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 vasculopathy, 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.5

In chronic inflammatory conditions, T cell activation is restricted to prevent unwarranted inflammatory side effects. Activation of antigen-specific CD4+ 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.8 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 leukocyte antigen genes (HLAs) corresponding to MHC class II confer susceptibility to SSc.9 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.9

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 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 TGFβ1, XCLI1, OSM, CCLA4, IL-4, IL-17, 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.12 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 cell cytotoxic functions are restricted by inhibitory receptors.13–15 Therefore, we compared expression of known T and NK cell cytotoxic functions are restricted by inhibitory receptors.13–15 Therefore, we compared expression of known T and NK cell activating, 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).

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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 naïve), 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; Thprm, hypofunctional tissue resident T cells; Tncm, naive/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 FindConservedMarker function implemented in Seurat (to find features that are conserved between the groups, ie, healthy donors and SSc). This approach confirmed enrichment of cytotoxic 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 myeloid 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 (PTGDS) (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 late 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+ cells were detected compared with healthy individuals (18% of total CD8+ 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 granulocyte 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 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
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 ninefold 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/maturity 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 %CD8+Ki67+ 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 contractures. 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.

**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.27 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.5 8 28 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.29 Because of its fibrotic clinical manifestations, SSc has been considered a T helper type 2 (Th2)-mediated disease.30 31 However, epigenetic studies revealed gene transcription in cytotoxic T cells and NK cells in SSc patients with disease risk loci.5 Also, SSc skin was found to be predominantly infiltrated by cytotoxic T cells, in proximity to preapoptotic endothelial cells.4 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.10 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 TGFB1, 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.32 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 CD7.18 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-4.33 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-3.7 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.34 35 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,
CTTs in SSc skin are characterised by an activating rather than an exhausted profile.

This study reconfirms the importance of autoimmunity 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 depleting nature, anti-CD3/CD7-IT is administered as a single treatment and that further supports its favourable safety profile. In line with this notion, CD7 targeting therapeutic approaches have shown clinical efficacy and safety in kidney transplantation patients.36,37 Previously, we showed that anti-CD3/CD7 imunotoxin 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 rates in patients with acute GvHD.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+ CTTs. 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 found 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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