CD7 activation regulates cytotoxicity-driven pathology in systemic sclerosis, yielding a target for selective cell depletion

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ABSTRACT

Objectives Cytotoxic T cells and natural killer (NK) cells are central effector cells in cancer and infections. Their effector response is regulated by activating and inhibitory receptors. The regulation of these cells in systemic autoimmune diseases such as systemic sclerosis (SSc) is less defined.

Methods We conducted ex vivo analysis of affected skin and blood samples from 4 SSc patient cohorts (a total of 165 SSc vs 80 healthy individuals) using single-cell transcriptomics, flow cytometry and multiplex immunofluorescence staining. We further analysed the effects of costimulatory modulation in functional assays, and in a severely affected SSc patient who was treated on compassionate use with a novel anti-CD3/CD7 immunotoxin treatment.

Results Here, we show that SSc-affected skin contains elevated numbers of proliferating T cells, cytotoxic T cells and NK cells. These cells selectively express the costimulatory molecule CD7 in association with cytotoxic, proinflammatory and profibrotic genes, especially in recent-onset and severe disease. We demonstrate that CD7 regulates the cytolytic activity of T cells and NK cells and that selective depletion of CD7+ cells prevents cytotoxic cell-induced fibroblast contraction and inhibits their profibrotic phenotype. Finally, anti-CD3/CD7 directed depletive treatment eliminated CD7+ skin cells and stabilised disease manifestations in a severely affected SSc patient.

Conclusion Together, the findings imply costimulatory molecules as key regulators of cytotoxicity-driven pathology in systemic autoimmune disease, yielding CD7 as a novel target for selective depletion of pathogenic cells.

INTRODUCTION

Systemic sclerosis (SSc) is a systemic autoimmune disease that is characterised by vascularopathy, inflammation and progressive fibrosis of the skin and internal organs. Autoimmunity in SSc is directed against nuclear autoantigens, which can be aberrantly presented by endothelial cells and fibroblasts due to hypoxic stress and serve as antigenic targets. This is exemplified by the development of a dysregulated Raynaud’s phenomenon as the first and principal disease manifestation. T lymphocytes have been detected in SSc-affected tissues and multiple studies have suggested their potential involvement in the observed fibrosis and vasculopathy through...
the production of cytokines such as interleukin (IL)-4, IL-13 and IL-17. Unexpectedly, a recent study showed a prominent role for cytotoxic T cells in mediating SSc skin pathology. Furthermore, an epigenetic study implicated natural killer (NK) and CD8$^+$ T cells in SSc pathogenesis. 

In chronic inflammatory conditions, T cell activation is restricted to prevent unwarranted inflammatory side effects. Activation of antigen-specific CD8$^+$ T cells is regulated by professional antigen-presenting cells via major histocompatibility complex (MHC) class II-controlled processes. Regulatory mechanisms are less defined for cytotoxic T cells and NK cells because these depend on non-MHC class II receptors and these are expressed ubiquitously in inflamed tissue. In chronic infections and malignancies, activation of cytotoxic T cells and NK cells is regulated by an interplay between costimulatory and inhibitory receptors. Animal models indicate that similar mechanisms may operate in cytotoxic autoimmunity. Still, the exact role of T cells in SSc pathogenesis is yet to be defined. On the one hand, genetic studies have proven that human leucocyte antigen genes (HLAs) corresponding to MHC class II confer susceptibility to SSc. On the other hand, treatment with the T cell directed drug cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) immunoglobulin (abatacept) has shown limited clinical efficacy.

Here, we hypothesise that costimulatory receptors, independent of CTLA-4, regulate cytotoxic cell-driven pathologic processes in SSc. Furthermore, we hypothesise that these processes can be alleviated by therapeutic targeting of such receptors. We conducted analyses of affected skin, lungs and blood at the single cell, protein and spatial level in four separate SSc patient cohorts. Furthermore, we analysed the effects of costimulatory modulation in ex vivo functional assays, and in a severely affected SSc patient who was treated on compassionate use with a novel combination of anti-CD3/CD7 immunotoxins (CD3/CD7-IT).

**Methods**

Detailed methods are provided in online supplemental methods.

**Results**

SSc skin contains increased numbers of activated cytotoxic T and NK cells with a cytolytic proinflammatory and profibrotic signature

T and NK cell subsets may upregulate costimulatory receptors to direct the autoimmune inflammatory process in SSc. To gain a comprehensive profiling of skin infiltrating lymphocytes, we analysed T and NK cell clusters (n=5061 cells) from a scRNAseq dataset of affected skin of 97 SSc patients compared with healthy skin from 56 individuals, as part of a larger dataset that was published recently (GSE195452) (online supplemental figure 1). To enhance confidence, we comparatively analysed another single-cell RNA transcriptome dataset containing 2126 cells from 9 healthy and 12 SSc skin biopsies (GSE138669) (online supplemental figure 2).

First, we analysed T cells and NK cells in skin single-cell datasets based on differential gene expression of known lineage-specific genes (GSE195452, GSE138669). Among the transcriptionally distinct cell subtypes that were detected (online supplemental figures 1A and 2A), the following three were significantly expanded (q<0.05 for all comparisons) in SSc compared with healthy skin in both datasets: proliferating T cells, CD8$^+$ cytotoxic T cells and NK cells (figure 1A,B). We verified the presence of these T and NK cell subsets at the protein level in SSc-affected skin in an additional cohort of 24 SSc patients (figure 1C). In addition, increased infiltration of cytotoxic CD8$^+$ T cells and CD56$^+$ NK cells was further apparent in biopsies from the affected compared with matched non-affected skin in 71% and 83% of SSc patients, respectively (n=24, p=0.06 and p<0.001, respectively) (figure 1D,E). In the affected SSc skin, cytotoxic T cells and NK cells were primarily present in perivascular areas while a smaller amount of these cells was infiltrated around blood vessels of the non-affected skin (figure 1D, online supplemental figure 4C).

Next, we analysed the potential function of these enriched cell populations in SSc skin. For this, we used gene set enrichment analyses based on each cluster’s differentially expressed genes with Wiki pathways as reference dataset. Both the skin cytotoxic T and NK cell clusters from each sc-dataset were not only associated with cell cytolytic pathways, but were also the only clusters from SSc skin that were specifically enriched for gene sets related to lung fibrosis, proinflammatory and profibrotic manifestations relative to healthy skin (figure 1F, online supplemental figure 2C). These pathways included profibrotic genes such as TGFB1, XCLI, OSM, CCL4, IL4, IL17, FGF and PDGF (for a complete overview see online supplemental figure 3). This indicates that cytotoxic cells are not only involved in cytotoxicity but also in directing profibrotic pathophysiological processes.

In recent studies in chronic inflammatory conditions, CD8$^+$ T cells were shown to mainly exert a cytokine-mediated function instead of their conventional cytotoxic effects with an important role of granzyme K. Therefore, we performed a focused analysis of CD8$^+$ T cells. In skin, at the sc-RNAseq level, the following CD8$^+$ subclusters were formed: naïve, proliferating, skin resident exhausted like, granzyme K (GZMK$^+$) and granzyme B (GZMB$^+$) positive effector cells. Of these, only the subset of CD8 effector GZMB$^+$ cells were significantly enriched in SSc skin (figure 1G, online supplemental figure 1D). Flow cytometry analysis in blood also showed increased (twofold) presence of CD8$^+$ GZMB$^+$ cells in SSc compared with healthy donors (figure 1H).

Expanded CD8$^+$ T and NK cells in the affected skin and lungs of SSc patients are characterised by upregulation of the CD7 costimulatory molecule

The activity of cytotoxic T cells and NK cells is closely regulated by an interplay between activating and inhibitory cell surface receptors. In chronic infection and malignancies, T and NK cytotoxic functions are restricted by inhibitory receptors. Therefore, we compared expression of known T and NK cell activating and inhibitory receptors between immune cell populations in SSc skin. For this, we used gene set enrichment analyses based on each cluster’s differentially expressed genes with Wiki pathways as reference dataset. Both the skin cytotoxic T and NK cell clusters from each sc-dataset were not only associated with cell cytolytic pathways, but were also the only clusters from SSc skin that were specifically enriched for gene sets related to lung fibrosis, proinflammatory and profibrotic manifestations relative to healthy skin (figure 1F, online supplemental figure 2C). These pathways included profibrotic genes such as TGFB1, XCLI, OSM, CCL4, IL4, IL17, FGF and PDGF (for a complete overview see online supplemental figure 3). This indicates that cytotoxic cells are not only involved in cytotoxicity but also in directing profibrotic pathophysiological processes.

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Figure 1  Increased frequency of profibrotic cytotoxic T cells and NK cells in SSc skin. (A) Frequencies of T cell and NK cell clusters in the skin of (n=56) healthy donors (HD) compared with (n=97) patients with SSc (GSE195452). (B) Frequencies of T cell and NK cell clusters in the skin of (n=9) HD compared with (n=12) patients with SSc (GSE138669). For both panels (A, B), values are represented as variation in cell counts (in %) and statistics were performed with Wilcoxon-Test, corrected for multiple comparisons. Only the adjusted p values (q) of the statistically significant comparisons are shown, *q<0.05, **q<0.01. (C) Representative multicolor immunofluorescence composite image of T helper CD3+CD8− (red), cytotoxic CD8+ (cyan), regulatory FOXP3+ (green) T cells and CD56+CD3− (yellow) NK cells in SSc-affected skin. (D) Immunofluorescence composite images of infiltrated cytotoxic CD8+ (cyan) T cells and CD56+CD3− (yellow) NK cells of the non-affected versus the affected skin from a representative SSc patient with early diffuse cutaneous disease. (E) Percentages (%) of cytotoxic T (CD3+CD8+) and NK cells (CD56+CD3−) in matched non-affected versus affected SSc skin (n=24 SSc patients). Values are represented as % of CD3+CD8+ or CD56+CD3− cells compared with all cells (DAPI+) present in each biopsy (excluding the rich in keratinocytes epidermis layer). Statistics were performed with non-parametric Wilcoxon test, ***p<0.001. (F) (left) Gene set enrichment analysis of skin T cell and NK cell clusters with Wiki pathways as reference dataset. Examples of top pathways (p<0.001) represented by NK and CTL clusters are shown. Statistics were performed with Kolmogorov-Smirnov (KS) test. (Right) Comparison of the enrichment scores of the "Overview of proinflammatory and profibrotic mediators" (q=0.025) and "Lung fibrosis" (q=0.0002) pathways in HD (green) vs SSc (red) skin T and NK cell clusters (GSE195452). (G) (Left) UMAP displaying 5 transcriptionally different CD8+ T cell clusters in skin of (n=56) HD and (n=97) SSc, n=977 cells. Based on the top differentially expressed genes, clusters were annotated as naive (T naive), Granzyme K+ (GZMK+), Granzyme B+ (GZMB+) exhausted (Texh) and proliferating (Tprolif). (right) Cell frequency of CD8+ T cell clusters between HD and SSc. (H) Percentage of Granzyme B (GZMB) expressing CD8+ T cells in peripheral blood of (n=15) HD and (n=30) SSc. Values are represented as percentage of total live peripheral blood mononuclear cells, *p<0.05. CTLs, cytotoxic T cells; BV, blood vessel; HF, hair follicle; NK, natural killer; Ssc, systemic sclerosis; Thpm, hypofunctional tissue resident T cells; Tncm, naïve/central memory; Tprolif, proliferating T cells; Tqcm, quiescent tissue-resident T cells; Tregs, regulatory T cells; Trm, tissue-resident memory T cells.
Figure 2  CD7 upregulation associates with activation of cytotoxic T and NK cells in SSc-affected skin and lungs. (A) Two-dimensional dot plots comparing the gene expression of selected activating and inhibitory costimulatory receptors in Tprolif, CD8+GZMB+ and NK clusters between HD and SSc (circle size shows the percentage of cells expressing each gene and colour intensity depicts average expression while numbers indicate average of normalised counts), *p<0.05 and **p<0.01. (B) (Top) UMAPs representing positive (red) and negative (grey) gene expression of CD7 among CD8+ T cells (left) and CD56+ NK cells (right). (Bottom) Intensity of CD7 normalised gene expression between HD and SSc among CD8+ T cells (left) and CD56+ NK cells (right). (C) Scatter plot with gene expression values highlighting genes that are specifically enriched in skin T and NK cells of patients with SSc compared with HD. (D) Representative photos of CD7 immunohistochemistry (IHC) staining of the affected and non-affected skin biopsies from an SSc patient, accompanied by quantification of CD7 IHC scores (n=20). Non-parametric sign test, *p<0.05. (E) Immunofluorescence microscopy showing coexpression of CD7 with CD8+ T and CD56+ NK cells in early dSSc skin. A representative experiment is depicted in scale of 100 µm. (F) (Left) UMAP displaying T and NK cells from control (healthy) (n=6) and SSc (n=7) lung tissues from patients with interstitial lung disease (GSE128169). (Middle) Density plots showing gene expression density of CD7, NCAM1 (CD56), CD4 and CD8A. (Right) CD7 gene expression counts (normalised) between control and SSc lung T and NK cells (each dot represents the average CD7 expression per donor). SSc, systemic sclerosis.
cell activation between healthy and SSc individuals, we used an alternative unbiased approach based on the FindConserved-Marker function implemented in Seurat (to find features that are conserved between the groups, ie, healthy donors and SSc). This approach confirmed enrichment of cytokotoxic genes and CD7 in cytotoxic T cells and NK cells of SSc patients compared with healthy controls. No other activating or inhibitory receptors were enriched in SSc in this analysis (figure 2C). These observations suggest that CD7 costimulation may be involved in SSc skin T and NK cell activation.

To validate these results at the protein level, we performed CD7 and CD3 immunohistochemistry in SSc skin tissue. The total amount of CD3+ T cells was higher even though statistically non-significant in the affected SSc skin (mean number of CD3+ T cells: 15.8 affected vs 6.1 in non-affected) (online supplemental figure 4A,B). Strikingly, an increased infiltration of CD7+ cells was specifically found in the perivascular areas (online supplemental figure 4C) of affected compared with the non-affected SSc skin (figure 2D). Furthermore, in SSc skin, CD7 was found to be coexpressed with CD8 and CD56 positive cells, while no expression on CD3+CD8− cells could be observed (figure 2E).

Recently, an increased presence of tissue-resident cytotoxic T and NK cells was also described in SSc lungs. Thus, we next evaluated CD7 expression in SSc lung tissue compared with healthy. In accordance with our data in skin, CD7 was selectively expressed in lung cytotoxic T cells and NK cells and its expression in SSc CD8+ T cells and CD56+ NK cells was significantly higher (twofold increase) compared to healthy counterparts (figure 2F). In conclusion, CD7 is a costimulatory receptor that is significantly upregulated in disease-related cytotoxic immune cell populations in both the affected skin and lungs of patients with SSc.

**CD7 costimulation is involved in T and NK cell cytotoxic and profibrotic processes**

CD7 is upregulated after TCR ligation and activated by its ligand, SECTM1. SECTM1 is a transmembrane protein produced by thymic epithelial cells and fibroblasts and induced by IFN-γ in professional antigen-presenting cells. CD7 activation by SECTM1 has been shown to augment CD4+ and CD8+ T cell effector functions. To gain insight on the function of CD7 in SSc, we analysed expression of SECTM1 in skin stromal and immune cells. In our dataset, SECTM1 as expected was primarily detected in skin myoid cells including monocytes, macrophages and dendritic cells. Furthermore, SECTM1 was also expressed by cells in the fibroblast cluster characterised by increased expression of Prostaglandin D Synthase (PGD) (figure 3A). Interestingly, it was previously reported that this fibroblast subtype is marked by high expression of MHC class I genes compared with other skin fibroblast subsets, suggesting that the SECTM1-CD7 axis may be important in T and NK cell activation (online supplemental figure 5A). Notably, T cell and NK cell CD7 expression was positively correlated with IFNG, while expression of its receptor (IFNGR1) positively correlated with SECTM1 in fibroblasts and antigen presenting cells (online supplemental figure 5B,C). This suggests an IFN-γ-driven SECTM1-CD7 axis in SSc skin.

From a clinical perspective, SSc is a heterogeneous disease with various disease subtypes and phases. Thus, we next analysed CD7 gene expression in subgrouping of SSc patients with limited (ISSc) versus diffuse (dSSc) cutaneous and early (≤3 years from first non-Raynaud symptom) versus later disease. We found that CD7 was significantly upregulated in early diffuse SSc compared with late disease (figure 3B) and CD7 expression was further associated with patients exhibiting increased skin score (p=0.03) (figure 3C). CD7 skin expression was not associated with the presence of interstitial lung disease. Furthermore, CD7 expression was similar between treatment naïve and patients who were receiving immunosuppressive medication, suggesting that currently used therapeutic approaches do not seem to directly target this activation axis (figure 3D).

To further explore the function of CD7+ T cells, we analysed the response to activation of cells purified from blood. In SSc blood, a larger fraction of CD8−CD7+ T cells were detected compared with healthy individuals (18% of total CD3+ cells in SSc vs 12% in HD) (figure 3E). The CD7+CD8+ T cells from SSc patients on short (t=4 hours) stimulation with phorbol myristate acetate and ionomycin produced significantly more granzyme B (MFI: 40 000 in SSc vs 34 000 in HD). In addition, SSc CD8−CD7+ T cells were also characterised by increased coexpression of the profibrotic cytokines IL-4 and IL-13 (among CD8+ T cells: 2.5% IL-13+ and 40% IL-4+) compared with CD8−CD7− cells of healthy controls (among CD8+ T cells: 1% IL-13+ and 30% IL-4+) (figure 3F). Taken together, these data indicate that CD8+ T and NK cells that exhibit cytotoxic and profibrotic properties in SSc, are characterised by increased CD7 expression.

To test the involvement of CD7 in T and NK cell cytotoxicity, we cocultured healthy peripheral blood mononuclear cells (PBMCs) (n=6) with K562 cancer cells and evaluated T and NK cell cytolytic activity by measuring the release of lactate dehydrogenase from the damaged target cells. Interestingly, while blockage of the CD7 receptor did not affect the cell viability of T and NK cells, it was accompanied by significant reduction in their cytolytic capacity towards K562 cells (figure 3G). This observation suggests that CD7 costimulation is important for an efficient cytotoxic response.

In addition, we observed above that SSc skin cytotoxic T and NK cells showed disease-related enrichment of pathways associated with lung fibrosis and proinflammatory/profibrotic manifestations. To explore the potential involvement of CD7 in the observed profibrotic manifestations of the cytotoxic skin cells, we obtained and merged the gene lists associated with those pathways and performed pairwise correlations with CD7 (online supplemental figure 3). Notably, CD7 gene expression in SSc-affected skin was positively correlated with expression of profibrotic mediators such as XCL1 and CCL5 in cytotoxic lymphocytes (CTLs) and TGFB1 and OSM in NK cells (figure 3H). From these observations, it is suggested that CD7 costimulation regulates both T and NK cell mediated cytotoxicity and fibrosis.

**In vitro elimination of the expanded and activated CD7+ T and NK cell subsets by targeted immunotoxin treatment halts fibroblast contraction**

The selective upregulation of CD7 expression in cytotoxic T and NK cells in SSc skin can serve as target for therapeutic modulation but also selective depletion of these cells. For this, we used a combination of anti-CD3/CD7 immunotoxins (CD3/CD7-IT) developed to target alloreactive activated T cells and NK cells in graft versus host disease (GvHD). In cultured PBMCs isolated from patients’ blood, a significant killing efficacy (>85% cells eliminated) of CD3/CD7-IT was only observed towards the activated T cells and NK cells (figure 4A). The combination of CD3 and CD7 immunotoxins had an additive effect on the killing...
Figure 3  CD7 costimulation plays an essential role in T and NK cell cytotoxic and profibrotic manifestations. (A) SECTM1 log normalised gene expression among skin immune and stromal cell subsets (GSE195452). Annotation of the depicted cell clusters were retrieved from Gur et al.10
(B) Subgroup analysis of CD7 normalised gene expression among healthy individuals (HD), and systemic sclerosis (SSc) patients with early versus late limited cutaneous SSc (iSSc) or diffuse cutaneous (dSSc) disease. Early disease was defined as ≤3 years from initial diagnosis. One-way ANOVA with Tukey’s multiple comparisons test, *p<0.05, ***p<0.001. (C) (Left) Scatter plot showing correlation of CD7 normalised gene expression with skin score. Each circle represents a single SSc patient. Spearman’s r=0.34, p=0.07. (Right) Normalised CD7 gene expression between SSc patients with low versus high skin scores. The distinction between low and high skin score was as described previously.10 (D) Normalised CD7 gene expression between SSc patients who were treatment naïve or treated with immunosuppressive medication and between SSc patients with or without the presence of interstitial lung disease (ILD). (E) Percentage of CD8⁺CD7⁺ and CD8⁺CD7⁻ T cells in peripheral blood of (n=15) HD and (n=30) SSc. Values are represented as percentage of total CD3⁺ T cells, *p<0.05. (F) Expression (mean fluorescence intensity, MFI) of Granzyme B (GZMB) and percentage of IL-4⁺/IL-13⁺ cells between HDs and SSc CD8⁺CD7⁺ T cells. Expression levels of GZMB are presented as MFI and values of IL-4⁺/IL-13⁺ cells are represented as percentage of positive cells among the CD8⁺CD7⁺ T cell compartment. Student’s t-test, *p<0.05. (G) Cytolytic activity of T and NK cells in a coculture with K562 target cells was quantified by measuring lactate dehydrogenase (LDH) release of the target cells. T cells and NK cells were stimulated with anti-CD3/CD28 and IL-2/IL-15, respectively, and anti-CD7 was added to block CD7 costimulation. Unstim refers to control cells that were not stimulated. Statistical comparisons between groups were performed with ordinary one-way ANOVA with Tukey’s multiple comparisons test *p<0.05, **p<0.01, ***p<0.001. (H) Pairwise correlation plots between CD7 and XCL1, TGB1, OSM, MMP9 gene expression within the NK or cytotoxic T cell (CTLs) clusters in SSc-affected skin (GSE195452). ANOVA, analysis of variance; SSc, systemic sclerosis.
Figure 4  Targeted immunotoxin mediated depletion of activated CD7+ T cells and CD7+ NK cells prevents fibroblast contraction and decreases myofibroblast phenotype (A) Flow cytometric quantification of CD3/CD7-IT induced cell death exhibiting absolute cell counts (cells/µl) of CD2+ T and CD56+ NK cells in PBMCs isolated from (n=5) SSc patients. (B) Percentage of normalised cell viability of CD3+ T and CD56+ NK cells isolated from SSc (n=3) PBMCs. (C) Flow cytometric quantification of CD3/CD7-IT-induced cell death illustrating absolute cell counts (cells/µl) of CD8+GZMB+ T and CD56+GZMB+ NK cells in PBMCs isolated from (n=5) SSc patients. (D) Flow cytometric quantification of CD3/CD7-IT-induced cell death towards CD8+ T and CD56+ NK cells in ex vivo skin explants (n=4). Paired t-test, *p<0.05, **p<0.01. (E) Schematic representation of our in vitro hydrogel collagen contraction assay in the developed three-dimensional (3D) model with cocultured primary skin fibroblasts and PBMCs. The level of contraction was quantified compared with no-cells control and plotted graphically on the right (n=3). Bars are mean±SD. An image of a representative experiment is depicted on the bottom of this panel. (F) The percentage of proapoptotic cytotoxic T (CD8+7-AAD−Annexin V+) and NK (CD56+7-AAD−Annexin V+) cells in the depicted conditions was measured with flow cytometry of the enzymatically digested collagen plugs (n=5). (G) IgG or CD3.CD7-IT treated PBMCs were cocultured with primary dermal fibroblasts in the developed 3D hydrogel collagen coculture model and fibroblasts were analysed for expression of genes reflective of a myofibroblast phenotype. Values represent relative gene expression (−ΔCt) as measured with qPCR. GAPDH and RPS27A were used as reference genes. Data represent mean ± SEM. Statistical comparisons between three or more groups were performed with ordinary one-way ANOVA with Tukey’s multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ANOVA, analysis of variance; IT, immunotoxin; PBMCs, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; SSc, systemic sclerosis.
efficacy towards T cells, while NK cells (CD3-CD56+CD7+) as expected were predominantly targeted by the CD7-IT (figure 4B). Of note, treatment with CD3/CD7-IT also effectively depleted the potentially pathogenic CD8+GZMB+ T cells and CD56+GZMB+ NK cells (figure 4C). IL-2 production was ninetofold decreased on treatment (online supplemental figure 6A), supporting that anti-CD3/CD7-IT treatment selectively depleted the activated T cells and NK cells. The surviving CD8+ T cells in the CD3/CD7-IT treated condition exhibited a clear alteration in their memory/maturaton status: decreased CD8 effector and increased memory and naïve phenotype, showing killing specificity towards effector cells (online supplemental figure 6B). Additionally, on post-treatment stimulation with PHA, the CD8+ T cells that survived treatment showed diminished cell proliferation (decreased 96% CD8-Ki-67+ cells) and production of cytotoxic (GZMB) and profibrotic molecules (IL-4) compared with their non-treated counterparts (online supplemental figure 6C). Importantly, treatment with anti-CD3/CD7-IT had no effect on the number nor the cell viability of CD19+ B cells and CD14+ M2 monocytes/macrophages (online supplemental figure 6D,E). Next, we used ex vivo whole skin cultures and showed that on treatment with anti-CD3/CD7-IT, both numbers of CD8+ T cells and CD56+ NK cells were significantly reduced compared with the untreated condition (figure 4D).

As we achieved specific elimination of the potentially pathogenic CD7+ T cells and NK cells, we next evaluated whether this depletion exhibits therapeutic relevance. Fibrosis accompanied by skin tightening is the main disease hallmark of SSc, so we developed a novel 3D collagen fibroblast: immune cell coculture hydrogel model that enables to study fibroblast contractility (figure 4E). In this model, spontaneous fibroblast contraction happened in the presence of allogeneic PBMCs and the level of contraction was significantly larger in the presence of PHA-activated PBMCs. PHA upregulates CD3 and CD7 expression on T cells and CD7 on NK cells (online supplemental figure 6F,G), so this model mimics the effector functions of the potentially pathogenic immune cell subsets on fibroblasts in vitro. Fibroblasts that were cocultured with sorted CD7+ T cells and NK cells exhibited increased contractility and a higher expression of IL-6, collagen type I and alpha-smooth muscle actin compared with fibroblasts cocultured with CD7- cells (online supplemental figure 7). Next, we pretreated PHA-activated PBMCs with 0.33 nM a-CD3/CD7 antibodies or CD3/CD7-IT and showed that only on immunotoxin treatment, fibroblast contraction was significantly reduced compared with PHA-activated PBMCs (figure 4E). Under these conditions (24 hours of coculture), the percentage of necrotic CD8+ or CD56+ cells was not (yet) significantly affected (online supplemental figure 6H). However, we observed a sharp increase in apoptotic CD8+ and CD56+ cells (figure 4F). Interestingly, fibroblasts that were cocultured with CD3/CD7-IT treated PBMCs exhibited a decreased gene expression of COL1A1, FN1 and ACTA2 (figure 4G), indicating a lowered profibrotic phenotype.

**Administration of bispecific CD3/CD7-IT treatment in the first patient with SSc effectively eliminates pathogenic CD7+ cells in blood and skin**

A 34-year-old male patient with severe diffuse cutaneous SSc showed disease progression following autologous hematopoietic stem cell transplantation (ASCT) that did not respond to treatment with mycophenolate mofetil, prednisone and rituximab. The patient had developed severely invalidating diffuse skin fibrosis (a modified Rodnan skin score of 27), joint contractures, high inflammation parameters with ESR 49 mm/hour (<15 mm/hour) and C reactive protein (CRP) 78 mg/L and joint contractions. He was bedridden with a very poor prognosis and was, therefore, treated with CD3/CD7-IT as last resort. Treatment resulted in a depletion of circulating and skin-resident T cells and NK cells, and a normalisation of CRP levels from 131 mg/L to 27 mg/L after 4 weeks, which CRP levels then further decreased to normal after 5 months. His functional status stabilised, with an observed increase in quality of life, yet with a persistent invalidation due to severe skin tightening and joint contractures that proved irreversible. The patient died 1.5 years after CD3/CD7-IT treatment from disease complications.

Biological responses to CD3/CD7-IT treatment were measured with flow cytometry in patient’s blood and multiplex immunofluorescent staining in skin predrug and postdrug administration. Consistent with the expected in vitro effect, treatment with CD3/CD7-IT directed a profound elimination of circulating T cells and NK cells. Already 1 week after administration, the amount of circulating T cells and NK cells was reduced by 86% and 77%, respectively (figure 5A). CD8+ T cells were preferentially targeted by CD3/CD7-IT compared with CD4+ T cells. More specifically, the percentage of CD8+ T cells exhibited a 37-fold decrease while CD4+ T cells showed an 8-fold reduction (figure 5B). We further explored the killing efficacy of CD3/CD7-IT towards effector cytotoxic T cell and NK cell populations. Effector cytotoxic T cells were characterised as CD8+ Perforin+ and effector NK cells as CD56+Perforin+. Interestingly, both CD8+ Perforin+ and CD56+ Perforin+ cell populations were completely depleted (100%) in patient’s blood (figure 5C). The therapeutic effectiveness towards skin-resident T and NK cells was then evaluated with multiplex immunofluorescence staining of skin biopsies before and after treatment. Post-treatment, skin biopsies showed a remarkable reduction in immune cell infiltration (figure 5D). More specifically, absolute cell counts of CD3+ T cells, CD8+ T cells and CD56+ NK cells were all considerably reduced post-treatment. Importantly, the numbers of CD3+FOXP3+ regulatory T cells and CD20+ B cells were not affected (figure 5E). While the treatment outcome was considered positive and clinically meaningful, we expect even greater benefit when CD3/CD7-IT is applied earlier in the course of the disease, when the inflammatory component is more prominent and the fibrotic process not yet irreversible.

**DISCUSSION**

Here, we show that SSc-affected skin contains increased numbers of proliferating T cells, cytotoxic T cells and NK cells. These cells exhibit a cytotoxic, proinflammatory and profibrotic gene signature. When focusing on their costimulatory and inhibitory molecule expression, these cells express the costimulatory molecule CD7 in association with proinflammatory and profibrotic genes, especially in recent-onset and severe disease. Furthermore, we show that CD7 regulates cytolytic activity of cytotoxic T cells and NK cells and that selective depletion of CD7+ cells prevents cytotoxic cell-induced fibroblast contraction by halting their profibrotic phenotype. Finally, CD3/CD7 directed depletive treatment depleted CD7+ cells and stabilised disease manifestations in a severely affected SSc patient.

The role of T cells in mediating the pathology of SSc has been a subject of controversy. The importance of the immune system, however, is highlighted by recent observations indicating that in treatment with ASCT, long-term remission of SSc disease manifestations can be achieved.47 CD4+ T cells have been considered as main effector cells since genetic studies indicated that some
MHC class II polymorphisms confer a risk of acquiring SSc. Recently, however, MHC class II polymorphisms were shown to confer not so much risk on SSc incidence as on the development of disease-related autoantibodies that precede the development of clinical disease in a proportion of cases. Because of its fibrotic clinical manifestations, SSc has been considered a T helper type 2 (Th2)-mediated disease. However, epigenetic studies revealed gene transcription in cytotoxic T cells and NK cells in SSc patients with disease risk loci. Also, SSc skin was found to be predominantly infiltrated by cytotoxic T cells, in proximity to preapoptotic endothelial cells. Another recent study associated increased infiltration of IFN-γ-producing effector T cells and NK cells in SSc skin to fibrotic activation of fibroblast subsets. Our study confirms these data, and our functional analyses suggest that SSc skin disease is driven by T cells and NK cells that produce cytotoxic proteins such as granzyme B and perforin, induce fibroblast contractility and myofibroblastic phenotype, and produce well described profibrotic mediators such as TGFβ1, XCL1, CCL3 and OSM. This suggests that increased cytotoxicity in SSc skin may be associated with induction of the fibrotic pathology of the disease.

Our study addresses the question how the cytotoxic immune response in SSc is regulated. Cytotoxic T cells and NK cells are central effector cells in cancer and infections. Their effector response is tightly regulated by the expression of activating and inhibitory surface receptors. Here, we find that cytotoxic cells in SSc consistently express high levels of CD7. Of interest, IFN-γ, a key cytokine in cytotoxic immune responses, is the main inducer of SECTM1, the ligand of CD7. This suggests that SECTM1-CD7 interaction is part of an IFN-γ-driven feedback loop that enhances cytotoxic responses in SSc skin.

The other side of the coin is that in chronic viral infection and cancer cytotoxic cells develop reduced and altered effector functions due to a process termed exhaustion. Exhaustion involves increased expression of inhibitory receptors such as PD-1, LAG-3, TIM-3 and CTLA-4. The extent of exhaustion varies from dysfunction to anergy or clonal deletion and is determined by factors such as antigen abundance and TCR affinity. The mechanisms of autoimmunity are less certain. In a model of autoimmunity activation of autoreactive CD8+ cytotoxic T cells was restrained by LAG-3. T cell exhaustion in patients with systemic autoimmune disease has mainly been investigated and described in peripheral blood samples and not in tissues where autoantigen presentation occurs. We found that in SSc skin compared with healthy skin a subset of cytotoxic T cells expressed LAG3, suggesting a restrained phenotype. Only a few cytotoxic T cells expressed PD-1 in conjunction with FOXP3, suggesting they are regulatory T cells. Taken together,
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CTLs in SSc skin are characterised by an activating rather than an exhausted profile.

This study reconfirms the importance of autoimmune in driving SSc pathology. This is clinically relevant since ASCT can cure the disease, but is a high-risk procedure and only applicable to a very restricted group (<10%) of SSc patients.25 Other currently used broad immunosuppressive treatments do not cure the disease and can only slow down fibrosis to a limited extent. Selective targeting of activated lymphocytes may represent a more selective and safer treatment for SSc. Thus, we used a novel combination of anti-CD3/CD7-IT that has been developed to deplete activated alloreactive T cells and NK cells for the treatment of GvHD.16 We gave proof of concept that treatment with a-CD3/CD7-IT, can selectively deplete the activated cytotoxic T cells and NK cells in blood and SSc-affected skin. Because of its depletive nature, anti-CD3/CD7-IT is administered as a single treatment and that furthers support its favourable safety profile. In line with this notion, CD7 targeting therapeutic approaches have shown clinical efficacy and safety in kidney transplantation patients.4 17 Previously, we showed that anti-CD3/CD7 immuno­toxin treatment was well tolerated and increased survival rates in patients with acute GvHD. Similarly to ASCT, a significant increase in the diversity of T cell repertoires that entailed new polyclonal T cell populations was observed, suggesting the efficacy of our treatment approach in rebalancing the immune composition.16

Our study comes along with some limitations. First, the analysed scRNA seq datasets lack T cell receptor (TCR) sequencing and this hampers the investigation of (auto) antigen-specific T cell responses. In future studies, it is of importance to examine whether the cytotoxic T cells are clonally expanded and autoreactive or bystander-activated cells. Second, our results suggest that prevention of fibro­blast contraction is mediated by CD7+ CTLs. However, additional research is needed to investigate if autoantibodies and other immune cell subsets such as macrophages contribute to this process. Finally, the safety and clinical efficacy of the CD3/CD7-IT for treatment of SSc needs to be investigated in a well-designed and prospective study. Given the large SSc heterogeneity, and since CD7 upregulation was profound in patients with early diffuse disease, our results suggest that this SSc subpopulation is expected to benefit from such a therapeutic approach in particular.

In conclusion, we found that CD7 activation regulates cellular cytotoxicity-driven pathological processes in SSc. Together the findings imply costimulatory molecules as key regulators of cytotoxicity-driven pathology in systemic autoimmune disease, yielding a flag for selective depletion of pathogenic cells.

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REFERENCES
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 revi ewal 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 we were 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 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-μ-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-μ-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...
was stimulated by adding 20 ng/ml rhM-CSF (R&D Systems, cat# 216-MC) and 10 ng/ml rhIL-4 (Biolegend, cat# S00815). 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 ø 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 xylol 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% H202 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 (Bright vision Poly-HRP, Immunologic DPVOSSHRP) 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-Fischer, 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# 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

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
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 (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 (DEGs) within each cluster, which were subsequently annotated based on the characteristics of these DEGs. An R package heatmap (Kolde, R. (2019). heatmap: Pretty Heatmaps (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 (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 (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 (NES) between control and SSc group was tested using Kolmogorov-Smirnov (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 (R package version 4.8.0)). An R package ComplexHeatmap (Gu, Z. (2016). ComplexHeatmap: Making Complex Heatmaps in R (R package version 2.10.0)) was used to visualize the correlations.

**Flow cytometry analysis**

Per donor, 1 x 106 PBMCs were first labeled with ViaKrome 808 fixable viability dye (1.5:1000 in PBS) for 30 min at 4 °C to exclude dead cells and then stained for 20 minutes at RT with fluorescently labeled extracellular antibodies (supplemental table 1). For intracellular stainings (supplemental table 2), cells were fixed with permeabilized using the Cyto-Fast™ Fix/Perm Buffer Set (Biolegend) according to manufacturer’s guidelines. To facilitate detection of intracellular cytokines, cells were pre-stimulated with 12.5 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma), 500ng/ml ionomycin (Merck) and 5 µg/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 inequal 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 (Galios) 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# M500KCAF0Y) 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 (Biolegio, 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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<th>Antigen</th>
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<th>Dilution</th>
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<th>Supplier</th>
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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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<td>TGCGAGTCTTCC-TACTGCTA</td>
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References


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


Supplementary Figure 1. Annotation of skin T and NK cell clusters from single cell RNA sequencing dataset GSE195452
Unsupervised Uniform Manifold Approximation and Projection (UMAP) to visualize clustering of 5,061 cells detects 8 transcriptionally distinct cell clusters: tissue-resident memory T cells (Trm), cytotoxic T cells (CTLs), regulatory T cells (Tregs), hypofunctional tissue resident T cells (Thprm), naïve/central memory (Tncm), proliferating T cells (Tprolif) and NK cells (NK) and a cluster containing a mixture of MAIT, INKT CD8⁺ T and γδT cells (T mix). B UMAP showing the cells belonging to healthy individuals (control) or patients (SSc) C Heatmap illustrating the top 5 differentially expressed genes in each distinguished cell cluster: Trm (CD69, ZFP36L2, CXCR4, IL7R), CTLs (GZMK, IFNG, CCL5, CCL4, CD8A), Tregs (CD4, FOXP3, CTLA4, IL2RA), Thprm (NR4A1, CD69, CXCR4, DUSP1), Tncm (TCF7, SELL, IL7R), Tprolif (MKI67), NK (NKG7, FCGR3A, FGFBP2, KLRD1, GZMB, PRF1) and Tmix (CD8A, CCL5, TRGC2, NKG7, GZMB, PRF1, FCGR3A, FGFBP2, KLRD1). D Heatmap demonstrating the top 10 upregulated genes in each of the 5 different clusters of isolated CD8⁺ T cells; naïve (Tn): IL7R, Granzyme K⁺ (GZK⁺): GZMK, IFNG, CCL4, Granzyme B⁻ (GZB⁻): PRF1, GNLY, NKG7, GZMB, GZMZ, GZMH, exhausted (Tex): NR4A2, NR4A3, and proliferating (Tprolif): LASP1, TMPO, ANP32B. E UMAP representing positive (red) and negative (grey) gene expression of PDCD1 (PD-1) among CD8⁺ T cell clusters.

Supplementary Figure 2. Single cell RNA sequencing analysis of dataset GSE138669

A Unsupervised Uniform Manifold Approximation and Projection (UMAP) clustering of 2,126 cells determines 7 transcriptionally distinct cell clusters: quiescent tissue resident T cells (Tqcm), cytotoxic T cells (CTLs), regulatory T cells (Tregs), hypofunctional tissue resident T cells (Thprm), naïve/central memory (Tncm), proliferating T cells (Tprolif) and NK cells (NK). UMAP displaying the cells belonging to healthy individuals (control) or patients (SSc) is depicted on the top of this panel. B Heatmap of the top 10 differentially expressed genes in each distinguished cell cluster: Tqcm (CD69,
IL7R, TCF7, SEL1, ANXA1), CTLs (CD8A, GZMK, GZMA), Tregs (CD4, CD27, CTLA4, IL2RA), Thprm (NR4A1, DUSP1), Tncm (TCF7, IL7R), Tprolif (MKI67), NK (NKG7, FCGR3A, KLRD1, PRF1). C (left) Gene set enrichment analysis with Wiki pathways as reference dataset. Examples of top pathways (p <1e-10) represented by NK and CTL clusters are shown. Statistics were performed with Kolmogorov-Smirnov (KS) test. (right) Comparison of the enrichment scores of the overview of proinflammatory and profibrotic (q=0.68) and lung fibrosis (q=6e-15) pathways in HD versus SSc skin T and NK cell clusters (here for GSE138669, Fig 1 for GSE195452).

Supplementary Figure 3. Pairwise correlations of CD7 with pro-fibrotic genes

The Wiki gene pathways lung fibrosis and pro-inflammatory and pro-fibrotic manifestations were merged and potential correlation of CD7 gene expression with the included genes was evaluated separately for the cluster of A cytotoxic T cells (CTLs) and B NK cells. Statistical significance for every
comparison was corrected for multiple comparisons and is presented as adjusted p value, *p<0.05, **p<0.01.

Supplementary figure 4. CD3 and CD7 immunohistochemistry in SSc affected versus non-affected skin of patients with systemic sclerosis. CD7 gene expression in skin immune and stromal cells is also depicted.

A Representative images of CD3 immunohistochemistry (IHC) staining of the affected and non-affected skin biopsies from one SSc patient. Scale is 100 µm. B Quantification of CD3+ T cells in the...
affected vs non lesion SSc skin (n=20). Bars are mean ±SD. Non-parametric Wilcoxon test, p=0.19. C In SSc affected skin large infiltration of CD7+ cells is found in perivascular areas while in matched non-affected skin, a smaller number of CD7+ cells is present around blood vessels. Scale is 100 µm. Here, representative images of one SSc patient with early diffuse disease are depicted. D 2-D dot plot comparing CD7 gene expression in skin immune and stromal cells. Cell cluster annotations were retrieved from metadata information as have been described in the single-cell RNA sequencing dataset GSE195452. Circle size shows the percentage of cells expressing CD7 and color intensity depicts average expression. Numbers indicate average of normalized counts.

Supplementary Figure 5. SECTM1-CD7 axis in activation of cytotoxic T and NK cells in SSc affected skin.

A Schematic model for the proposed involvement of SECTM1-CD7 axis in cytotoxic T cell and NK cell activation (Created with BioRender.com). In SSc affected skin, CD7 and IFNG is predominantly expressed in cytotoxic T and NK cells while its receptor IFNGR and SECTM1 in antigen presenting cells (APCs) and stromal cells (mainly fibroblasts). This suggests a cytokine-mediated positive feedback loop in the communication between CD7+ cytotoxic immune cells and SECTM1 producing APCs and fibroblasts, with IFN-γ being a key cytokine. B 2-D dot plots comparing expression levels of selected genes in skin immune (myeloid and lymphoid) and stromal cell populations. Cell cluster annotations were retrieved from metadata information as have been described in the single-cell RNA sequencing dataset GSE195452. Circle size shows the percentage of cells expressing each gene and color intensity depicts average expression. Numbers indicate average of normalized counts. C Pair-wise correlation plots in SSc affected skin (GSE195452) show a positive correlation between CD7/IFNG and SECTM1/IFNGR1. Abbreviations; TCR: T cell receptor, NKR: NK cell receptor, MHC: major histocompatibility complex, APC: antigen-presenting cell.
Supplementary Figure 6. CD3/CD7-IT specifically eliminates only the activated cytotoxic T and NK cells in vitro.
**A** Concentration of IL-2 (pg/ml) was measured in cell supernatant of cells with or without treatment with α-CD3/CD7-IT. **B** Pie charts illustrating the proportion of effector (CD8+CD45RA−CD27+), memory (CD8+CD45RA−CD27+) and naïve (CD8+CD45RA−CD27+) cells among the CD8+ T cell population in the depicted stimulation and treatment culture conditions (percentages in the pie charts are mean values of n=6 SSc patients). **C** Response to TCR mediated (PHA) restimulation of cells treated with α-CD3/CD7-IT was evaluated by intracellular flow cytometry. Values are represented as fold change of the re-stimulated compared to the values before stimulation. **D** (Bottom) Comparison of absolute counts (cells/µl) of CD19+ B cells after in-vitro treatment with CD3/CD7-IT compared to non-treated peripheral blood mononuclear cells (n=6). (top) Representative flow cytometry plots of one experiment. **E** Normalized cell viability, of M2 macrophages and CD19+ B cells that were isolated from SSc patients’ blood for the depicted different culture/treatment conditions. Cycloheximide was used as a positive control. **F** Flow cytometric histograms of one representative experiment exhibiting elevated expression of CD3 and CD7 in CD8−GZMB+ cells and CD7 in CD56−GZMB+ NK cells upon stimulation with phytohemagglutinin (PHA) that is further quantified in **G**. CD3 and CD7 expression is presented as mean fluorescence intensity (MFI). Statistics were performed with Student’s t-test, **p<0.01, ****p<0.0001. **H** The percentage of necrotic cytotoxic T (CD8+7-AAD−Annexin V+) and NK (CD56+7-AAD−Annexin V+) cells in the depicted conditions was measured with flow cytometry of the enzymatically digested collagen plugs (n=5).
Supplementary Figure 7. Fibroblasts co-cultured with CD7+ T cells and NK cells exhibit increased contractility that is accompanied by an elevated myofibroblast-like phenotype.

A) Collagen contraction assay
B) Flow cytometry analysis of cell types
C) Graph showing percentage of cells compared to control
D) Graph showing absolute number of positive cells
E) Graph showing percentage of α-SMA+ fibroblasts
F) Graph showing mean fluorescence intensity (MFI) of α-SMA
G) Graph showing relative gene expression
A Schematic representation of the experimental design in the developed 3D in-vitro collagen contraction fibroblast : immune cell co-culture model. B Flow cytometry gating strategy that was used to sort CD7⁺ versus CD7⁻ T cell and NK cell populations from healthy peripheral blood (n=3). First, we gated on the lymphoid cell population based on cell size (FSC) and granularity (SSC). Following, we excluded dead cells based on 7-AAD⁺ staining and we then sorted CD19⁺CD14⁻ lymphoid cells that were either positive or negative for CD7 expression. C The level of fibroblast contraction was quantified compared to no-cells control and plotted graphically (n=3). Bars are mean ±SD. An image of a representative experiment is depicted on the right part of this panel. D (left) Representative images of Collagen type 1 immunohistochemistry of the collagen plugs that contained only fibroblasts or fibroblasts co-cultured with CD7⁺ T cells and NK cells. (right) Quantification of Collagen type 1 positive fibroblasts in control versus fibroblasts that were co-cultured with either CD7⁻ or CD7⁺ cells (n=3). Bars are mean ±SD. E The percentage of CD45⁻ a-SMA⁻IL-6⁺ fibroblasts in the depicted conditions was measured with flow cytometry of the enzymatically digested collagen plugs (n=3). F Expression levels (mean fluorescence intensity-MFI) of a-SMA in CD45⁻a-SMA⁺ fibroblasts in the depicted conditions was measured with flow cytometry of the enzymatically digested collagen plugs (n=3). (right) Flow cytometry histograms of one representative experiment exhibiting increased expression of α-SMA in fibroblasts co-cultured with CD7⁺ compared to CD7⁻ T cells and NK cells is shown. G CD7⁺ versus CD7⁻ T and NK cells (n=3) were co-cultured with primary dermal fibroblasts in the developed 3D hydrogel collagen co-culture model and fibroblasts were analyzed for expression of genes reflective of a myofibroblast phenotype such as COL1A1 and ACTA2. Values represent relative gene expression (ΔCt) as measured with qPCR. GAPDH and RPS27A were used as reference genes. Data represents mean ± SEM.
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 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-μ 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 XVIVOTM 15 medium (Lonza, cat# 04-4180) at a density of 50,000 cells/well in 96-well μ 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 XVIVOTM 15 medium that was supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin and 2% human pooled serum. Differentiation towards M2-like macrophages...
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 ø 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 xylol 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-Fischer, 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 I (Goat Anti-Type I Collagen-UNLB, Southern Biotech, cat# 4777, clone 236A/E7, 1:100) with Opal520, anti-FOXP3 (eBioscience Affymetrix, 14–01). Slides were then scanned 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

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
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 (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 (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 (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 (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 (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 (NES) between control and SSC group was tested using Kolmogorov-Smirnov (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 (R package version 4.8.0)). An R package ComplexHeatmap (Gu, Z. (2016). ComplexHeatmap: Making Complex Heatmaps in R (R package version 2.10.0)) was used to visualize the correlations.

Flow cytometry analysis

Per donor, 1 x 10^6 PBMCs were first labeled with ViaKrome 808 fixable viability dye (1:5:1000 in PBS) for 30 min at 4 °C to exclude dead cells and then stained for 20 minutes at RT with fluorescently labeled extracellular antibodies (supplemental table 1). For intracellular stainings (supplemental table 2), cells were fixed with permeabilized using the Cyto-Fast™ Fix/Perm Buffer Set (Biolegend) according to manufacturer’s guidelines. To facilitate detection of intracellular cytokines, cells were pre-stimulated with 12.5 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma), 500ng/ml Ionomycin (Merck) and 5 μg/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 ug/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 (Gallios) 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# M500KCAF0Y) 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 Table 1: List of antibodies used for cell surface staining

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<th>Supplier</th>
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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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</table>
References


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


Supplementary Figure 1. Annotation of skin T and NK cell clusters from single cell RNA sequencing dataset GSE195452
Unsupervised Uniform Manifold Approximation and Projection (UMAP) to visualize clustering of 5,061 cells detects 8 transcriptionally distinct cell clusters: tissue-resident memory T cells (Trm), cytotoxic T cells (CTLs), regulatory T cells (Tregs), hypofunctional tissue resident T cells (Thprm), naïve/central memory (Tncm), proliferating T cells (Tprolif) and NK cells (NK) and a cluster containing a mixture of MAIT, INKT CD8+ T and γδ T cells (T mix).

B UMAP showing the cells belonging to healthy individuals (control) or patients (SSc)

C Heatmap illustrating the top 5 differentially expressed genes in each distinguished cell cluster: Trm (CD69, ZFP36L2, CXCR4, IL7R), CTLs (GZMK, IFNG, CCL5, CCL4, CD8A), Tregs (CD4, FOXP3, CTLA4, IL2RA), Thprm (NR4A1, CD69, CXCR4, DUSP1), Tncm (TCF7, SELL, IL7R), Tprolif (MKI67), NK (NKG7, FCGR3A, FGFBP2, KLRL1, GZMB, PRF1) and Tmix (CD8A, CCL5, TRGC2, NKG7, GZMB, PRF1, FCGR3A, FGFBP2, KLRL1).

D Heatmap demonstrating the top 10 upregulated genes in each of the 5 different clusters of isolated CD8+ T cells; naïve (Tn): IL7R, Granzyme K+ (GZK+): GZMK, IFNG, CCL4, Granzyme B+ (GZB+): PRF1, GNLY, NKG7, GZMB, GZMZ, GZMH, exhausted (Tex): NR4A2, NR4A3, and proliferating (Tprolif): LASP1, TMPO, ANP32B.

E UMAP representing positive (red) and negative (grey) gene expression of PDCD1 (PD-1) among CD8+ T cell clusters.

Supplementary Figure 2. Single cell RNA sequencing analysis of dataset GSE138669

A Unsupervised Uniform Manifold Approximation and Projection (UMAP) clustering of 2,126 cells determines 7 transcriptionally distinct cell clusters: quiescent tissue resident T cells (Tqcm), cytotoxic T cells (CTLs), regulatory T cells (Tregs), hypofunctional tissue resident T cells (Thprm), naïve/central memory (Tncm), proliferating T cells (Tprolif) and NK cells (NK). UMAP displaying the cells belonging to healthy individuals (control) or patients (SSc) is depicted on the top of this panel.

B Heatmap of the top 10 differentially expressed genes in each distinguished cell cluster: Tqcm (CD69,
IL7R, TCF7, SELL, ANXA1), CTLs (CD8A, GZMK, GZMA, ), Tregs (CD4, CD27, CTLA4, IL2RA), Thprm (NR4A1, DUSP1), Tncm (TCF7, IL7R), Tprolif (MKI67), NK (NGK7, FCGR3A, KLRD1, PRF1).

C (left) Gene set enrichment analysis with Wiki pathways as reference dataset. Examples of top pathways (p <1e-10) represented by NK and CTL clusters are shown. Statistics were performed with Kolmogorov-Smirnov (KS) test.(right) Comparison of the enrichment scores of the overview of proinflammatory and profibrotic (q=0.68) and lung fibrosis (q=6e-15) pathways in HD versus SSc skin T and NK cell clusters (here for GSE138669, Fig 1 for GSE195452).

Supplementary Figure 3. Pairwise correlations of CD7 with pro-fibrotic genes

The Wiki gene pathways lung fibrosis and pro-inflammatory and pro-fibrotic manifestations were merged and potential correlation of CD7 gene expression with the included genes was evaluated separately for the cluster of A cytotoxic T cells (CTLs) and B NK cells. Statistical significance for every
comparison was corrected for multiple comparisons and is presented as adjusted p value, *p<0.05, **p<0.01.

Supplementary figure 4. CD3 and CD7 immunohistochemistry in SSc affected versus non-affected skin of patients with systemic sclerosis. CD7 gene expression in skin immune and stromal cells is also depicted.

A Representative images of CD3 immunohistochemistry (IHC) staining of the affected and non-affected skin biopsies from one SSc patient. Scale is 100 µm. B Quantification of CD3+ T cells in the
affected vs non lesion SSc skin (n=20). Bars are mean ±SD. Non-parametric Wilcoxon test, p=0.19. In SSc affected skin large infiltration of CD7+ cells is found in perivascular areas while in matched non-affected skin, a smaller number of CD7+ cells is present around blood vessels. Scale is 100 µm. Here, representative images of one SSc patient with early diffuse disease are depicted. D 2-D dot plot comparing CD7 gene expression in skin immune and stromal cells. Cell cluster annotations were retrieved from metadata information as have been described in the single-cell RNA sequencing dataset GSE195452. Circle size shows the percentage of cells expressing CD7 and color intensity depicts average expression. Numbers indicate average of normalized counts.

Supplementary Figure 5. SECTM1-CD7 axis in activation of cytotoxic T and NK cells in SSc affected skin.

A Schematic model for the proposed involvement of SECTM1-CD7 axis in cytotoxic T cell and NK cell activation (Created with BioRender.com). In SSc affected skin, CD7 and IFNG is predominantly expressed in cytotoxic T and NK cells while its receptor IFNGR and SECTM1 in antigen presenting cells (APCs) and stromal cells (mainly fibroblasts). This suggests a cytokine-mediated positive feedback loop in the communication between CD7+ cytotoxic immune cells and SECTM1 producing APCs and fibroblasts, with IFN-γ being a key cytokine. B 2-D dot plots comparing expression levels of selected genes in skin immune (myeloid and lymphoid) and stromal cell populations. Cell cluster annotations were retrieved from metadata information as have been described in the single-cell RNA sequencing dataset GSE195452. Circle size shows the percentage of cells expressing each gene and color intensity depicts average expression. Numbers indicate average of normalized counts. C Pairwise correlation plots in SSc affected skin (GSE195452) show a positive correlation between CD7/IFNG and SECTM1/IFNGR1. Abbreviations; TCR: T cell receptor, NKR: NK cell receptor, MHC: major histocompatibility complex, APC: antigen-presenting cell.
Supplementary Figure 6. CD3/CD7-IT specifically eliminates only the activated cytotoxic T and NK cells in vitro.
A Concentration of IL-2 (pg/ml) was measured in cell supernatant of cells with or without treatment with α-CD3/CD7-IT. B Pie charts illustrating the proportion of effector (CD8+CD45RA−CD27+), memory (CD8+CD45RA−CD27−) and naïve (CD8+CD45RA−CD27−) cells among the CD8+ T cell population in the depicted stimulation and treatment culture conditions (percentages in the pie charts are mean values of n=6 SSc patients). C Response to TCR mediated (PHA) restimulation of cells treated with α-CD3/CD7-IT was evaluated by intracellular flow cytometry. Values are represented as fold change of the re-stimulated compared to the values before stimulation. D (Bottom) Comparison of absolute counts (cells/µl) of CD19+ B cells after in-vitro treatment with CD3/CD7-IT compared to non-treated peripheral blood mononuclear cells (n=6). (top) Representative flow cytometry plots of one experiment. E Normalized cell viability, of M2 macrophages and CD19+ B cells that were isolated from SSc patients’ blood for the depicted different culture/treatment conditions. Cycloheximide was used as a positive control. F Flow cytometric histograms of one representative experiment exhibiting elevated expression of CD3 and CD7 in CD8+GZMB+ cells and CD7 in CD56+GZMB+ NK cells upon stimulation with phytohemagglutinin (PHA) that is further quantified in G. CD3 and CD7 expression is presented as mean fluorescence intensity (MFI). Statistics were performed with Student's t-test, **p<0.01, ****p<0.0001. H The percentage of necrotic cytotoxic T (CD8+7-AAD−Annexin V+) and NK (CD56+7-AAD−Annexin V+) cells in the depicted conditions was measured with flow cytometry of the enzymatically digested collagen plugs (n=5).
Supplementary Figure 7. Fibroblasts co-cultured with CD7+ T cells and NK cells exhibit increased contractility that is accompanied by an elevated myofibroblast-like phenotype.

A) Collagen contraction assay
B) Flow cytometry analysis of CD7+ and CD7- cells
C) Graph showing % of no control cells
D) Graph showing absolute number positive cells
E) Graph showing % of CD45+α-SMA+ fibroblasts
F) Graph showing MFI (α-SMA)
G) Graph showing relative gene expression (ΔCt) for COL1A1 and ACTA2
A Schematic representation of the experimental design in the developed 3D in-vitro collagen contraction fibroblast : immune cell co-culture model. B Flow cytometry gating strategy that was used to sort CD7+ versus CD7- T cell and NK cell populations from healthy peripheral blood (n=3). First, we gated on the lymphoid cell population based on cell size (FSC) and granularity (SSC). Following, we excluded dead cells based on 7-AAD+ staining and we then sorted CD19+CD14- lymphoid cells that were either positive or negative for CD7 expression. C The level of fibroblast contraction was quantified compared to no-cells control and plotted graphically (n=3). Bars are mean ±SD. An image of a representative experiment is depicted on the right part of this panel. D (left) Representative images of Collagen type 1 immunohistochemistry of the collagen plugs that contained only fibroblasts or fibroblasts co-cultured with CD7+ T cells and NK cells. (right) Quantification of Collagen type 1 positive fibroblasts in control versus fibroblasts that were co-cultured with either CD7- or CD7+ cells (n=3). Bars are mean ±SD. E The percentage of CD45−α-SMA−IL-6+ fibroblasts in the depicted conditions was measured with flow cytometry of the enzymatically digested collagen plugs (n=3). F Expression levels (mean fluorescence intensity-MFI) of α-SMA in CD45-α-SMA+ fibroblasts in the depicted conditions was measured with flow cytometry of the enzymatically digested collagen plugs (n=3). (right) Flow cytometry histograms of one representative experiment exhibiting increased expression of α-SMA in fibroblasts co-cultured with CD7+ compared to CD7- T cells and NK cells is shown. G CD7+ versus CD7- T and NK cells (n=3) were co-cultured with primary dermal fibroblasts in the developed 3D hydrogel collagen co-culture model and fibroblasts were analyzed for expression of genes reflective of a myofibroblast phenotype such as COL1A1 and ACTA2. Values represent relative gene expression (−ΔCt) as measured with qPCR. GAPDH and RPS27A were used as reference genes. Data represents mean ± SEM.