Online supplemental methods

Study design

The objective of this study was to delineate the role of co-stimulatory receptors in regulating cytotoxic cell driven pathologic processes in the affected skin of patients with systemic sclerosis (SSc) and examine whether therapeutic targeting of such receptors halts SSc pathology. To address these questions, we performed single-cell RNA sequencing analysis of two separate SSc cohorts (total of n= 109 SSc and n= 65 healthy individuals) containing skin cells from SSc and healthy individuals and used multiplex immunohistochemistry for spatial imaging (n=24) and multi-color flow cytometry for protein level confirmation. We further analyzed the effects of costimulatory modulation in functional assays using (i) stimulation/inhibition of primary lymphocytes from SSc individuals with recombinant proteins, (ii) blocking antibodies in co-cultures of lymphocytes with K562 target cells and (iii) in a fibroblast/immune cell co-culture collagen contraction assay that serves as a disease-relevant in-vitro model to mimic SSc tight and hard skin. Treatment efficacy of a novel combination of bispecific anti-CD3/CD7 targeting immunotoxin was evaluated (i) in lymphocytes from SSc individuals blood, (ii) ex vivo skin cultures and (iii) in a severely affected SSc patient who was treated on compassionate use with a novel anti-CD3/7 immunotoxin (CD3/CD7-IT) treatment. Functional experiments were performed with multiple biological and technical replicates as mentioned in each figure’s legend and in each assay’s methods description.

Patient and public involvement

This research incorporated the active participation of patients in its design and execution. Two patient research partners were proactively involved in the design of primary research questions and methods of patient recruitment by structured interviews and regular, interactive discussions. Patient research partners were trained in the context of STAP (“Key To Active Participation”), an initiative of the department of rheumatic diseases of the Radboud University Medical Centre (Nijmegen, the Netherlands) to establish a patient panel within the hospital setting to provide support for rheumatology research (1). The involvement of patients and their families in disseminating the results of this study in patient organizations played a central role in motivating community engagement both during and after the study.

Patients

Our study was approved by the local research ethics committee of Radboud University Medical Center, the Netherlands (study numbers: NL57997.091.16, NL67672.091.18). All procedures regarding patient participation followed the Declaration of Helsinki principles were performed in accordance with the relevant Dutch legislation regarding reviewal by an accredited research ethics committee, with the file number 2021-8193. All patients (aged >18) that donated whole blood and skin biopsies, were diagnosed with established systemic sclerosis disease according to the ACR 1980 preliminary classification criteria(2). SSc patients with overlapping syndromes were not included in our study. Blood samples from age and sex matched healthy volunteers were collected from Sanquin bloodbank (project number: NVT 0397-02) from individuals that consented on donating blood for medical research. All patients agreed to participate in the study before blood withdrawal or skin biopsy acquisition. For analyses were we examined the relationship between CD7 normalized mean gene expression and selected patient clinical characteristics, SSc patients’ clinical data were received as part of a previous publication (3).

Immunotoxins
The anti-CD3/CD7 combination of immunotoxins (CD3/CD7-IT) as referred to in this article contains a 1:1 mixture (w/w) of the murine monoclonal antibodies SPV-T3a (anti-CD3) and WT1 (anti-CD7) that are both conjugated to recombinant ricin toxin A as has been previously described (4, 5).

Peripheral blood mononuclear cell (PBMC) isolation, cryopreservation and culture

PBMCs were isolated from patients' (n=30) and healthy donors' (n=15) peripheral blood by Ficoll Pacque PLUS density centrifugation and cultured in complete RPMI medium 1640+ GlutaMAX (Gibco, ref 72400-021) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, 100 mg/l sodium pyruvate and 10% human pooled serum. PBMCs that were not processed immediately were cryopreserved and stored in liquid nitrogen until further use. To generate phytohemagglutinin (PHA)-activated T cells, PBMCs were first seeded in 96-well-u bottom plates (Greiner) at a cell density of 100,000 cells per well and then stimulated with 5 µg/ml PHA (Roche, cat# 11082132001) for 24 hours at 37 °C, 5% CO2. To evaluate production of cytokines, prior to flow cytometric staining, PBMCs were stimulated for 4 hours at 37 °C, 5% CO2, with 12.5 ng/ml phorbol myristate acetate (Sigma), and 500 ng/ml ionomycin (Merck) in the presence of 5 µg/ml brefeldin A (Merck).

Collection and cell culture of primary fibroblasts

Half piece of 4 mm diameter skin biopsies were placed in 24 well plates containing 2 ml DMEM Glutamax medium (Gibco, Waltham, MA, USA) that was supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 100 mg/L sodium pyruvate and 20% fetal calf serum. Plates were incubated in regular culture conditions (5% CO2, 37 °C, 95% humidity) for 2 weeks in which primary skin fibroblasts spontaneously grew out. Medium was refreshed every 3-4 days. After outgrowth, primary fibroblasts were cultured in DMEM Glutamax medium (Gibco) that was supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 100 mg/L sodium pyruvate and 10% fetal calf serum and used in experiments after passage 5.

Isolation, culture and cell viability of T cells, B cells and NK cells from SSc peripheral blood

Cryopreserved PBMCs from patients with systemic sclerosis were thawed and washed as previously described to isolate specific immune cell populations. CD3+ T cells were isolated with a magnetic negative selection according to the manufacturer’s instructions (MojoSort pan CD3+ T cell isolation kit; Cat# 480021). CD19+ B cells were also isolated with negative selection using the MojoSort TM Human Pan B cell Isolation Kit (cat# 480082). Isolation of untouched CD56+ NK cells from SSc PBMCs was performed by using NK isolation kit (Miltenyi Biotec, cat# 130-092-657), according to manufacturer’s protocol. After isolation, enriched CD3+ T cell, CD19+ B cell and CD56+ NK cell fractions exhibited more than 95% purity as evaluated by flow cytometry staining for CD3, CD19, CD56 markers. The isolated immune cell populations were cultured with XVIVO15 medium (Lonza, cat# 04-418Q) at a density of 50,000 cells/well in 96-well u bottom plates (Greiner). To evaluate cell viability of the cells after different stimulation (24 hours) and treatment conditions (48 hours), the CellTiter-Glo® 2.0 Cell Viability Assay (Promega) was used as per manufacturer’s instructions. Cells were also treated with 5 mM cycloheximide (Sigma, cat# 01810-1G) as positive control. Luminescence was measured with the use of CLARIOstar Plus (BMG LABTECH). For every experimental condition, 4 technical replicates were used and the average of them was used in further analysis. Experimental values were corrected for medium luminescence and were normalized to the control unstimulated and untreated condition.

Monocytes isolation and differentiation to M2 macrophages

CD14+ monocytes were isolated from PBMCs with positive selection kit (Miltenyi Biotec, cat# 130-050-201) according to manufacturer’s instructions. Monocytes were then seeded in 6-well plates at a cell density of 1 million cells per well at a volume of 2 ml in XVIVO15 medium that was supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin and 2% human pooled serum. Differentiation towards M2-like macrophages

M2...
was stimulated by adding 20 ng/ml rhM-CSF (R&D Systems, cat# 216-MC) and 10 ng/ml rhIL-4 (Biolegend, cat# 500815). Duration of culture was 7 days and medium with cytokines was refreshed at day 3. Cell viability of M2-like macrophages was evaluated with CellTiter-Glo® 2.0 Cell Viability Assay (Promega) as previously described.

**Immunohistochemistry**

Immunohistochemical analysis was performed on formalin fixed paraffin embedded (FFPE) skin biopsies of 20 patients with systemic sclerosis. Skin biopsies were obtained from both a affected and non-affected area of the forearm as diagnosed by an expert clinician via surgical excision with a 6-mm punch biopsy. In all cutaneous specimens staining for CD3 was used as a marker to evaluate T cell infiltration and CD7 to assess infiltration of activated T lymphocytes and NK cells. For the CD3 staining, slides were deparaffinized with xyol wash and rehydrated with ethanol. Antigen was retrieved in 10 mM sodium citrate buffer (pH 6.0) room temperature (RT). Blocking of the peroxidase activity was conducted by incubation with 3% H2O2 in or 30 min. Then, sections were incubated with the primary mouse CD3 anti-human monoclonal antibody (1:200 dilution in PBS containing 1% BSA; Clone F7.2.38; Dako; Cat# M7254) overnight at RT. Next, tissues were incubated with secondary antibody (BrightVision Poly-HRP, Immunologic DPVO55HRP) for 60 minutes at RT. 3’3’-diaminobenzene was used to visualize antibodies (bright DAB, Immunologic). Nuclei in all slides were counterstained with hematoxylin and mounted with a cover slip (Permount, Thermo-Fisher, Waltham, MA, USA). CD7 was immunohistochemically evaluated with the use of the Omnis automatic immunostainer (DAKO) according to manufacturer’s standard procedures. In brief, FFPE tissues were deparaffinized, rehydrated and subjected to heat-mediated antigen retrieval (30 min at 97°C). Following, endogenous peroxidase was blocked and the primary mouse CD7 anti-human monoclonal antibody (ready to use, diluted in Envision Flex Antibody Diluent, clone CBC.37, DAKO; Cat# GA64361-2) was added for 20 min at RT. Secondary antibody (Envision Flex HRP, DAKO) was then applied for 20 min at RT. Antibody complex was developed with Envision Flex Substrate Working solution (DAKO) and nuclei were counterstained with hematoxylin. Human synovial/tonsil specimens were used as positive controls and skin sections without the primary antibodies as negative controls. Cellular infiltrates were examined through the whole surface of all sections (n=4) mounted per donor and condition and imaged with CaseViewer (v2.3.0.99276). CD3 positive cells were counted by 2 independent observers in four randomly selected fields and total number of positive cells was plotted as mean ± SD. CD7 positive staining was assessed using an arbitrary 0-4 semiquantitative scoring system of positively stained areas. This scoring was performed blindly by 2 independent observers. Expression of collagen type 1 (Goat Anti-Type I Collagen-UNLB, Southern Biotech, cat# 1310-01) in fibroblasts was also evaluated. Staining was performed similarly to CD3 marker with the exception that incubation with a secondary rabbit biotinylated anti-goat IgG antibody (Vector Laboratories, PK-6101) was performed for 30 minutes at room temperature. Values illustrated in the graphs represent mean ± SD.

**Multiplex immunohistochemistry staining and imaging of SSc skin**

For multiplex immunofluorescent staining, 5 µm thick sections from matched affected and non-affected skin of 24 SSc patients were included. Slides were stained by an automated platform with the use of Opal 7-color Automation IHC kit (NEL801001KT; PerkinElmer) on the BOND RX IHC & ISH Research platform (Leica Biosystems) as it has been previously described(6). Incubation with primary and secondary antibodies was for 1 hour and 30 min respectively at RT. For the detection of skin lymphocyte cell populations the following antibodies were used; anti-CD56 (Cell Marque, 156R-94, clone MRQ-42) with Opal620, anti-CD8 (Dako, M7103, clone C8/144B, 1:200) with Opal690, anti-CD7 (Dako, GA64361-2, clone CBC.37, 1:30) with Opal480, anti-CD3 (Thermo Fisher, RM-9107, clone RM-9107, 1:200) with Opal520, anti-FOXP3 (eBioscience Affymetrix, 14–4777, clone 236A/E7, 1:100) with Opal570 and anti-CD20 (ThermoFisher, MS-340, clone L26, 1:600) with Opal570. Slides were stained with DAPI for 5 minutes, washed and mounted with Fluoromount-G (SouthernBiotech, 0100–01). Slides were then scanned by the Automated Quantitative Pathology Imaging System (Vectra V.3.0.4, PerkinElmer) with using an overview of 4x magnification. Annotation of multispectral images of skin tissue was performed with Phenochart (V.1.0.9, PerkinElmer) and scanned at 20x magnification. Spectral unmixing of the Opal fluorophores was done by InForm software (V.2.4.2, PerkinElmer) and the multichannel images were then digitally merged. For quantitative

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analysis, digital scans containing whole skin biopsies \((n=3\) sections per biopsy per donor and condition\) were quantified by QuPath-0.4.4 (7).

**Single-cell RNA sequencing analysis**

The single-cell count matrix \((\text{Cell by Gene})\) was obtained from two publicly available datasets, namely GSE195452, GSE138669 and GSE128169. Preprocessing of the data was performed using Seurat (version 4.3.0) (8). Quality control measures were implemented by filtering out cells with a high content of mitochondrial genes (>5%) and cells with gene counts per cell values below 200 or above 2000. Subsequently, CD3+ and/or CD7+ cells were sorted, resulting in the recovery of 2126 and 5061 high-quality cells from both datasets, respectively. Later, the CD8+ subset of cells was sorted from these cells for separate analyses.

For primary dimension reduction, non-negative matrix factorization was employed, followed by the application of UMAP: Uniform Manifold Approximation and Projection (9) with Louvain clustering, as previously described by Singh et al. (10). The FindAllMarkers function of Seurat was then utilized to identify differentially expressed genes \((\text{DEGs})\) within each cluster, which were subsequently annotated based on the characteristics of these DEGs. An R package pheatmap (Kolde, R. (2019). pheatmap: Pretty Heatmaps \((\text{R package version 1.0.12})\)) was used to visualize the DEGs across the cell types/groups.

To assess DEGs between healthy and diseased individuals, the FindConservedMarker function of Seurat was employed. Additionally, the Wilcox-Test (11) was applied to test for differences in cell frequencies between healthy individuals and those with systemic sclerosis \((\text{SSc})\).

**Single-Sample Gene Set Enrichment and Correlation analyses**

To gain insights into the functional characteristics of each cell type, we performed single-sample gene set enrichment analysis \((\text{ssGSEA})\) using the escape R package (12) with WikiPathways from MsigDB (13) as the reference gene set collection. An R function called `geom_tile` from ggplot2 package (Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis (2nd ed.). Springer) was used to visualize the pathways across different cell types/groups. The difference in the distribution of Normalized Enrichment Scores \((\text{NES})\) between control and SSc group was tested using Kolmogorov-Smirnov \((\text{KS})\) test (14). To capture pathways associated with fibrosis and inflammation, we retrieved the gene list related to these processes and further augmented it by including CD7.

Performing correlations at the single-cell level can be noisy and biased by technical factors. Hence, we constructed a meta-cell object from previously described Seurat object comprising CD3/CD7+ cells, by employing the WGCNA R package (15). This object contains framework weighted gene co-expression to identify modules of highly correlated genes. Subsequently, from this meta-cell object was used to obtain pairwise correlations and p-values were computed using the Hmisc function from the Hmisc R package (Harrell Jr., F. E., & with contributions from Charles Dupont and many others (2020). Hmisc: Harrell Miscellaneous \((\text{R package version 4.8.0})\)). An R package ComplexHeatmap (Gu, Z. (2016). ComplexHeatmap: Making Complex Heatmaps in R \((\text{R package version 2.10.0})\)) was used to visualize the correlations.

**Flow cytometry analysis**

Per donor, \(1 \times 10^6\) PBMCs were first labeled with ViaKrome 808 fixable viability dye \((1.5:\text{1000 in PBS})\) for 30 min at \(4^\circ\text{C}\) to exclude dead cells and then stained for 20 minutes at RT with fluorescently labeled extracellular antibodies \((\text{supplemental table 1})\). For intracellular stainings \((\text{supplemental table 2})\), cells were fixed with permeabilized using the Cyto-Fast™ Fix/Perm Buffer Set \((\text{Biolegend})\) according to manufacturer’s guidelines. To facilitate detection of intracellular cytokines, cells were pre-stimulated with \(12.5\ \text{ng/ml phorbol 12-myristate 13-acetate (PMA)}\) \((\text{Sigma})\), \(500\text{ng/ml Ionomycin (Merck)}\) and \(5\ \mu\text{g/ml brefeldin A (Merck)}\) before staining. Samples were acquired on a Beckman Coulter Cytoflex LX 21-color flow cytometer immediately after staining.
Multi-parameter flow cytometric quantification of CD3/CD7-IT-induced cell death

To evaluate the killing efficacy of CD3/CD7-IT towards activated T and NK cells in vitro, we developed a model in which a 24-hour PHA (Roche) stimulation of PBMCs was used to mimic disease-related T cell activation. PHA stimulation was accompanied by elevated surface expression of CD3 (2-fold increase in MFI) and CD7 (3-fold increase in MFI) antigens on cytotoxic CD8+GZMB+ T cells and CD7 (2-fold increase in MFI) on CD56+GZMB+ NK cells (Supp. Figure 6A, B). Non-activated or PHA-activated (5 µg/ml) PBMCs were cultured for 24 hours at 37°C, 5% CO₂ before treated with CD3/CD7-immunotoxin (IT) for 48 hours. Based on previous studies, the in vitro clinically therapeutic concentration was between 1-5 nM. We titrated drug concentration (0-10 nM) based on its killing efficacy towards primary T cells and we chose the lowest concentration exerting maximum killing efficacy. Concentration of the drug that was used in in-vitro experiments was 0.33 nM. Post treatment, cells were collected in 15 ml conical tubes, washed with PBS and processed for flow cytometric staining. Staining protocol for live/dead, extracellular and intracellular markers followed as it was previously described. CD2 was used to identify and characterize T cell populations, instead of CD3, due to possible modulation of the CD3 antigen from the CD3/CD7-IT treatment. To enable quantification of absolute cell counts, a fixed amount of counting beads (Precision Count BeadsTM, Biolegend, cat# 424902) was added in each sample prior to acquisition. Samples were acquired on a Beckman Coulter Cytoflex LX 21-color flow cytometer immediately after staining.

Ex vivo skin culture

Full thickness 6 mm diameter skin punch biopsies were obtained from the abdomen of 4 healthy individuals that underwent plastic surgery. All patients signed informed consent that their surgical leftover material will be used for research purposes. From each skin tissue, 4-6 punch biopsies were received and cut in half. To account for a potentially unequal infiltration of immune cells between skin biopsies, all skin pieces were pooled together and then distributed equally in the different experimental conditions. The skin tissue was cultured in 24-well plates in 1 ml of RPMI medium 1640 with 100 IU/ml rhIL-2 (Thermo Fischer, cat# 16-7027-85), 5 µg/ml PHA (Roche), growth supplements and antibiotics. Twenty-four hours later, samples were treated with 0.33 nM a-CD3/CD7-IT. After 48 hours, the skin pieces of each condition were used to obtain single-cell suspensions containing skin infiltrating lymphocytes for functional assays. Protocol that was used combines mechanical and enzymatic dissociation of the skin tissue and has been extensively described by He et al. (16).

Apoptosis assay

To distinguish early apoptotic cells from non-apoptotic and cells in late apoptosis/necrosis, cells were first stained extracellularly with monoclonal antibodies of interest for 20 minutes at RT. Cells were then washed twice with cold PBS and resuspended in 100 µl of a buffer containing 5 µl 7-AAD (eBioscience, cat# 00-6993-50), 5 µl Annexin V:FITC labeled (BD Pharmigen) and 0.15 µl CaCl₂ (1 M) in PBS. Samples were incubated in the dark at RT for 10 minutes and were acquired by flow cytometry (Galilios) immediately after staining. Cells being 7-AAD+AnnexinV+ are referred as late apoptotic/necrotic cells while cells being 7-AAD-AnnexinV+ as early apoptotic. Live cells are negative for both 7-AAD and AnnexinV.

Cytokine measurements

Quantification of human cytokines and chemokines in culture supernatants were measured by Luminex. The Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad, cat# M500KCAFOY) was used following the manufacturer’s instructions. Samples were analyzed with BioPlex Manager 4 software (Bio-Rad Laboratories, Hercules, CA, USA).

LDH cytotoxicity assay

To assess cytotoxic capacity of cytotoxic T and NK cells, Lactate dehydrogenase (LDH) was measured in PBMC and K562 cell co-culture supernatants using a LDH-cytotox kit according to manufacturer’s protocol (Biolegend #426401). PBMCs and K562 cells were seeded into 96-well plates (F-bottom) in a 10:1 ratio, using triplicate wells. To augment cytotoxic function of CD8+ T cells and NK cells, PBMCs were stimulated overnight with 1 µg/ml anti-CD3/CD28 (Biolegend, cat# 317326, 302913) or 500 IU/ml IL-2 (Thermo Fischer, cat# 16-7027-85), 10 ng/ml IL-15 (Gibco, PHC9154) respectively. To evaluate the involvement of CD7 receptor in T and NK cell cytotoxicity 330 nM of anti-CD7 (WT1) blocking antibody was used. Percentage of cytotoxic capacity was calculated according to the formula: %cytotoxicity= (experimental value-low control value)/(high control value-low control value) x 100. Low and high control values correspond to LDH levels of K562 cells alone without or after addition of lysis solution respectively.

Fibroblast and immune cell in vitro co-culture collagen contraction assay

Primary healthy human fibroblasts were detached with trypsin and were then brought to a cell density of 2 x 106 cells/ml. PBMCs from 5 healthy individuals were thawed and washed as previously described and stimulated/treated with the different experimental conditions mentioned in the results section. Cell suspension containing a mixture of PBMCs and fibroblasts in a 5:1 ratio was then prepared. To create the 3D collagen hydrogels, for every plug, 20 µl Minimal Essential Medium (Sigma-Aldrich, Saint Louis, CA, USA), 10 μL sodium bicarbonate (Gibco, Waltham, MA, USA), 150 μL soluble collagen (PureCol, type 1 collagen) and 90 μL cell suspension were sequentially mixed in a different tube and the respective order. After the suspension was delicately homogenized, 250 µl was added per well of 48-well plates. Thereafter, 750 µl of complete RPMI medium was added and the plugs were incubated under standard conditions for 24 or 48 hours. Spontaneous fibroblast contraction was macroscopically evaluated by scanning plates on a standard office flat-bed scanner. To quantify the area of contraction, generated images were analyzed with Fiji ImageJ. To further study the phenotype and function of this model’s lymphocytes and fibroblasts, after macroscopic evaluation, the collagen plugs were enzymatically digested with a mixture of collagenase D, Dispase and DNase in plain RPMI medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin for 1 hour at 37 °C on a roller. Reaction was stopped with the addition of complete RPMI medium containing 10% HPS and single-cell suspensions were then washed twice with PBS and used for flow cytometry analysis. To co-culture fibroblasts with CD7+ or CD7− T and NK cells (Supp. Figure 7) these cells were FACS sorted from PBMCs and seeded in the 3D collagen hydrogels.

RNA isolation and quantitative real-time PCR

RNA isolation was performed with the use of 500 µl of TRIzol (Sigma-Aldrich), according to the manufacturer’s guidelines. After isolation, RNA concentration was quantified with a Nanodrop photospectrometer (Thermo Scientific, Waltham, MA, USA) and any genomic DNA was removed using DNase I. Next, a maximum of 1 µg of RNA was reverse-transcribed into cDNA in a single step reverse transcriptase PCR at 37°C with the use of oligo dT primer and 200U M-MLV Reverse transcriptase (All Life Technologies) using a thermocycler. Gene expression in this cDNA was measured using 0.25 mM of validated primers (Biologio, Nijmegen, the Netherlands: see supp. table 3) and SYBR green master mix (Applied Biosystems, Waltham, MA, USA) in a quantitative real-time polymerase chain reaction (qPCR). The relative gene expression (-ΔCt) was calculated based on the average of the following reference genes: GAPDH and RPS27A.

Statistics

Data visualization of the results and comparisons for statistical significance between experimental groups were performed with R Studio (version 4.1.3) and the Prism software (Graphpad 9.0.0, San Diego, CA, USA). The exact statistical tests performed in every analysis/experiment are indicated in the figure legends.
## Supplementary materials

### Supplementary Table 1: List of antibodies used for cell surface staining

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### Supplementary Table 2: List of antibodies used for intracellular staining

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### Supplementary Table 3: List of primer sequences used

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References


10. Singh P, Zhai Y. Deciphering hematopoiesis at single cell level through the lens of reduced dimensions. Genomics [Preprint].


