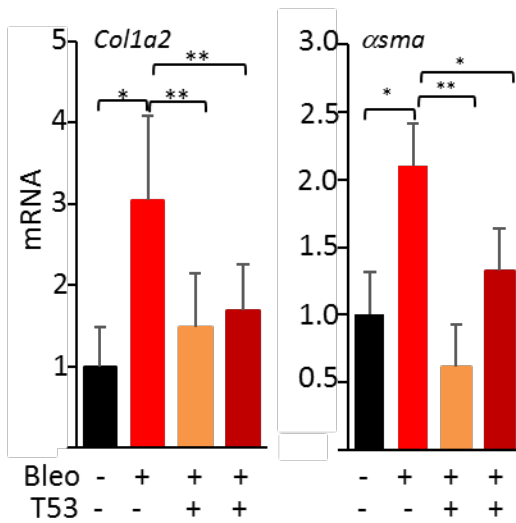
**A**



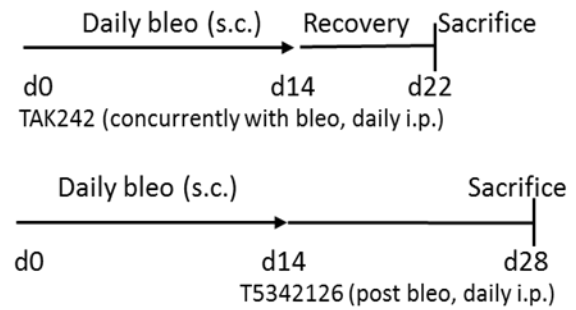
**B**



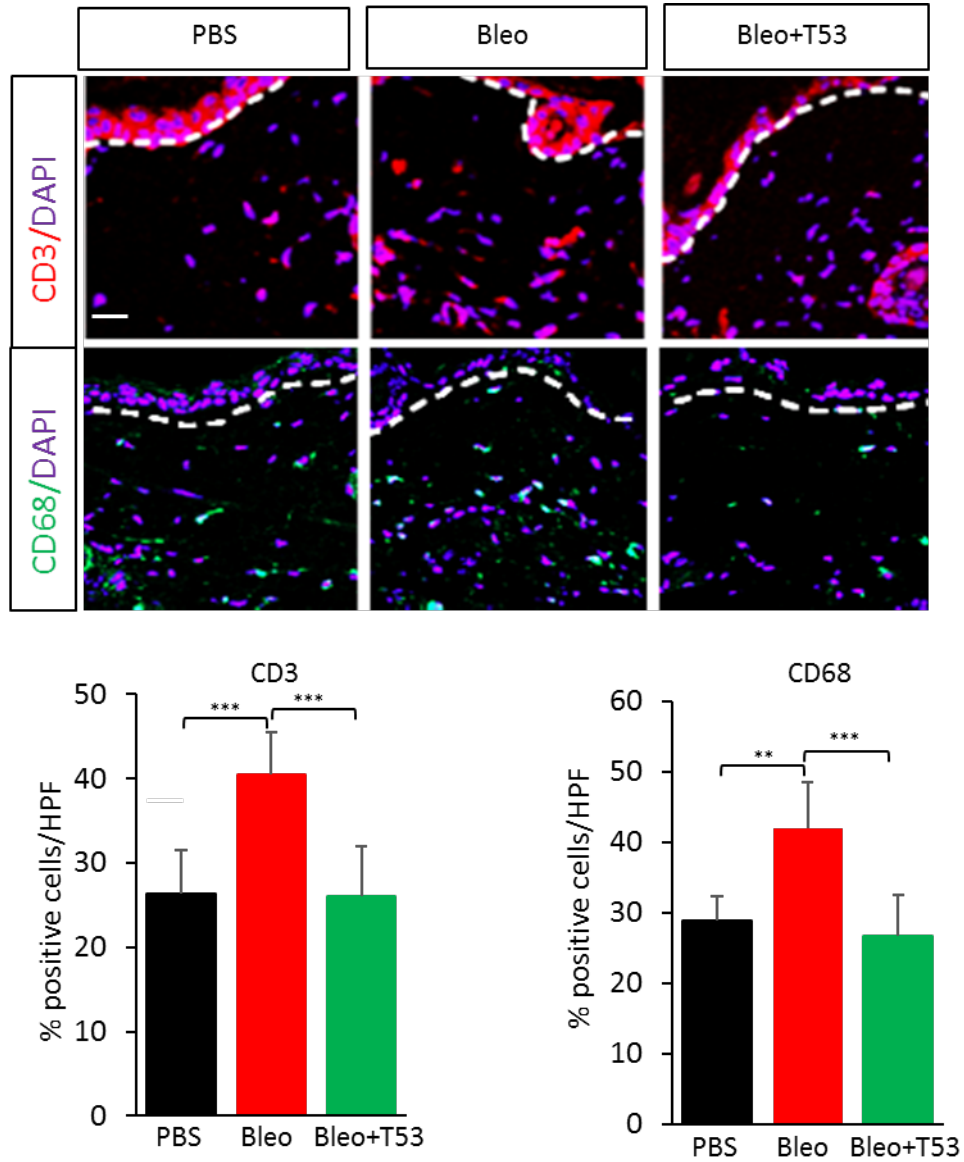
**C**



**D**

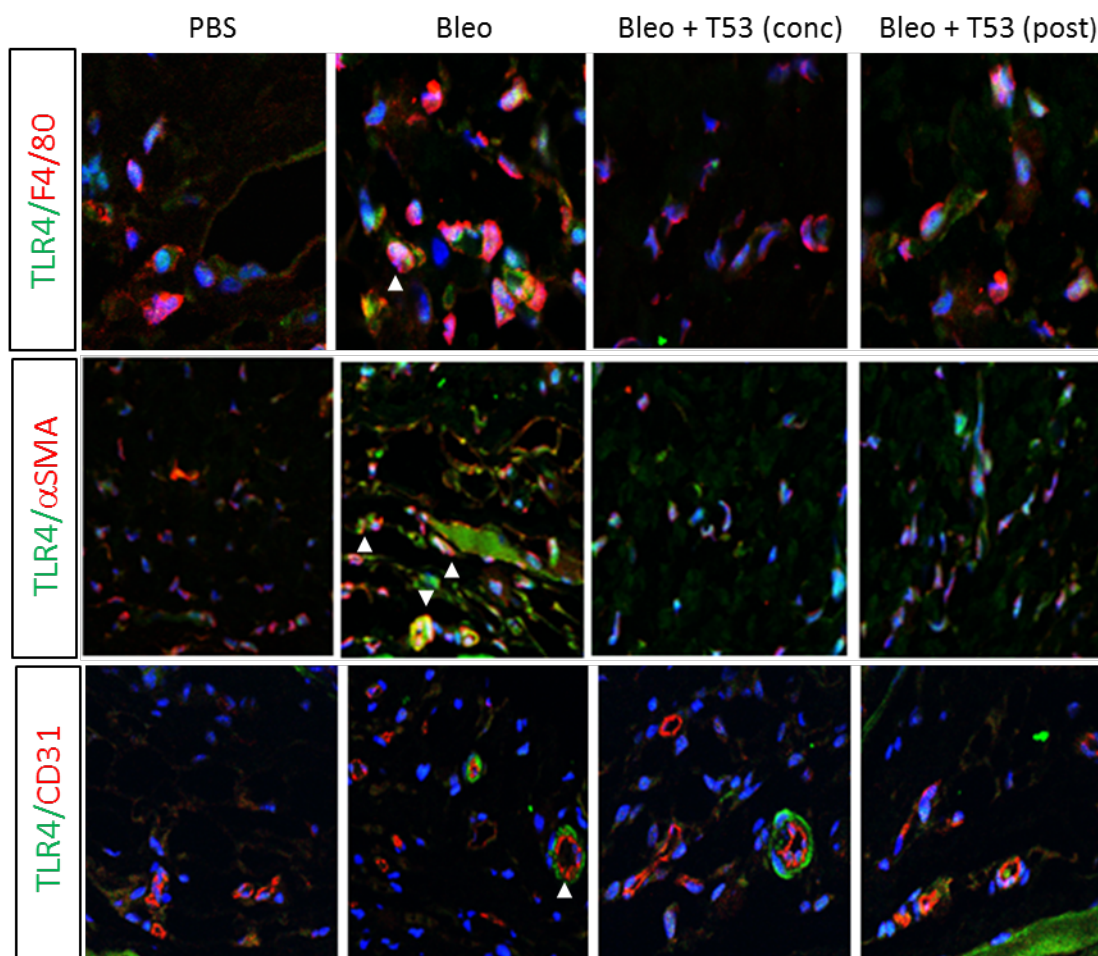


**Supplementary Fig. 3**

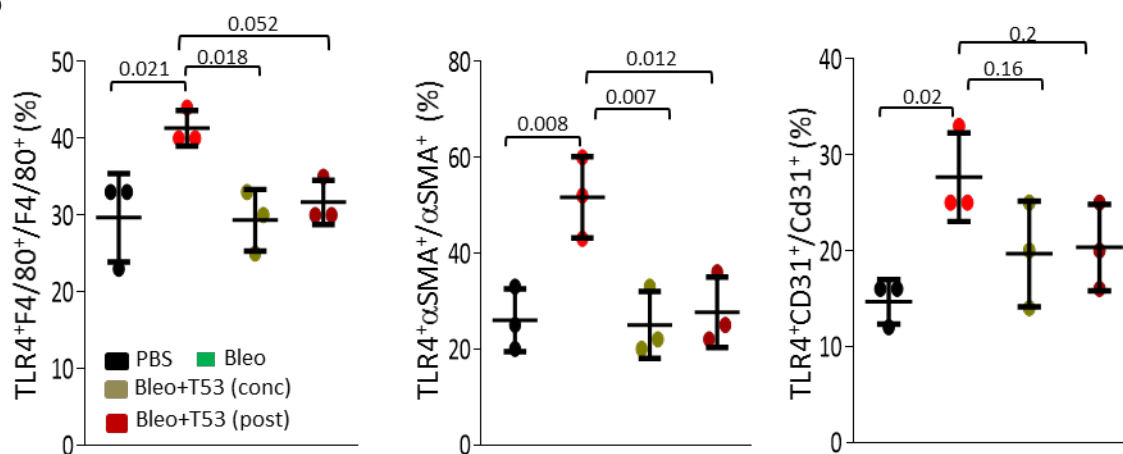


Supplementary Fig. 4

A

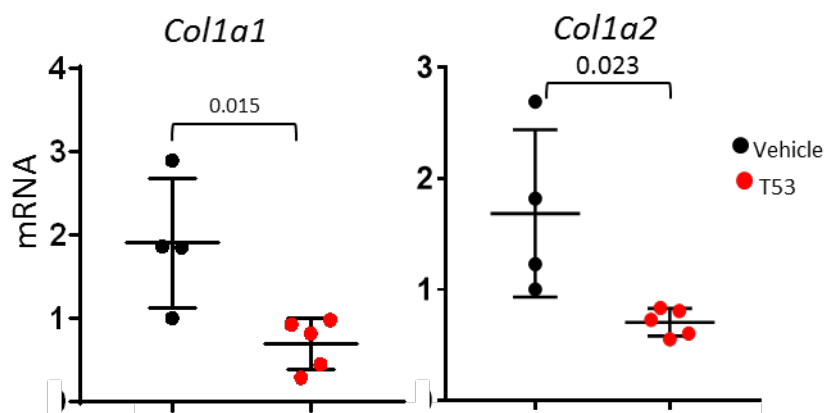


B



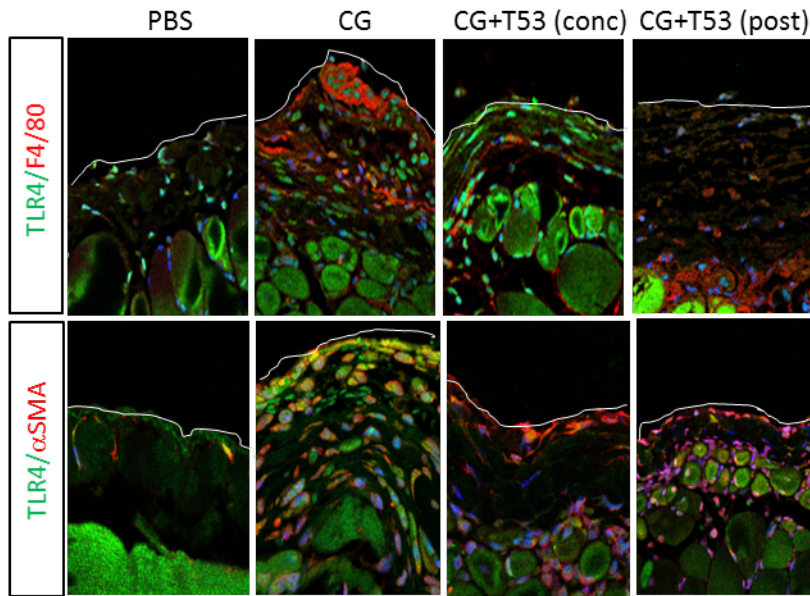


Supplementary Fig. 5

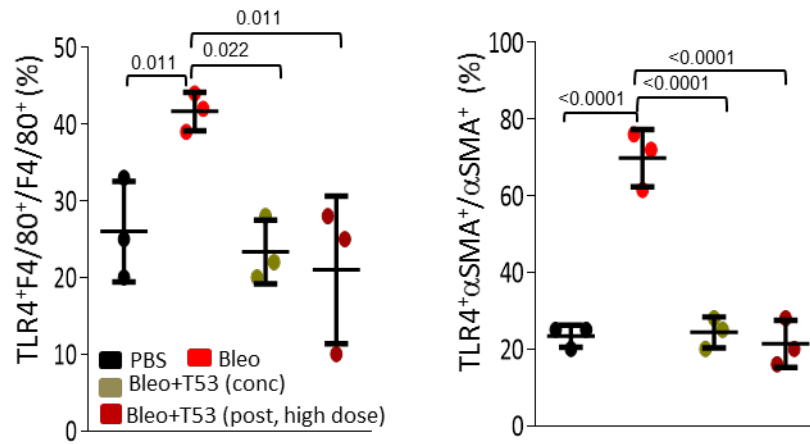


Supplementary Fig. 6

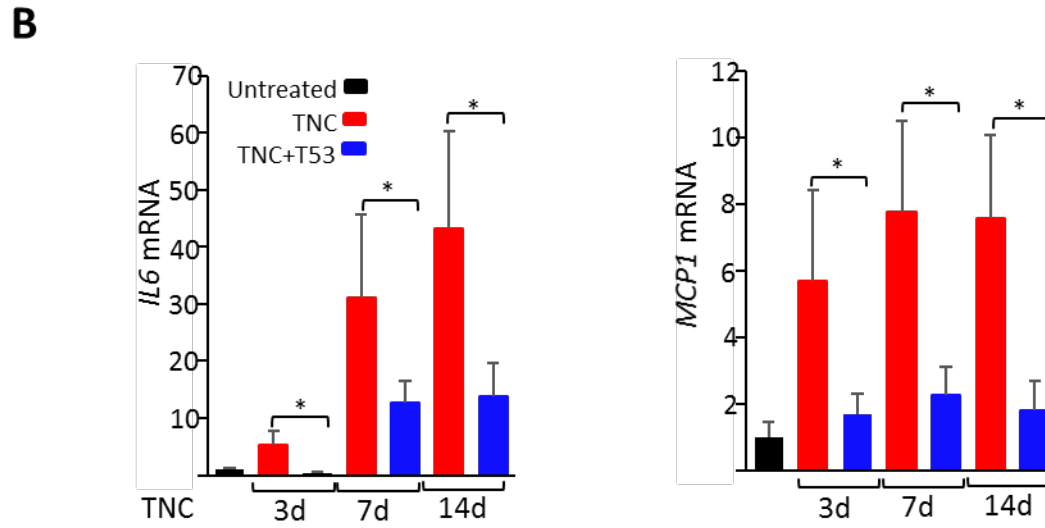
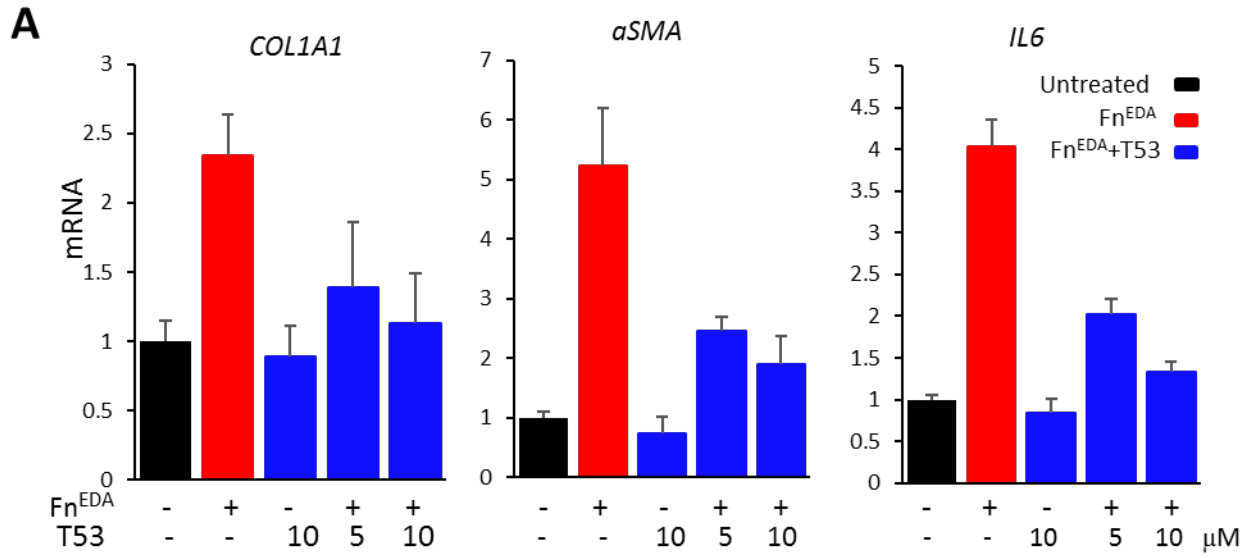
A



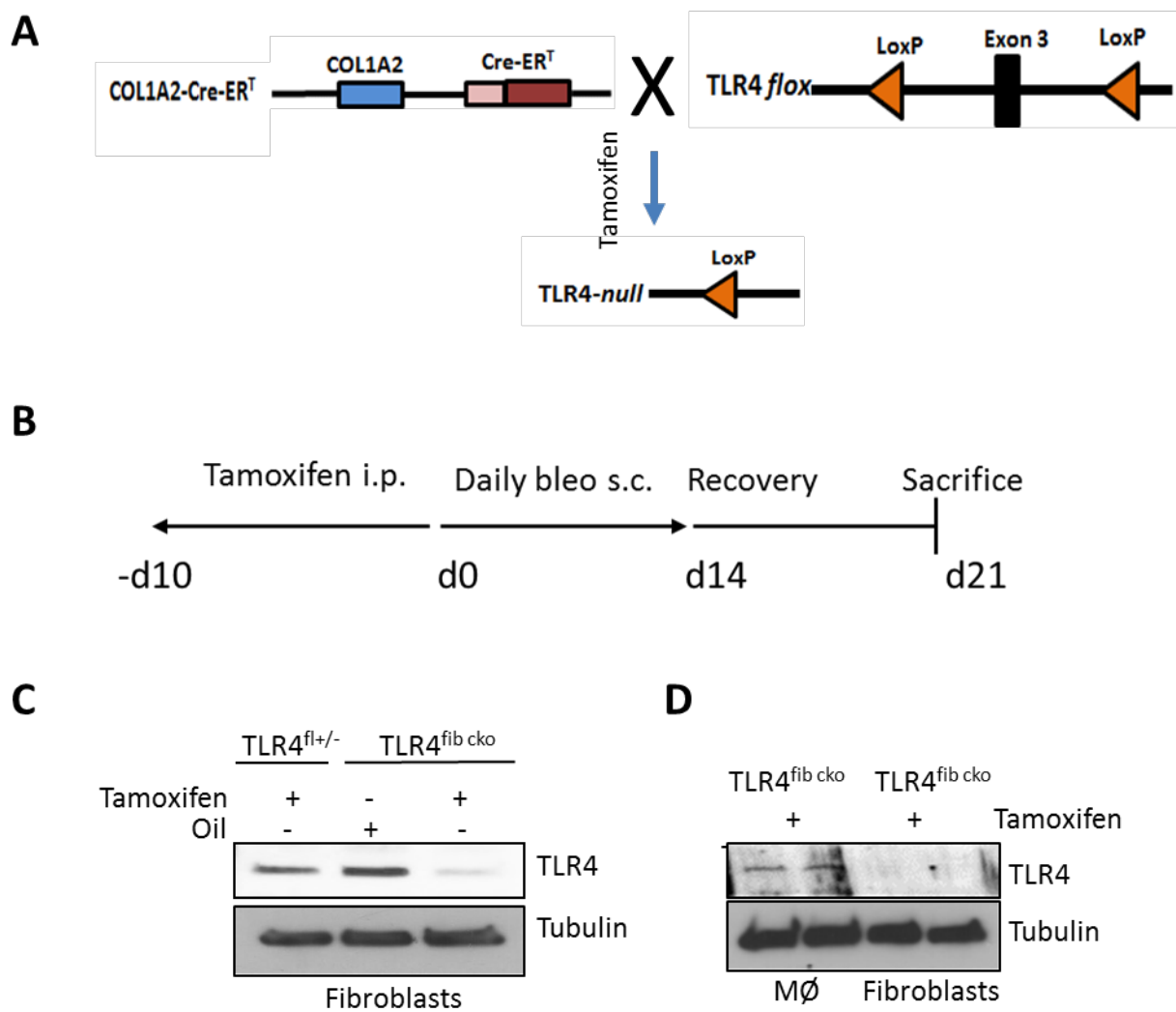
B



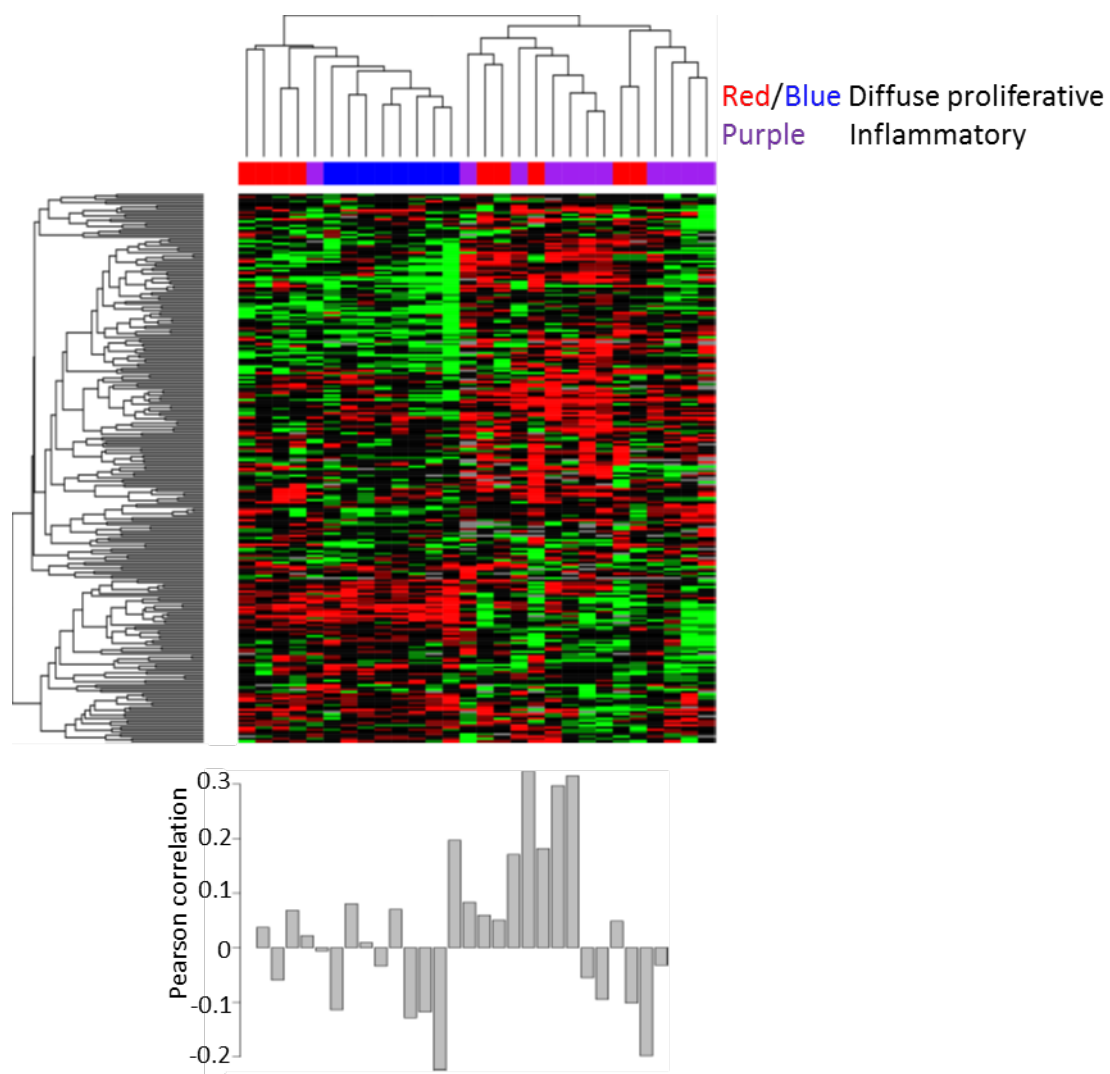
Supplementary Fig. 7



Supplementary Fig. 8



Supplementary Fig. 9



**Supplementary Table 1. Top 20 LPS-induced genes**

Gene name	Log fold-change	t test p value
<b><i>CXCL10</i></b>	11.229	9.86968E-05
<b><i>IFI44L</i></b>	8.89025	0.000400789
<b><i>CXCL1</i></b>	6.5465	0.000988075
<b><i>CCL2</i></b>	6.4495	7.74726E-05
<b><i>IFIT1</i></b>	6.238	8.15715E-05
<b><i>IL8</i></b>	6.11175	0.002863702
<b><i>CXCL9</i></b>	5.2635	0.010857731
<b><i>NFKB1</i></b>	5.12625	4.75E-06
<b><i>CXCL2</i></b>	5.11125	0.000738173
<b><i>TNFAIP3</i></b>	4.6025	5.12264E-05
<b><i>TNF</i></b>	4.482833333	0.066688041
<b><i>IL6</i></b>	4.3375	0.001656301
<b><i>IFI44</i></b>	4.32225	1.05085E-05
<b><i>NFKBIA</i></b>	2.694	0.000389462
<b><i>IRF7</i></b>	2.5815	0.000370207
<b><i>CXCL9</i></b>	2.35925	7.75E-05
<b><i>IL6R</i></b>	2.329	2.4792E-06
<b><i>IL7R</i></b>	2.27825	2.03774E-05
<b><i>IRAK1</i></b>	2.27125	0.000160828
<b><i>MYD88</i></b>	2.24625	4.74985E-06

Confluent human skin fibroblasts were treated with LPS (1 µg/ml) for 28 h. At the end of incubation, total RNA was isolated and subjected to genome-wide transcriptome analysis (27).

**Supplementary Table 2. Overview of *in vivo* experiments presented in this manuscript**

Experiment	Mouse strain; sex	Intervention	Time course
<b>LPS-induced inflammatory response (IL6); <i>in vivo</i> bioluminescence imaging</b>	C57BL/6; females	Concomitant injection of LPS and T5342126	6 h
<b>Bleomycin-induced skin inflammation and fibrosis</b>	C57BL/6; Females	Prevention and reversal by treatment with T5342126	7, 21 and 28 days
<b>Bleomycin-induced skin inflammation and fibrosis</b>	DBA/1; Females	Prevention and reversal by treatment with T5342126	21 and 28 days
<b>Bleomycin-induced lung inflammation and fibrosis</b>	TLR4 <sup>fibKO</sup> ; Females	Prevention in mice with TLR4 ablation	21 days
<b>Spontaneous skin fibrosis</b>	TSK1/+; males and females	Reversal by treatment with T5342126	6 weeks
<b>CG-induced peritoneal fibrosis</b>	C57BL/6; males and females	Prevention and reversal by treatment with T5342126	21 days

### Supplementary Table 3.

Enriched biological processes with TLR4-regulated genes, GO analysis (Gene Answers software)

GO Terms	GO IDs	TLR4-Empty
response to wounding	GO:0009611	101 (4.91e-15)
response to organic substance	GO:0010033	109 (3.39e-11)
cell migration	GO:0016477	64 (8.3e-11)
defense response	GO:0006952	86 (4.45e-10)
extracellular matrix organization	GO:0030198	22 (8.86e-10)
system development	GO:0048731	195 (2.3e-09)
programmed cell death	GO:0012501	107 (2.56e-09)
response to cytokine stimulus	GO:0034097	41 (3.25e-09)
inflammatory response	GO:0006954	45 (3.31e-09)
circulatory system development	GO:0072359	56 (1.11e-08)

Dermal fibroblasts were transfected with constitutively-active TLR4 or empty vector. At the end of 48 h incubation, total RNA was isolated and subjected to genome-wide transcriptome analysis using Illumina Microarray Chips.



**Supplementary Table 4. Primer sequences used in the present studies**

**Human**

<b>hCOL1A1</b>	Forward	5'-TGGTGTGCAAGGTCCC-3'
	Reverse	5'-CATTCCCTGAAGGCCAG-3'
<b>hCOL1A2</b>	Forward	5'-CGGACGACCTGGTGAGAGA-3'
	Reverse	5'-CATTGTGTCCCCTAATGCCTT-3'
<b>h-<i>α</i>SMA</b>	Forward	5'-CAGGGCTGTTTTCCCATCCAT-3'
	Reverse	5'-GCCATGTTCTATCGGGTACTTC-3'
<b>hIL6</b>	Forward	5'-AAATTCGGTACATCCTCGACGG-3'
	Reverse	5'-GGAAGGTTCAAGTTGTTTTCTGC-3'
<b>hTNC</b>	Forward	5'-ACTGTGGACGGAACCAAGAC-3'
	Reverse	5'-TGTGGTGAATGACCCTGAGA3'-3'
<b>hGAPDH</b>	Forward	5'-CATGAGAAGTATGACAACAGCCT-3'
	Reverse	5'-AGTCCTTCCACGATACCAAAGT-3'
<b>hTLR4</b>	Forward	5'-TGAGGACTGGGTGAGAAATGAG-3'
	Reverse	5'-TGGATGATGTTGGCAGCAATG-3'
<b>hIL1b</b>	Forward	5'-AAACCTCTTCGAGGCACAAG-3'
	Reverse	5'-GTTTAGGGCCATCAGCTTCA-3'
<b>hCOL3A1</b>	Forward	5'-TGGTCCCCAAGGTGTCAAAG-3'

	Reverse	5'-GGGGGTCCTGGGTTACCATTA-3'
--	---------	-----------------------------

<b>hTGfb1</b>	Forward	CCCTGCCCCCTACATTTG
	Reverse	CGGGTTATGCTGGTTGT

<b>hMCPI</b>	Forward	5'-ACTGAAGCTCGTACTCTC-3'
	Reverse	5'-CTTGGGTTGTGGAGTGAG-3'

**Mouse**

<b>mCol1a2</b>	Forward	5'-CCGTGCTTCTCAGAACATCA-3'
	Reverse	5'-CTTGCCCCATTCATTTGTCT-3'

<b>masma</b>	Forward	5'-ATGCAGAAGGAGATCACAGC-3'
	Reverse	5'-GTATTCCTGTTTGCTGATCCAC-3'

<b>mFneda</b>	Forward	5'-AGTCAGTGTGGTTGCCTTG-3'
	Reverse	5'-CTGAACACTGGGTGCTATCC-3'

<b>mTgfb1</b>	Forward	5'-ACCGCAACAACGCCATCTAT-3'
	Reverse	5'-GTAACGCCAGGAATTGTTGC3'

<b>mIl6</b>	Forward	5'-GTACTCCAGAAGACCAGAGG-3'
	Reverse	5'-TGCTGGTGACAACCACGGCC-3'

**mGapdh** Forward 5'-ATCTTCTTGTGCAGTGCCAGC-3'

Reverse 5'-GTTGATGGCAACAATCTCCAC-3'

**mTlr4** Forward 5'-TTCAGAACTTCAGTGGCTGGATT-3'

Reverse 5'-CCATGCCTTGTCTTCAATTGTTT-3'

**mI1b** Forward 5'-CGAGGCTAATAGGCTCATCT-3'

Reverse 5'-GTTTGGAAGCAGCCCTTCAT

**mMd2** Forward 5'-CCTGCATAAGACTGAGGGGA-3'

Reverse 5'-CCATGGCACAGAACTTCCTT-3'

**mMcp1** Forward 5'-AAAACACGGGACGAGAAACCC-3'

Reverse 5'-ACGGGAACCTTTATTAACCCCT-3'

**mColla1** Forward 5'-AGCCGCAAAGAGTCTACATG-3'

Reverse 5'-CTTAGGCCATTGTGTATGCAG-3'