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Presence of CD4+CD8+ Double-Positive T Cells With Very High Interleukin-4 Production Potential in Lesional Skin of Patients With Systemic Sclerosis

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Objective. Fibrotic skin changes in systemic sclerosis (SSc) are preceded by the appearance of an inflammatory infiltrate rich in T cells. Since no direct comparison with T cells in normal skin has been performed previously, this study was undertaken to functionally characterize T cells in the skin of patients with early active SSc and in normal skin.

Methods. We characterized coreceptor expression, T cell receptor (TCR) usage, cytokine production, and helper and cytolytic activity of T cell lines and clones established from skin biopsy specimens from 6 SSc patients and 4 healthy individuals. Immunofluorescence analysis of skin biopsy and peripheral blood samples was performed to confirm the presence of specific subsets *in vivo*.

Results. A distinct subset expressing both CD4 and CD8 α/β coreceptors at high levels (double-positive [DP]) was present in T cell lines from SSc and normal skin. DP T cells actively transcribed both accessory molecules, exerted clonally distributed cytolytic and helper activity, and expressed TCR clonotypes distinct

from those in CD4+ or CD8+ single-positive (SP) T cells. In SSc skin, DP T cells produced very high levels of interleukin-4 (IL-4) compared with CD4+ SP T cells. Furthermore, DP T cells were directly identified in SSc skin, thus providing evidence that they are a distinct subset *in vivo*.

Conclusion. The present findings show that T cells with the unusual CD4+CD8+ DP phenotype are present in the skin. Their very high level of IL-4 production in early active SSc may contribute to enhanced extracellular matrix deposition by fibroblasts.

The expression of CD4 coreceptors and CD8 α/β coreceptors on mature T cells is considered to be mutually exclusive and to reflect subset-related, specific functions (helper versus cytolytic) and differences in major histocompatibility complex (MHC) restriction for antigen recognition. However, CD4+CD8+ double-positive (DP) T cells have been described in several conditions, mainly of inflammatory origin (1). The CD4 coreceptor is a protein encoded by a single gene that is uniformly expressed on the T cell surface as a monomer. In contrast, the CD8 coreceptor exists as an α/α homodimer or an α/β heterodimer. CD8+ single-positive (SP) cytolytic T cells express the heterodimeric form, while intestinal intraepithelial T cells, natural killer T cells, and T cell receptor γ/δ (TCR γ/δ)-positive T cells express the homodimeric form (2,3). DP T cells represent a heterogeneous population with regard to their expression of CD4 and CD8 coreceptors, probably related to differences in origin.

A subset of CD4+ SP T cells, when cultured in the presence of interleukin-4 (IL-4), acquires CD8 α/α (CD4^{high}CD8^{low} DP T cells) and becomes competent for cytotoxicity (4–6). TCR analysis of circulating CD4+CD8 α/α + DP T cells from healthy aging donors

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and from 1 patient with the acquired immunodeficiency syndrome revealed sharing of their TCR with CD4+, but not with CD8+, SP T cells (7,8), thus indicating that CD4+ SP T cells may acquire the CD8 α/α coreceptor *in vivo*. Similarly, activation of CD8 α/β + SP T cells can lead to the expression of low levels of CD4 (CD4^{low}CD8^{high} DP T cells) (9). In addition to CD4^{high}CD8^{low} DP T cells and CD4^{low}CD8^{high} DP T cells, DP T cells that express both CD4 and CD8 at high levels (CD4^{high}CD8^{high} DP) have been found in the peripheral blood of patients with multiple sclerosis (10) and in the skin and peripheral blood of patients with atopic dermatitis (11). However, no data are available on the composition of the CD8 coreceptor in these conditions.

Systemic sclerosis (SSc) is a connective tissue disease of presumed autoimmune inflammatory origin, characterized by fibroproliferative vasculopathy and fibrosis of the skin and internal organs. In SSc, T cells are present at an early stage at sites undergoing fibrosis (12–14). Collagen synthesis is up-regulated in fibroblasts in the proximity of infiltrating T cells (15–17), which suggests that T cells may directly or indirectly dysregulate the metabolism of fibroblasts and participate in the development of fibrosis. According to this perspective, Th2 cells may act by releasing profibrotic cytokines (IL-4, IL-13) (18–20) that induce fibroblasts to produce extracellular matrix components, and Th1 cells by releasing antifibrotic cytokines (interferon- γ [IFN γ]) that inhibit extracellular matrix production (21). In addition, T lymphocytes may act directly on fibroblasts by cell–cell contact (21–23), and their effect may be mediated by other cells such as macrophages (24).

When characterizing T cells cultured from lesional skin of SSc patients, we found TCR α/β + CD4^{high}CD8^{high} DP T cells in addition to the previously described CD4+ and CD8+ SP T cells (25). In the present study, we aimed to functionally characterize DP T cells, analyze their coreceptor composition, and directly assess whether they are present in skin undergoing fibrosis in patients with SSc.

MATERIALS AND METHODS

Reagents. RPMI 1640 medium, fetal calf serum (FCS), β -mercaptoethanol, phorbol myristate acetate, and brefeldin A were from Sigma (St. Louis, MO). Ionomycin and Pronase were from Calbiochem (San Diego, CA). Phosphate buffered saline (PBS), penicillin, streptomycin, L-glutamine, nonessential amino acids, sodium pyruvate, and pokeweed mitogen (PWM) were from Life Technologies (Paisley, UK). Human AB serum was obtained from the blood bank of Geneva

University Hospital. Ficoll-Paque was from Pharmacia (Uppsala, Sweden). Phytohemagglutinin (PHA) was from EY Laboratories (San Mateo, CA). DNase I was from Roche (Reutkreuz, Switzerland). Anti-IFN γ was a gift from G. Garotta (Sero Research Institute, Geneva, Switzerland). Recombinant human IL-2 was from Biogen (Cambridge, MA), recombinant human IL-4 from Sandoz (Basel, Switzerland), and recombinant human IL-12 from R&D Systems (Minneapolis, MN). Anti-IL-4 monoclonal antibody (mAb) 25D2.11, mAb anti-CLA, and OKT3 were from American Type Culture Collection (Manassas, VA). Anti-CD4, anti-CD8 α , anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD45RO, and isotype-matched irrelevant controls were from Dako (Copenhagen, Denmark). Anti-CD8 β , biotinylated anti-CD3, and biotinylated anti-CD8 α were from Beckman Coulter (Brea, CA). Anti-TCR α/β , anti-TCR γ/δ , streptavidin–phycoerythrin–Cy5, anti-IFN γ , and anti-IL-4 for flow cytometry were from BD Pharmingen (San Diego, CA).

Patients and controls. Five patients with SSc were recruited at the Department of Rheumatology, Lund University Hospital and 1 at the Department of Immunology and Allergy, Geneva University Hospital. All 6 patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of SSc (26) and had clinical features of diffuse cutaneous involvement (27), with initial sclerodermatous changes noted <3 years before samples were obtained for this study (mean 1.7 years). There were 3 men and 3 women, with an age range of 49–74 years (mean 58.7 years). Four of the 6 patients were positive for antinuclear antibodies. One of the 6 was receiving immunosuppressive therapy. Punch skin biopsy specimens were obtained from affected skin from the forearm in all patients and from the leg in 1 patient. Peripheral blood was obtained from all 6 patients. In addition, skin biopsy specimens from 4 healthy individuals undergoing cosmetic abdominal surgery, and peripheral blood samples from 6 other healthy individuals matched for age and sex, were obtained. Permission to perform this investigation was obtained by the ethics committees of the institutions involved, and all participants provided informed consent.

Generation of skin-derived T cell lines. Punch biopsy specimens were cut into several small fragments, each cultured individually in 24-well plates in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 5% heat-inactivated AB human serum (complete medium) to which IL-2 (20 units/ml) was added. Thereafter, growing T cell lines were expanded by adding 1.5×10^6 irradiated allogeneic peripheral blood mononuclear cells (PBMCs) (feeder cells) and PHA (0.7 μ g/ml) every 15–20 days (28). In most cases, the number of T cells was sufficient to enable phenotypic and functional characterization 4 weeks after culture initiation.

Generation of CD4+CD8+ DP and CD4+ or CD8+ SP skin-derived T cell lines. Pure SP and DP T cell lines were generated with a stepwise approach. First, CD4+ T cells from skin-derived T cell lines were positively selected using anti-CD4 mAb coupled with Dynabeads (Dyna, Oslo, Norway). In a second step, after 72–96 hours of culture, positively selected CD4+ T cells were enriched in CD8-expressing T cells by addition of anti-CD8 α mAb coupled to Dynabeads, thus yielding DP T cells. CD8+ SP T cells were negatively selected during the first step, and CD4+ SP cells negatively selected

during the second step of this procedure. The purity of the T cells lines thus generated (mean \pm SD) was $98.3 \pm 2.5\%$ for CD4+ cells, $97.4 \pm 1.9\%$ for CD8+ cells, and $98.8 \pm 1.1\%$ for CD4+CD8+ T cells ($n = 5$ cell lines for each).

Generation of polarized T cell lines. Naive CD4+ T cells from peripheral blood were obtained by negative selection using Dynabeads, as previously described (25). Naive CD4+ T cells were activated by CD3 crosslinking in the presence of IL-12 and anti-IL-4 to obtain Th1 cells or in the presence of IL-4, anti-IFN γ , and anti-IL-12 to obtain Th2 cells (29). T cell clones of each T cell subset were generated by limiting dilution (28).

Flow cytometry. PBMC expression of CD4, CD8 β , and CD3 was determined by 3-color fluorescence-activated cell sorter (FACS) analysis using a FACSCalibur, and data were analyzed with CellQuest software (Becton Dickinson, Mountain View, CA). Four-color flow cytometry was used to simultaneously detect surface expression of accessory molecules and intracellular accumulation of cytokines, as previously described (25).

Pronase treatment. T cells were incubated twice (15 minutes apart) with 0.04% Pronase and 100 μ g/ml DNase I in PBS at 37°C (30). The enzymatic reaction was stopped by adding an equal volume of FCS. Expression of CD4, CD8 β , and CD3 was assessed by FACS analysis before treatment, immediately after treatment, and after 16 hours of culture in complete medium at either 37°C or 4°C.

Helper assay. B cells positively sorted from normal PBMCs were plated at 0.5×10^5 cells/well in 96-well plates in 200 μ l of medium with or without extensively washed T cells of various origins at either 1×10^5 , 0.5×10^5 , or 0.25×10^5 cells/well, in the presence or absence of PWM (12.5 μ g/ml). After 7 days of culture, supernatants were harvested and IgG levels determined by enzyme-linked immunosorbent assay (ELISA) (31).

Cytolytic assay. P815 target cells incubated for 90 minutes at 37°C in the presence of 100 μ Ci 51 Cr (Amersham Biosciences, Little Chalfont, UK), were plated at 0.5×10^5 cells/well in 96-well U-bottomed plates with or without T cells and in the presence or absence of 1 μ g/ml OKT3, at either 2×10^5 , 1×10^5 , 0.5×10^5 , 0.25×10^5 , or 0.125×10^5 cells/well. After 4 hours at 37°C, the supernatants were assessed for the presence of 51 Cr, using a gamma counter (Clini-Gamma-1272; Wallac PerkinElmer, Boston, MA) (32). The results were expressed as the percent of lysis, according to the following formula: % lysis = (test 51 Cr - control 51 Cr)/(maximum 51 Cr - control 51 Cr) \times 100. Lytic units were then computed as previously described (33).

Cytokine production assay. T cell lines or clones were cultured for 48 hours at 37°C in 200 μ l of 10% FCS-RPMI 1640 medium at 5×10^4 cells/well in 96-well plates, after which some lines were activated by CD3 crosslinking. Production of IFN γ and IL-4 in T cell supernatants was assessed by ELISA.

Reverse transcriptase-polymerase chain reaction (RT-PCR). RNA extraction, reverse transcription, and complementary DNA (cDNA) amplification were performed as previously described (34). Briefly, cDNA was obtained by lysing 10×10^3 T cells in 15 μ l of buffer provided with the Superscript II reverse transcriptase kit (Gibco BRL, Grand Island, NY) supplemented with 0.12% Triton X-100 (Fluka, Buchs, Switzerland), 80 units/ml rRNasin (Promega, Madison, WI), 200

ng/ml oligo(dT) T20 (Amplimmun, Madulain, Switzerland), 50 nM dNTP (Gibco BRL), and 3 μ g total RNA (Boehringer Mannheim, Rotkreuz, Switzerland), followed by 50 minutes of incubation at 42°C. For PCR amplification, primers were added to 2 μ l of cDNA in 19 μ l of Taq DNA polymerase buffer (Gibco BRL) (35 cycles of 50 seconds at 94°C, 60 seconds at 59°C, and 60 seconds at 72°C). Primers were as follows: GAPDH forward 5'-GGACCTGACCTGCCGTCTAG-3', reverse 5'-GGCCATGTGGGCCATGAGGTC-3'; CD3 forward 5'-CGTTCAGTTCCTCCTTTTCTT-3', reverse 5'-GATTAGGGGGTTGGTAGGGAGTG-3'; CD4 forward 5'-CTAGCCCAATGAAAAGCAGGAG-3', reverse 5'-GCTGAGCTTGAAACTGGAGAACA-3'; CD8 α forward 5'-TCCATCATGTACTIONCAGCCACT-3', reverse 5'-TGCAGTAAAGGGTGATAACCAG-3'; CD8 β forward 5'-GATGCTGCTCGGAGGCTAAAAT-3', reverse 5'-CTTGGTGGGCTGGGCAGTGGTG-3'.

Spectratype analysis. All procedures, as well as the sequences of oligonucleotides corresponding to the 21 variable segments of the TCR β chain (TCRBV) used in this study, have been reported previously (35). Briefly, total RNA and cDNA were prepared from 5,000 CD4+ SP, CD8+ SP, or CD4+CD8+ DP T cells, with the use of RNeasy kits (Qiagen, Hilden, Germany). PCR was performed with 6-FAM-, HEX-, and TET-5'-labeled primers (Amplimmun), allowing amplification and analysis of multiple TCRBV segments in the same reaction. Data analysis was performed with GeneScan analysis software (PerkinElmer, Rotkreuz, Switzerland). T cell lines were derived from a single donor. Spectratype analysis was performed in 3 replicates of each T cell subset, and a given TCRBV was considered to be present when the corresponding amplification product was found in all of the replicates.

Confocal analysis. Immunofluorescence analysis of skin sections was performed as previously described (36). Briefly, individual 5- μ m cryosections were fixed with cooled methanol and acetone, dried, and rehydrated in PBS, 0.2% gelatin, and 0.05% Tween 20. Triple staining involved stepwise incubation with anti-CD4, Texas Red-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, Hamburg, Germany), fluorescein isothiocyanate (FITC)-conjugated anti-CD8 α , and Topro-3 (Molecular Probes, Leiden, The Netherlands). Immunofluorescence images were acquired using an LSM510 laser scanning microscope (Zeiss, Wetzlar, Germany). For each section, the inflammatory infiltrate present in the dermis was located and analyzed. Within this inflammatory area a minimum of 10 fields (images) were collected. Image analysis and processing were performed with the Image J and Metamorph software packages. The total number of cells (blue staining with Topro-3), as well the total number of FITC- and Texas Red-SP and DP cells, were counted in each field. The number of single- or double-stained cells was expressed as a percentage of total cells. The counts were performed under blinded conditions.

Statistical analysis. For data on T cell lines from SSc patients and healthy individuals, taking into account the lack of independence between cell lines obtained from the same subject, we compared the 2 groups using a generalized estimating equation (GEE) (37). These models allow for a non-null correlation between clustered observations. We used a linear regression GEE model, in which each person defined a cluster, and the correlation matrix was set to "exchangeable,"

as implemented on Stata 8.2 (StataCorp., College Station, TX). Student's 2-tailed *t*-test was used when appropriate. Data are reported as the mean \pm SD.

RESULTS

Characterization of T cell lines derived from normal and SSc skin. We have previously shown that T cell lines and clones can be generated in the presence of IL-2 from the involved skin of patients with SSc (25).

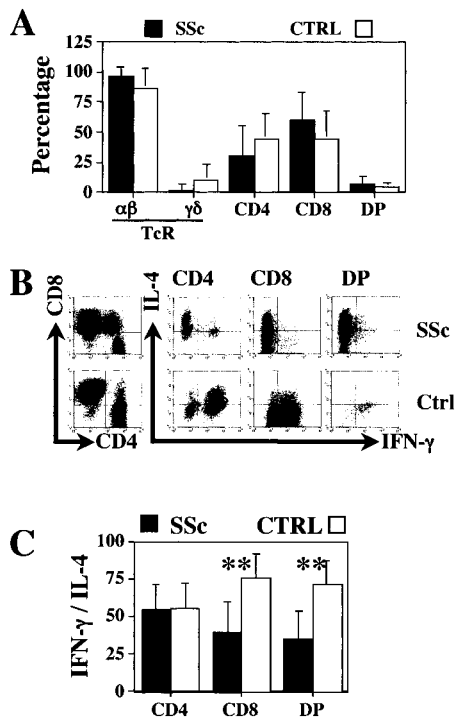


Figure 1. T cell receptor (TCR) CD4 and CD8 surface expression and interferon- γ (IFN γ) and interleukin-4 (IL-4) production potential in T cell lines generated from the skin of systemic sclerosis (SSc) patients and healthy controls. Skin fragments were cultured in medium supplemented with IL-2. Outgrowing T cells were expanded and subjected to fluorescence-activated cell sorter (FACS) analysis. Twenty-six T cell lines were generated from 7 skin biopsy samples from 6 SSc patients, and 47 T cell lines from 4 skin biopsy samples from 4 healthy controls. **A**, Surface expression. **B**, Representative 4-color FACS analysis of CD4 and CD8 expression and IFN γ and IL-4 production potential. **C**, IFN γ /IL-4 production index. Subsets were defined by surface expression of T cell lineage-specific markers CD4 and CD8. The potential for IFN γ and IL-4 production was evaluated at the single-cell level by intracellular staining upon T cell activation. The IFN γ /IL-4 production index was computed according to the formula [MFI IFN γ /(MFI IFN γ + MFI IL-4)] \times 100 (MFI = mean fluorescence intensity). With this formula, pure Th1-like cells have an index of 100 and pure Th2-like cells an index of 0. Values in **A** and **C** are the mean and SD. ** = $P < 0.0001$ as computed by the generalized estimating equation (37). DP = double-positive.

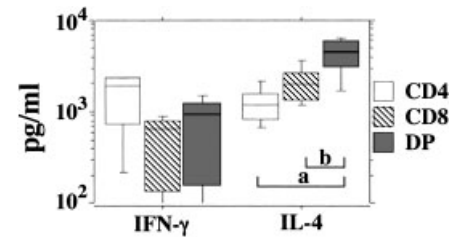


Figure 2. IFN γ and IL-4 production by CD4⁺, CD8⁺, and CD4⁺CD8⁺ DP T cells generated from the skin of SSc patients. Homogeneous subsets were generated from parental skin-derived T cell lines by immunomagnetic bead sorting as described in Materials and Methods. IFN γ and IL-4 levels in the supernatants of 5×10^4 T cells activated by CD3 crosslinking for 48 hours were determined by enzyme-linked immunosorbent assay. Data are shown as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the 50th percentile, and the lines outside the boxes represent the 10th and 90th percentiles. a = $P = 0.0076$; b = $P = 0.0447$. See Figure 1 for definitions.

Using the same protocol, we isolated T cell lines from 47 of 52 fragments from skin biopsy samples from 4 healthy individuals. In normal skin, the majority of T cells isolated were TCR α/β ⁺, while a mean \pm SD of $9.5 \pm 13\%$ were TCR γ/δ ⁺ (Figure 1A). The latter population was virtually absent from the biopsy specimens from 6 SSc patients. The percentage of CD4^{high}CD8^{high} DP T cells in SSc skin ($6.6 \pm 7.2\%$) tended to be higher than in normal skin ($4.5 \pm 3.6\%$) (Figure 1A). Interestingly, CD8⁺ SP T cells, as well as DP T cells, isolated from SSc skin produced significantly more IL-4 and significantly less IFN γ than T cells from normal skin, as assessed by intracellular staining ($P < 0.0001$ for both populations) (Figures 1B and C). This difference was not found for CD4⁺ T cells. Hence, the main difference between normal and SSc skin was the presence of CD8⁺ SP and CD4^{high}CD8^{high} DP IL-4-producing T cells, and as a result, the T cell population in SSc skin appeared to be considerably more biased toward a Th2-like phenotype than that in normal skin.

CD4^{high}CD8^{high} DP T cells from SSc skin produce high amounts of IL-4, provide help for B cells to produce IgG, and are able to kill P815 target cells. To further characterize skin-derived T cells, we sorted T cell lines generated from 5 distinct SSc skin biopsy specimens into homogeneous CD4⁺, CD8⁺, and CD4⁺CD8⁺ DP subsets and compared their capacity to produce IFN γ and IL-4 after stimulation with anti-CD3. DP T cells produced significantly more IL-4 than did CD4⁺ or CD8⁺ SP T cells ($P = 0.0076$ and $P = 0.0447$, respectively) (Figure 2A). No significant difference in their capacity to produce IFN γ was observed. Similar

results were obtained with 18 CD4+ SP, 37 CD8+ SP, and 34 CD4^{high}CD8^{high} DP individual T cell clones generated from the respective T cell lines (data not shown).

We further assessed the helper and cytolytic capacity of DP T cells at the clonal level. Three of 6 DP T cell clones tested (582G10, 693E6, and 711F5) were able to provide help to B cells for IgG production. This helper activity was dose dependent and required the presence of a mitogenic stimulus (PWM) (Figures 3A and B). Of note, the helper activity was as efficient as that of in vitro-polarized Th2 cells, indicating that at least a subset of DP T cells could operate as bona fide Th2 cells. Interestingly, 8 of 10 DP T cell clones tested were capable of killing P815 target cells (Figures 3C and D). This cytolytic activity was dose dependent, and 3 DP T cell clones (582C10, 701D11, and 711F5) were as potent as CD8+ SP T cells. Furthermore, clone 711F5 displayed both potent helper and cytolytic activities (Figures 3B and D). Overall, these results indicated that DP T cells produce very high levels of IL-4 and exert both helper and cytolytic activities in a clonally distributed manner.

CD4^{high}CD8^{high} DP T cells express CD4, CD8 α , and CD8 β chains at their surface and are clonally distinct from CD4+ SP and CD8+ SP T cells. CD4+ SP T cells may become DP after culture, in particular in the presence of IL-4 (4–6), and passive transfer of coreceptor molecules from CD4+ SP T cells to CD8+ SP T cells may cause the latter to become DP (38). To exclude the possibility that the DP T cells had become DP in vitro, we tested whether DP T cells cultured from skin lesions expressed CD8 β chains (since IL-4 induces only CD8 α on CD4+ SP T cells) and whether the DP T cells actually transcribed both coreceptors. As shown in Figure 4A, all CD4+CD8+ DP T cells generated from SSc and control skin simultaneously expressed CD4, CD8 α , and CD8 β . Furthermore, all DP T cell clones transcribed messenger RNA (mRNA) for CD4, CD8 α , and CD8 β genes as detected by RT-PCR (Figure 4B). In addition, CD8 β and CD4 mRNA were actively translated into protein in DP T cells; the CD4 and CD8 β coreceptors removed by Pronase treatment were reexpressed during culture at 37°C but not at 4°C (Figure 4C). During this procedure, all DP T cells remained DP, and SP T cells remained SP (Figure 4C). These results demonstrate that DP T cells transcribe and translate CD4, CD8 α , and CD8 β genes, thus indicating that they are a population distinct from CD4+ or CD8+ SP T cells. Furthermore, CD4+, CD8+, and CD4+CD8+

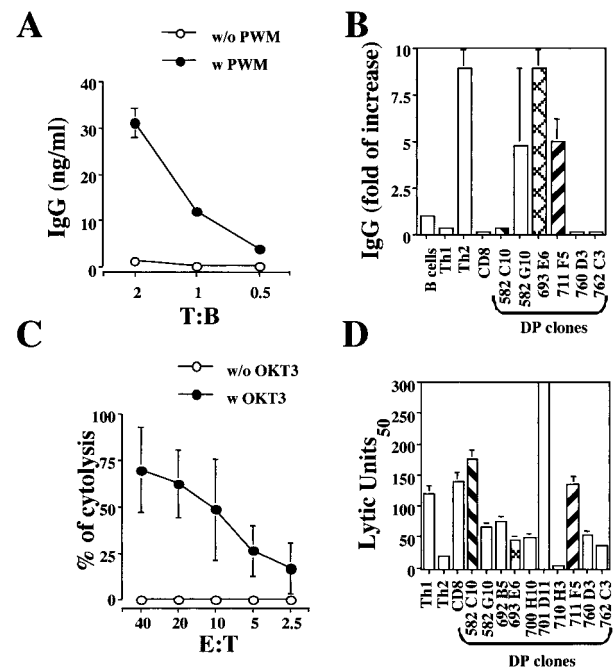


Figure 3. Helper and cytolytic function of CD4+CD8+ double-positive (DP) T cells generated from the skin of systemic sclerosis (SSc) patients. **A** and **B**, Allogeneic B cells were purified from normal peripheral blood mononuclear cells by immunomagnetic sorting and cultured at 5×10^4 cells/well for 7 days in the presence or absence of CD4+CD8+ DP T cell clones from SSc skin, in vitro-polarized Th1 and Th2 T cell lines, or CD8+ T cells from a healthy donor, with (w) or without (w/o) pokeweed mitogen (PWM). IgG production was evaluated by enzyme-linked immunosorbent assay. Dose-dependent production of IgG by B cells with CD4+CD8+ DP T cells in the presence of PWM is shown in **A**, and the fold increase in IgG production (compared with production in the absence of T cells [2 ng/ml]) in duplicate cultures of B cells and T cells cultured with PWM at a 1:1 cell ratio is shown in **B**. **C** and **D**, ^{51}Cr -labeled P815 cells were incubated for 4 hours in the presence or absence of CD4+CD8+ DP T cell clones from SSc skin, in vitro-polarized Th1 and Th2 T cell lines, or CD8+ T cells from a healthy donor, with or without anti-CD3 monoclonal antibody (OKT3). Cytolytic activity was expressed as the percent of lysis, as described in Materials and Methods. Dose-dependent P815 killing with CD4+CD8+ DP T cells in the presence of OKT3 is shown in **C**, and lytic units, computed by plotting the percent of lysis versus the log of the effector cell number to extrapolate the number of cells giving 50% lysis, are shown in **D**. Patterned bars in **B** and **D** indicate T cell clones with differential helper and cytolytic potential. Values are the mean \pm SD. E:T = effector:target.

DP T cell lines maintained in culture for up to 3 months maintained a stable function and phenotype.

We were able to provide further evidence of the distinct clonal origin of DP T cells by studying the TCR usage of the CD4+ SP, CD8+ SP, and DP T cells from the same skin biopsy specimen, by spectratyping (35). This technique measures the size heterogeneity of the

third complementarity-determining region of the TCRBV chains that is caused by random addition or removal of nucleotides during the rearrangement process. Whereas 5,000 cells from a normal T cell population would generate a Gaussian distribution of 9–11 bands per V_{β} chain, representing the different lengths of the respective TCR V–D–J regions and thus yield >200 different bands for 21 V_{β} chains tested (37), we detected only 2.6 ± 1.1 , 9.3 ± 2.5 , and 9.3 ± 2.5 bands (mean \pm SD) for the CD4+ SP, CD8+ SP, and CD4+CD8+ DP T cells, respectively. Hence, independent of their phenotype, the skin-infiltrating T cells are extremely oligoclonal. Table 1 depicts the TCR usage of the respective populations (only the major 11 bands present in the 3 cell types are shown). Clearly, none of the 7 TCRs used by the CD4+CD8+ DP T cells corresponded to any of the TCRs used by the SP T cells. Therefore, DP T cells

Table 1. TCR usage by CD4+CD8+ double-positive versus CD4+ or CD8+ single-positive T cells from the skin of patients with SSc*

T cell subset	TCRBV (size of amplification product, bp)
CD4+CD8+	$V_{\beta}9$ (170), $V_{\beta}13$ (172), $V_{\beta}17$ (172)
CD4+	$V_{\beta}5.1$ (150)
CD8+	$V_{\beta}1$ (201), $V_{\beta}6$ (164), $V_{\beta}6$ (179), $V_{\beta}8$ (176), $V_{\beta}13$ (167), $V_{\beta}20$ (211), $V_{\beta}24$ (156)

* Total RNA and DNA were prepared from 5,000 CD4+CD8+ double-positive or CD4+ or CD8+ single-positive T cells from the skin of a patient with systemic sclerosis (SSc). Spectratype analysis was performed in 3 replicates of each T cell subset, and a given variable segment of the T cell receptor β -chain (TCRBV) was considered to be present when the corresponding amplification product was found in all of the replicates. A similar analysis was performed with 5,000 T cells from normal peripheral blood, demonstrating polyclonal spectratypes within all V_{β} families (35).

infiltrating the skin of SSc individuals are oligoclonal, and their origin is distinct from that of SP T cells.

In vivo presence of DP T cells in SSc patients and healthy individuals. We next assessed whether DP T cells are present in vivo. We identified DP T cells within CD3 cells in the peripheral blood of healthy donors and, at higher frequency, in SSc patients (mean \pm SD $0.20 \pm 0.09\%$ versus $0.51 \pm 0.17\%$; $P < 0.005$). In addition, to ascertain whether DP T cells are present in the skin, we performed triple (CD4, CD8 α , DNA) immunofluorescence studies by laser scanning microphotography on skin samples from 3 SSc patients and 2 healthy donors. CD4+CD8+ DP T cells were observed in 2 of 3 SSc specimens and in none of the control specimens (Figure 5). CD4+CD8+ DP cells composed $2.3 \pm 1.0\%$ (mean \pm SD), CD4+ SP cells $52.0 \pm 12.1\%$, and CD8+ SP cells $10.0 \pm 2.6\%$ of the inflammatory infiltrate in SSc specimens and 0%, $23.3 \pm 12.7\%$, and $9.0 \pm 6.0\%$, respectively, in control specimens. The presence of DP T cells in lesional skin of SSc patients provides evidence of their existence in vivo and lends support to the hypothesis that they may be involved in the pathogenesis of SSc.

DISCUSSION

Our study was aimed at assessing the presence, function, and origin of CD4+CD8+ DP T cells in human skin. We were able to demonstrate that CD4+CD8+ DP T cell lines can be generated from the skin of patients with SSc as well as healthy individuals. These DP T cells have stable and high expression of CD4 and of the α/β isoform of CD8, are clonally endowed with cytolytic and helper functions, and their potential to produce IL-4 distinguishes SSc from control DP T cells. Furthermore, the DP T cells that can be

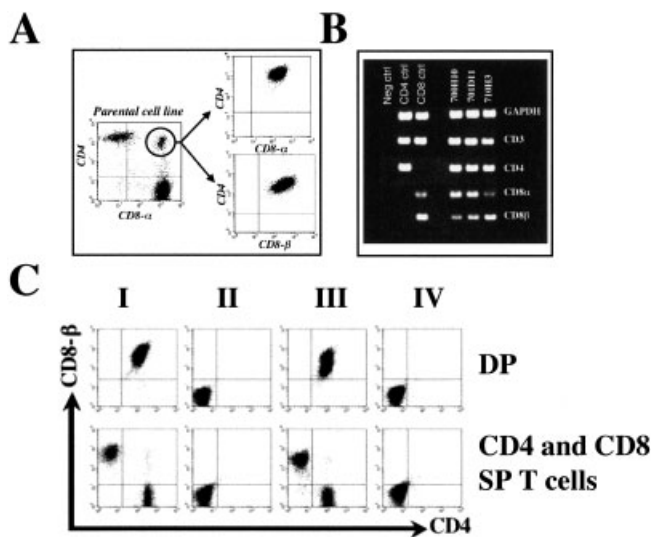


Figure 4. Expression, transcription, and active translation of CD4, CD8 α , and CD8 β in SSc skin-derived CD4+CD8+ DP T cells. **A**, DP T cells were purified from a parental skin-derived T cell line by immunomagnetic bead sorting as described in Materials and Methods. Simultaneous expression of CD4 with CD8 α and CD8 β by the purified T cells was then determined by FACS analysis. **B**, CD4, CD8 α , CD8 β , CD3, and GAPDH mRNA expression in 3 distinct SSc skin-derived DP T cell lines and purified CD4 and CD8 T cells from normal peripheral blood mononuclear cells was analyzed by reverse transcriptase-polymerase chain reaction. **C**, Simultaneous surface expression of CD4 and CD8 β by CD4+CD8+ DP T cells as well as by a T cell line composed of CD4+ or CD8+ single-positive (SP) T cells before (I) and after (II) Pronase treatment, which results in the shedding of CD4 and CD8 coreceptors, was determined by FACS analysis. Their expression was assessed again after 16 hours of culture at 37°C (III) or 4°C (IV). See Figure 1 for other definitions.

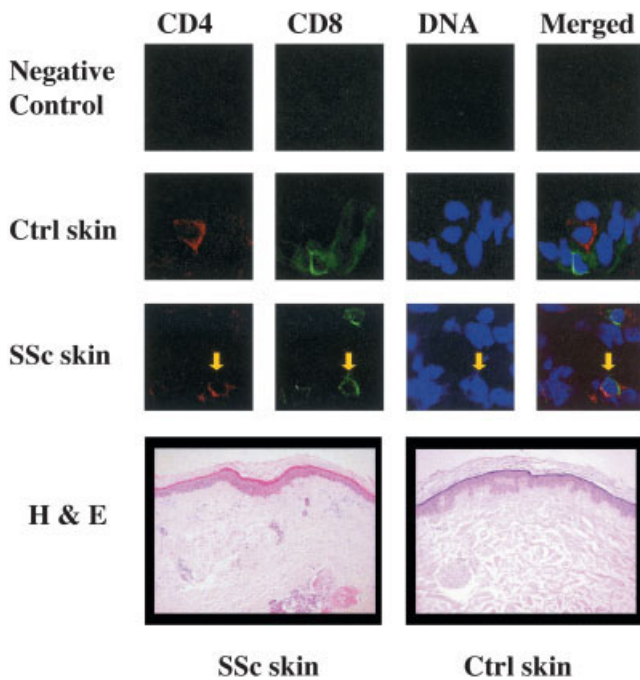


Figure 5. CD4+CD8+ double-positive T cells in lesional systemic sclerosis (SSc) skin. Frozen sections of skin from healthy controls and involved skin from SSc patients were stained and analyzed by laser scanning microphotography. CD4 appears red, CD8 α green, and nuclei blue. **Arrows** indicate cells simultaneously expressing CD8 α and CD4, which were found in 2 of 3 SSc specimens and in neither of 2 healthy control specimens. Representative photomicrographs are shown. Specimens were stained with hematoxylin and eosin (H&E) to document the inflammatory infiltration (original magnification \times 100).

microscopically identified in lesional skin of SSc patients appear to have a clonal origin distinct from that of CD4+ and CD8+ SP T cells as judged by their TCRBV usage.

Earlier studies have shown that the T cells infiltrating the skin of SSc patients are predominantly CD4+ and produce higher levels of IL-4 than IFN γ , and are therefore profibrotic (12,25,39). By allowing a direct comparison of IFN γ and IL-4 production potential between T cell subsets generated from healthy and SSc skin, our data highlight the point that CD4+CD8+ DP as well as CD8+ SP T cells, rather than CD4+ SP T cells, are the cells producing the high amounts of IL-4. Our results are reminiscent of the finding that the presence of CD8+ T cells producing IL-4 in the bronchoalveolar lavage fluid of patients with SSc correlates with development of lung fibrosis (40,41).

DP T cells have been observed in patients with various pathologic conditions as well as in normal do-

nors (1). Several features of the DP T cells described herein are notable. First, they express both CD4 and CD8 coreceptors at high levels, while in other studies expression of one or the other was dim. Second, they express the α/β chain of the CD8 molecule, suggesting that DP T cells in SSc skin are different from most DP T cells described, which express the CD8 α/α isoform typical of CD4 cells that acquire a CD8 coreceptor. Our findings also show that it is unlikely that DP T cells in SSc skin are derived from the CD8+ SP T cells in the sclerotic skin lesions, because DP T cells used a set of TCRs completely different from those used by the CD8+ SP T cells. Furthermore, double positivity was not due to passive transfer of the other coreceptor, since the expression of both CD4 and CD8 molecules was dependent on active synthesis and maintained in long-term cultures.

The identification of CD4+CD8+ T cells in SSc skin and in the peripheral blood of SSc patients and healthy subjects demonstrates unequivocally that DP T cells exist *in vivo* and are not a product of *in vitro* cultures. It is of note that the frequency of DP T cells was lower in the blood of controls than in that of SSc patients and that no DP T cells could be identified in healthy skin. Although the latter may simply be due to the low number of cells present in normal skin such that rare DP T cells remained undetected, we believe the difference is significant and implies involvement of DP T cells in SSc. The same holds true for high IL-4 production by the DP T cells in SSc skin, since IL-4 promotes collagen production and favors extracellular matrix deposition also by inhibiting metalloproteinase production by fibroblasts.

While DP T cells and CD8+ SP T cells in the SSc skin predominantly produced high amounts of IL-4, CD4+ SP T cells were more heterogeneous, and a substantial proportion of them produced both IFN γ and IL-4. This functional heterogeneity in lesional skin, which has previously been observed by others (19,39,42), may be inherent to SSc or to particular phases of the disease.

The TCRBV usage of the T cell lines we examined was extremely limited. Although the culture conditions may have favored the outgrowth of certain clones, we do not believe this influenced the results considerably, because oligoclonality in T cells infiltrating SSc skin has also been observed previously in studies using techniques not involving T cell culture (43), and T cells cultured from normal skin have been shown to be polyclonal (44,45). Thus, our results are consistent with the hypothesis that T cells are recruited in SSc skin in

response to a restricted number of antigens, possibly of autoantigenic nature.

The small number of patients and controls included in our study is a distinct limitation. However, patients were selected from a homogeneous subset with early disease and diffuse skin involvement. In addition, the number of T cells infiltrating the skin was, as expected, low.

The biologic significance of DP T cells in a sparse inflammatory infiltrate is obviously debatable, and their significance in autoimmune disorders such as SSc remains to be established. One might speculate that DP T cells that expressed only 1 coreceptor at the time of thymic selection were not properly deleted because the affinity of the TCR/coreceptor for the MHC-self antigen complex was too low (1). Such cells may become self-reactive after expression of a second coreceptor that would increase the affinity of the TCR/coreceptor for the autoantigen. Alternatively, SP T cells may become DP in the periphery after prolonged autoantigenic stimulation. Indeed, DP T cells have been described mainly in chronic inflammatory and chronic infectious disorders (1). Whatever their origin, DP T cells appear to be unique in their dual functionality, i.e., exerting cytolytic as well as helper activities and being high producers of cytokines with immunomodulatory potential. In addition, since cytolytic granules may induce apoptotic death and self protein fragments generated by granzyme B are recognized by autoantibodies in a subset of SSc patients (46,47), it is possible that DP T cells participate in posttranslational modifications of self protein with neopeptide formation, which would favor their antigenicity. Double-positive T cells may therefore play a major role in the development or perpetuation of inflammatory disorders such as SSc.

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AUTHOR CONTRIBUTIONS

Dr. Chizzolini had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Parel, Dayer, Roosnek, Chizzolini.

Acquisition of data. Parel, Aurrand-Lions, Scheja, Roosnek, Chizzolini.

Analysis and interpretation of data. Parel, Aurrand-Lions, Dayer, Roosnek, Chizzolini.

Manuscript preparation. Parel, Roosnek, Scheja, Chizzolini.

Statistical analysis. Parel.

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