Supplementary methods

Study protocol summary

Patients meeting eligibility requirements were randomized through the BUMC investigational pharmacy 2:1 to rilonacept or placebo. Patient and study physicians remained blinded to treatment assignment until after data lock.

Baseline laboratory testing included a complete blood count with differential (CBC), comprehensive metabolic panel, CRP, lipid profile, urinalysis, and HIV and HCV/HBV serologies.

All patients met the American College of Rheumatology criteria for SSc with diffuse cutaneous involvement, had a disease duration of <24 months since the onset of the first SSc manifestation other than Raynaud's phenomenon, had a MRSS of ≥15 and were ≥18 years of age. Patients were excluded from the study if they were on high dose steroids (>10mg/day prednisone or equivalent) or unstable steroid dose; under treatment with immunosuppressive (other than low dose steroids), cytotoxic or anti-fibrotic drug within 4 weeks of screening; positive for viral hepatitis B, hepatitis C or HIV serologies; with a known active infection within 4 weeks of screening; evidence of active or latent mycobateria infection; a history of malignancy within the past 5 years; moderate to severe or renal hepatic impairment; scleroderma renal crisis within 6 months; gastrointestinal involvement requiring total parenteral nutrition or hospitalization within the past 3 months for pseudo-obstruction; moderately severe pulmonary disease with FVC <60%, or DLCO <50% predicted; moderately severe cardiac disease with a history of significant arrhythmia, clinically significant heart failure, or unstable angina; or hemoglobin <8.5 gm/dl, white blood count <3,000/mm^3, total neutrophil count <1,500, or platelet count <100,000/mm^3. A negative pregnancy test was required for women of childbearing potential. Male and female patients of child-producing potential were required to use effective contraception while enrolled, and for at least 3 months after the last treatment.

Treatment was initiated with a loading dose of 320 mg delivered as two subcutaneous injections of 160 mg each given on study visit 1 (day 0) at two different sites. Dosing was continued with a once-weekly injection of 160 mg administered as a single subcutaneous injection for five additional weeks. Patients randomized to receive placebo received sterile saline administered in the same volumes.

At the baseline (day 0) and week 6 visits two, 3 mm punch skin biopsies were carried out at adjacent sites over the mid-forearm (~1 cm distance between biopsies). One biopsy was placed in formalin and the other in RNAlater. Samples in RNAlater were stored in the freezer until RNA preparation for biomarker skin score. The MRSS was assessed at each study visit and the Scleroderma Modified Health Assessment Questionnaire (SHAQ) was administered at baseline (day 0) repeated on week 6 and week 12.

Safety assessments were carried out at all follow study visits and included an interim medical history and physical examination, a CBC with differential, a comprehensive metabolic panel, a urinalysis and assessment of any adverse events. MRSS and CRP were assessed at each study visit; and serum, plasma and peripheral blood mononuclear cell RNA was banked for potential future analyses. All adverse events occurring after administration of the study drug were followed until resolution. The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 was be used for adverse event reporting. AEs were graded by study investigator by severity (mild, moderate, severe) and relationship to study drug (not related, unlikely related, possibly related and related).

Biomarker studies

RNA purification and skin expression of 2G SSc biomarker was assessed by nanostring as described (15). Microarray gene expression at week 6 was compared to day 0 gene expression by microarray analysis of skin RNAs. In addition, in rilonacept-treated patients, gene expression at week 6 was

Supplemental Table I. Rilonacept fails to inhibit IL-1 induced murine genes.

Gene name	Gene symbol	IL-1 induction*	Rilonacept Arm**	*p*-value***
S100 calcium binding protein A9	S100A9	5.60	-0.47	0.24
C-X-C motif chemokine receptor 2	CXCR2	5.45	-0.25	0.08
interleukin 1 beta	IL1B	5.39	-0.13	0.17
C-X-C motif chemokine ligand 1	CXCL1	5.21	-0.21	0.05
alpha fetoprotein	AFP	5.13	0.13	0.05
neurotensin	NTS	5.06	0.35	0.19
interleukin 6	IL6	4.92	0.20	0.18
mannose receptor C type 2	MRC2	4.58	0.12	0.13
ATP citrate lyase	ACLY	4.55	-0.13	0.19
S100 calcium binding protein A8	S100A8	4.38	-0.51	0.13
lipase G, endothelial type	LIPG	4.34	-0.44	0.06
anterior gradient 2, protein disulphide isomerase family member	AGR2	3.76	-0.47	0.15
TIMP metallopeptidase inhibitor 1	TIMP1	3.75	0.14	0.19
EH domain containing 2	EHD2	3.73	0.07	0.22
prostaglandin-endoperoxide synthase 2	PTGS2	3.70	-0.23	0.25
potassium voltage-gated channel subfamily J member 2	KCNJ2	3.68	-0.16	0.05
homeobox A11	HOXA11	3.64	0.11	0.11
semaphorin 3A	SEMA3A	3.53	0.17	0.09
solute carrier family 45 member 2	SLC45A2	3.45	0.18	0.01
zymogen granule protein 16	ZG16	3.31	-0.11	0.03
LIM domain containing 2	LIMD2	3.28	0.07	0.21
lipocalin 2	LCN2	3.20	-0.52	0.10
natriuretic peptide A	NPPA	3.19	0.09	0.12
transgelin 3	TAGLN3	3.07	-0.37	0.03
ankyrin repeat and SOCS box containing 7	ASB7	3.05	-0.07	0.19

*Log2 fold change from PBS treated mouse. **Log2 fold change of visit 3 compared to visit 1. ****p*-value calculated from the difference between visit 3 and visit 1.

Supplemental Table II. Rilonacept on Reactome IL1 signaling associated genes.

ProbeID	Symbol	Name	logFC	*p*-value	adj.*p*-value
1326_at	MAP3K8	mitogen-activated protein kinase 8	-0.21	0.04	0.79
3553_at	IL1B	interleukin 1 beta	-0.13	0.17	0.84
3552_at	IL1A	interleukin 1 alpha	-0.11	0.47	0.94
5604_at	MAP2K1	mitogen-activated protein kinase 1	-0.08	0.11	0.82
64127_at	NOD2	nucleotide binding oligomerization domain containing 2	-0.06	0.37	0.91
3551_at	IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	-0.06	0.32	0.89
6885_at	MAP3K7	mitogen-activated protein kinase 7	-0.05	0.29	0.89
7850_at	IL1R2	interleukin 1 receptor type 2	-0.04	0.78	0.98
3554_at	IL1R1	interleukin 1 receptor type 1	-0.04	0.48	0.94
1147_at	CHUK	conserved helix-loop-helix ubiquitous kinase	-0.04	0.56	0.96
57162_at	PELI1	pellino E3 ubiquitin protein ligase 1	-0.03	0.62	0.97
51135_at	IRAK4	interleukin 1 receptor associated kinase 4	-0.03	0.72	0.97
57161_at	PELI2	pellino E3 ubiquitin protein ligase family member 2	-0.02	0.78	0.98
54472_at	TOLLIP	toll interacting protein	-0.01	0.84	0.99
6416_at	MAP2K4	mitogen-activated protein kinase 4	0.00	0.99	1.00
11213_at	IRAK3	interleukin 1 receptor associated kinase 3	0.01	0.91	0.99
9978_at	RBX1	ring-box 1	0.05	0.42	0.93
7189_at	TRAF6	TNF receptor associated factor 6	0.05	0.38	0.91
3557_at	IL1RN	interleukin 1 receptor antagonist	0.06	0.51	0.95
10392_at	NOD1	nucleotide binding oligomerization domain containing 1	0.07	0.26	0.89
79155_at	TNIP2	TNFAIP3 interacting protein 2	0.08	0.19	0.85
3556_at	IL1RAP	interleukin 1 receptor accessory protein	0.08	0.18	0.85
10454_at	TAB1	TGF-beta activated kinase 1 (MAP3K7) binding protein 1	0.09	0.16	0.84
4215_at	MAP3K3	mitogen-activated protein kinase 3	0.09	0.16	0.84
5608_at	MAP2K6	mitogen-activated protein kinase 6	0.09	0.12	0.82
4615_at	MYD88	myeloid differentiation primary response 88	0.11	0.20	0.86
3654_at	IRAK1	interleukin 1 receptor associated kinase 1	0.15	0.01	0.71

Supplemental Table III. Rilonacept on BioCarta IL1R pathway associated genes.

ProbeID	Symbol	NAME	logFC	*p*-value	adj.*p*-value
3553_at	IL1B	interleukin 1 beta	-0.13	0.17	0.84
3552_at	IL1A	interleukin 1 alpha	-0.11	0.47	0.94
4214_at	MAP3K1	mitogen-activated protein kinase 1	-0.09	0.32	0.89
3551_at	IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	-0.06	0.32	0.89
6885_at	MAP3K7	mitogen-activated protein kinase 7	-0.05	0.29	0.89
1432_at	MAPK14	mitogen-activated protein kinase 14	-0.04	0.48	0.94
3554_at	IL1R1	interleukin 1 receptor type 1	-0.04	0.48	0.94
1147_at	CHUK	conserved helix-loop-helix ubiquitous kinase	-0.04	0.56	0.96
3725_at	JUN	Jun proto-oncogene, AP-1 transcription factor subunit	-0.04	0.71	0.97
4792_at	NFKBIA	NFKB inhibitor alpha	-0.03	0.62	0.97
54472_at	TOLLIP	toll interacting protein	-0.01	0.84	0.99
7040_at	TGFB1	transforming growth factor beta 1	0.01	0.92	0.99
11213_at	IRAK3	interleukin 1 receptor associated kinase 3	0.01	0.91	0.99
7124_at	TNF	tumor necrosis factor	0.03	0.74	0.98
7189_at	TRAF6	TNF receptor associated factor 6	0.05	0.38	0.91
5606_at	MAP2K3	mitogen-activated protein kinase 3	0.06	0.30	0.89
3557_at	IL1RN	interleukin 1 receptor antagonist	0.06	0.51	0.95
9020_at	MAP3K14	mitogen-activated protein kinase 14	0.07	0.39	0.92
3556_at	IL1RAP	interleukin 1 receptor accessory protein	0.08	0.18	0.85
10454_at	TAB1	TGF-beta activated kinase 1 (MAP3K7) binding protein 1	0.09	0.16	0.84
5608_at	MAP2K6	mitogen-activated protein kinase 6	0.09	0.12	0.82
4790_at	NFKB1	nuclear factor kappa B subunit 1	0.09	0.05	0.79
5599_at	MAPK8	mitogen-activated protein kinase 8	0.10	0.11	0.82
4615_at	MYD88	myeloid differentiation primary response 88	0.11	0.20	0.86
7043_at	TGFB3	transforming growth factor beta 3	0.13	0.17	0.85
3654_at	IRAK1	interleukin 1 receptor associated kinase 1	0.15	0.01	0.71
3569_at	IL6	interleukin 6	0.20	0.18	0.85

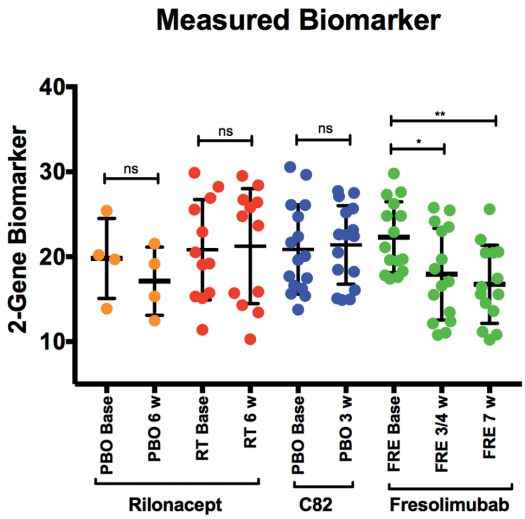
compared to gene expression at day 0. Changes in gene expression (from day 0 to week 6) in patients treated with rilonacept were compared to changes in gene expression (from day 0 to week 6) seen in patients treated with placebo.

Murine IL-1 administration

Osmotic pumps (Alzet: 2001) designed to deliver over 7 days, were sterilely implanted subcutaneously in 6- to 8-week-old C57BL/6J mice (Jackson laboratory) mice. Pumps were loaded with IL-1beta (Thermo Scientific, Recombinant Mouse IL-1beta) 0.1 mg/ml, leading to a release of 10 ng IL-1/hour. Local skin was dissected and gene expression analyzed by microarray as described (14).

Supplementary Table IV. Adverse Events in study participants.

Adverse event	Rilonacept (n=12)	Placebo (n=7)
Pain right shin		1 (14%)
Allergic reaction on abdomen	1 (8%)	
Digital ulcer worsening	1 (8%)	
Abcess on back	1 (8%)	
Left breast swelling	1 (8%)	
Malaise		1 (14%)
Injection site reaction (Grade 1)	2 (16.6%)	
Fatigue (Grade 1)	2 (16.6%)	
Infection, biopsy site (Grade 2)	1 (8%)	
Diarrhea	1 (8%)	



Supplemental Fig. 1. Rilonacept treatment does not show improvements in the 2-gene biomarker compared to other clinical trials.
PBO: placebo; RT: Rilonacept; w: week, FRE: Fresolimubab

Human microarrays

RNA from patient biopsies were isolated as previously described. Affymetrix HGU 133 2.0 array chips were used and processed as previously described. CEL files were processed and normalized using the Robust Multiarray Average (RMA) method in the "affy" package (version 1.52.0). Entrez Gene probeset mapping from Brainarray (version 21.0.0) was utilized. Where applicable differential expression analysis was carried out by using linear modeling within the "limma" package (version 3.30.12). A total of 8,137 were included in the analysis, after filtering genes that were 0.3 standard deviations away from the mean. All microarray analysis were carried out using the R statistical software (version 3.3.2). Clinical trial microarray data has been deposited on GEO (GSE119939).

ELISAS

Serum concentrations of CCL18 and IL-6 in study patients and healthy controls were quantified by commercially available enzyme-linked immunosorbent assays (ELISAS) The CCL18 assay minimum detection level was 18.5 pg/ml (R&D catalog number: DCL180B). The IL-6 assay minimum detection level was 6.25 pg/ml (ABCAM catalog number: ab46027).

Statistical methods

Continuous variables are presented as mean and standard deviation and categorical data is presented as percentages. Mann-Whitney test was used for the evaluation of quantitative variables and Pearson Chi-Square test exact Fisher's test was used for the evaluation of qualitative variables.

We calculated the difference in MRSS assessed at each follow up visits (*i.e.*, Visit 2, 3, and 4) from that at baseline (*i.e.*, Visit 1) for each subject. We compared the difference in means of MRSS change between rilonacept and placebo groups for each follow-up visit. Finally, we examined changes in MRSS over time between rilonacept and placebo groups using mixed effects regression model. We took the same approach to assess the effect of rilonacept on SHAQ and CRP, respectively.

Sample size calculation

For the primary outcome, a sample size of 16 rilonacept- and 8 placebo-treated subjects was calculated to have a power of 0.8 to detect a difference between groups of 5.23 at a $p<0.05$, assuming a standard deviation of 4 for the change in 2G SSc biomarker skin score at week 6 compared to baseline (day 0). Of these, the study recruited 12 patients into the rilonacept- and 7 patients into the placebo-treated groups. Once all subjects completed the study or discontinued prematurely, the final analyses were completed. Data lock was completed before unblinding and data analysis. Finally, we examined changes in MRSS over time between rilonacept and placebo groups using mixed effects regression model.

References

1. RICE LM, ZIEMEK J, STRATTON EA *et al.*: A longitudinal biomarker for the extent of skin disease in patients with diffuse cutaneous systemic sclerosis. *Arthritis Rheumatol* 2015; 67: 3004-15.
2. STIFANO G, AFFANDI AJ, MATHES *et al.*: Chronic Toll-like receptor 4 stimulation in skin induces inflammation, macrophage activation, transforming growth factor beta signature gene expression, and fibrosis. *Arthritis Res Ther* 2014; 16: R136.