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Systemic Sclerosis Th2 Cells Inhibit Collagen Production by Dermal Fibroblasts Via Membrane-Associated Tumor Necrosis Factor α

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Objective. In systemic sclerosis (SSc; scleroderma), T cells infiltrate organs undergoing fibrotic changes and may participate in dysregulated production of collagen by fibroblasts. The objective of this study was to functionally characterize T cells infiltrating skin lesions in early SSc and investigate their capacity to affect production of type I collagen and interstitial collagenase (matrix metalloproteinase 1 [MMP-1]) by dermal fibroblasts.

Methods. Four-color cytometric analysis was used to characterize subset distribution and production of interferon-γ (IFNγ) and interleukin-4 (IL-4) in T cell lines generated from the skin of patients with SSc. T cell clones were generated, and their capacity to modulate collagen and MMP-1 production by fibroblasts derived from patients with SSc and from normal individuals was assessed. Neutralizing reagents were used to identify T cell mediators involved in fibroblast modulation.

Results. The skin of individuals with early-stage SSc contained T cells preferentially producing high levels of IL-4. Cloned CD4+ Th2-like cells inhibited collagen production by normal fibroblasts. Th2 cell–dependent inhibition was, at least in part, contact-dependent, was essentially mediated by tumor necrosis factor α (TNFα), and was dominant over the enhancement induced by profibrotic IL-4 and transforming growth factor β cytokines. The simultaneous induction of MMP-1 production confirmed the specificity of these observations. To be inhibitory, Th2 cells required activation by CD3 ligation. Th2 cells were less potent than were Th1 cells in inhibiting collagen production by normal fibroblasts via cell-to-cell interaction, and SSc fibroblasts were resistant to inhibition.

Conclusion. These findings indicate that, despite their production of IL-4, Th2 cells reduce type I collagen synthesis by dermal fibroblasts because of the dominant effect of TNFα, and suggest that strategies based on TNFα blockade aimed at controlling fibrosis in SSc may be unwise.

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Systemic sclerosis (SSc) is a disease of undefined origin characterized by fibrosis of the skin (scleroderma) and internal organs, vascular abnormalities, and the presence of autoantibodies. Inflammation is a dominant aspect of the early phases of SSc, and it is thought to contribute to the development of fibrosis (1). Indeed, in SSc skin the presence of an inflammatory infiltrate has been described to precede development of characteristic microvascular changes and extracellular matrix deposition (2). Histologic examination of newly affected SSc skin reveals mononuclear cell infiltrates in which CD4+ T cells appear to outnumber CD8+ T cells (3,4). In addition, T cells belonging to the α/β T cell receptor (TCR)–positive subset appear to be oligoclonal, thus hinting at an antigen-driven local recruitment (5).

In some studies, T cells cloned from SSc skin biopsy specimens were exclusively CD4+ and produced interferon-γ (IFNγ) or glycosaminoglycan-stimulating factor (6). Furthermore, immunohistochemical and

polymerase chain reaction–based techniques of tissue analysis indicated that the majority of T cells infiltrating early active lesions produce IFNγ and tumor necrosis factor α (TNFα) (7). Other authors have reported that T cell clones generated from SSc skin are Th2-like CD4+ cells that produce high levels of interleukin-4 (IL-4) but no IFNγ (8), and in situ hybridization of infiltrated tissues revealed high levels of IL-4 but little or no expression of IFNγ messenger RNA (mRNA) (8,9).

In addition, a Th2-oriented profile of male-offspring T cells reactive against maternal major histocompatibility complex antigens present in the peripheral blood and skin of women with SSc has recently been documented (10). Furthermore, T cells bearing the TCRγ/δ are also present in perivascular infiltrates of SSc skin lesions (11), and γ/δ T cells of the $V_\delta 1+$ subset with a preferential Th1-like profile are increased in SSc peripheral blood (12). In patients with SSc who have lung involvement, the bronchoalveolar lavage (BAL) fluid contains CD8+ T cells expressing mRNA for IL-4 and IL-5, with or without equal amounts of IFNγ mRNA. In contrast, in normal BAL fluid, CD8+ T cells preferentially express IFNγ mRNA with little or no IL-4 or IL-5 mRNA (13). Thus, the data available on the characteristics of T cells infiltrating organs affected by SSc are heterogeneous. Some data point to the recruitment, on tissues undergoing fibrosis, of polarized T cells preferentially producing IFNγ and therefore belonging to the Th1-like subset (6,7). Other data indicate that T cells preferentially produce IL-4 and therefore belong to the Th2-like subset (8–11).

Fibroblasts are the main cells involved in extracellular matrix (ECM) deposition, and ECM is continuously renewed through balanced synthesis and degradation. In SSc, the rate of synthesis exceeds the rate of degradation (14). Whether SSc fibroblasts are set to a higher deposition rate because of intrinsic changes and/or are under the influence of inflammatory mediators or other stimuli has not yet been established. Cytokines play a major role in regulating ECM deposition by fibroblasts (15). With regard to polarized T cells, it is interesting to note that IFNγ inhibits, while IL-4 and IL-13 enhance, collagen synthesis (16–19). Thus, based on the effect of the prototypic cytokines produced by T cells on ECM deposition, Th1-like T cells have been predicted to decrease ECM deposition and Th2-like T cells to increase ECM deposition (1). Consistent with this hypothesis, we have documented that Th1-like T cells inhibit collagen deposition by dermal fibroblasts in a contact-dependent manner, and that membrane-associated IFNγ is the main mediator involved in the

inhibition (20). Additional inhibitory cytokines are membrane-associated IL-1 and TNFα (21). Of interest, SSc fibroblasts are resistant to inhibition when compared with control fibroblasts (20). However, no data exist as to the effect of Th2-like T cells on collagen synthesis by dermal fibroblasts.

The aim of the present study was to investigate whether T cells infiltrating the skin of patients with early-stage SSc belong to a defined functional subset, and whether they are capable of modulating collagen production by dermal fibroblasts. We report that SSc skin–infiltrating T cells are heterogeneous in terms of accessory molecule expression and cytokine production, with a sizable proportion of them producing high levels of IL-4. Of major interest, T cells, regardless of their functional phenotype, inhibit collagen while inducing collagenase production. Collagen inhibition is, at least in part, cell contact–dependent and, in Th2-like cells, is principally mediated by TNFα.

PATIENTS AND METHODS

Patients and controls. Five patients with SSc were recruited from the Department of Rheumatology at University Hospital in Lund, Sweden. All 5 patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of SSc (22) and had clinical features of early disease. None of the patients was receiving immunosuppressive therapy (23). In all 5 individuals, 2 skin punch biopsy specimens (3 mm in diameter) were obtained from the forearm in areas of affected skin; in 1 of these patients, specimens were also obtained from involved skin of the leg. One additional patient, who had aggressive, diffuse disease and was receiving immunosuppressants, was recruited from the Division of Rheumatology at the University of Basel, and a surgical biopsy specimen was obtained from the affected skin of the arm. Patients ranged in age from 24 to 64 years. Four of the 6 patients were women, and 4 of 6 had antinuclear antibodies.

The initial sclerodermatous changes were observed 4 years before entry in this study in 2 patients, 3 years before study entry in 2 patients, and 2 years before study entry in the other 2 patients. One skin punch biopsy specimen was obtained from each of 6 healthy age- and sex-matched individuals and provided control fibroblasts. Permission to perform this investigation was granted by the ethics committees of the various institutions involved. Informed consent was obtained from all subjects. Peripheral blood from healthy individuals was provided by the Blood Transfusion Center, Geneva University Hospital (Switzerland).

Reagents. Anti-CD4 monoclonal antibody (mAb) 6D10 and anti-CD8 mAb 733.18.1 were kindly supplied by Dr. E. Roosnek (Geneva, Switzerland). Anti-CD3 OKT3 and anti–cutaneous lymphocyte antigen (anti-CLA; HECA-451) mAb were obtained from American Tissue Culture Collection (Bethesda, MD). Anti-CD40 ligand (anti-CD40L) mAb (clone

5C8) was a gift from Dr. P. E. Lipsky (National Institutes of Health, Bethesda, MD). Anti-CD3 fluorescein isothiocyanate (FITC), anti-CD4 R-phycoerythrin (R-PE), and anti-CD8 FITC mAb were obtained from Dako (Glostrup, Denmark). Anti-$\alpha\beta$ FITC, anti-$\gamma\delta$ R-PE, anti–CD8-β R-PE, anti-IFNγ FITC, anti–IL-4 allophycocyanin (APC) mAb, streptavidin–Cy-Chrome, and Cytofix/Cytoperm were from Becton Dickinson (San Diego, CA). RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), glutamine, penicillin, streptomycin, trypsin, and fetal calf serum (FCS) were obtained from Gibco (Paisley, UK). Sucrose, phenylmethylsulfonyl fluoride, pepstatin, EDTA, iodoacetamide, Nonidet P40, phorbol myristate acetate (PMA), L-ascorbic acid, α-ketoglutarate, β-aminopropionic acid, indomethacin, and brefeldin A were from Sigma (St. Louis, MO). Pan Mouse IgG DynaBeads were from Dynal (Oslo, Norway); anti-human IFNγ mAb was a gift from Dr. G. Garotta (Serono Biomedical Research Institute, Geneva, Switzerland), recombinant human IL-4 (rHuIL-4) was from Schering-Plough (Dardilly, France), rHuIL-2 was from Biogen (Cambridge, MA), recombinant human TGFβ was from R&D Systems (Minneapolis, MN), and anti-CD54 (intracellular adhesion molecule 1 [ICAM-1]) was from PharMingen (San Diego, CA). Anti-TNF (recombinant methionyl soluble TNF receptor I [rsTNFRI] coupled to a polyethylene glycol molecule) and rHuIL-1 receptor antagonist (rHuIL-1Ra) were from Amgen (Thousand Oaks, CA).

Generation of skin-derived T cell lines. Skin punch biopsy specimens were cut with a scalpel, and fragments were individually cultured in 24-well plates in RPMI 1640 medium containing IL-2 (20 units/ml) supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), 5% human AB serum, and 10% FCS. After 15 days of culture, irradiated (3,500 rads) allogeneic peripheral blood mononuclear cells, phytohemagglutinin (1 μg/ml), and IL-2 (20 units/ml) were added. Growing cells were further expanded in IL-2–containing medium.

T cell clones and T cell membrane preparation. Skin-derived T cell clones were generated by limiting dilution cultures from selected skin-derived T cell lines. Prototypic Th1 and Th2 cell clones were generated from peripheral blood obtained from a normal individual upon antigen activation, as previously described (24), and were characterized for their capacity to produce IFNγ and IL-4. High IFNγ/low IL-4 producers were defined as Th1 cells, whereas low IFNγ/high IL-4 producers were defined as Th2 cells.

For the preparation of plasma cell membrane, 8×10^6 T cells cultured with plastic-adsorbed anti-CD3 mAb were activated in 6-well trays. Control cultures consisted of T cells cultured in medium alone. After 6 hours of culture, the supernatants were collected and frozen for further cytokine measurement. Cell membranes were prepared as previously described (24).

Flow cytometry. Four-color flow cytometry was performed on T cells using FACSCalibur (Becton Dickinson), and data were analyzed with CellQuest software (Becton Dickinson). Positivity was defined as fluorescence greater than that of negative controls (isotypic-matched irrelevant mouse mAb). T cells were first labeled with biotinylated anti-CD8 mAb (OKT8), washed, and incubated with streptavidin–Cy-Chrome. T cells were then cultured in RPMI 1640 medium supplemented with 10% FCS, in the presence of PMA (50 ng/ml), ionomycin (1 μM), and brefeldin A (2.5 μg/ml), for 270

minutes at 37°C. Upon fixation and permeabilization with Cytofix/Cytoperm (PharMingen), T cells were labeled with anti-IFNγ FITC and anti–IL-4 APC mAb, and finally were labeled with anti-CD4 R-PE.

Collagen production in skin fibroblast–lymphocyte cocultures. Fibroblasts derived from a fragment of the skin biopsy specimen were grown in DMEM supplemented with 10% FCS, penicillin, and streptomycin and were split when confluence was reached. All experiments with fibroblasts were performed at passages 4–9. To study collagen or matrix metalloproteinase 1 (MMP-1) production, fibroblasts were plated in 96-well trays at 2×10^4 cells/well in 200 μl of DMEM supplemented with 1% FCS. To favor normal fibrillogenesis and avoid procollagen precipitation, vitamin C (25 μg/ml), α-ketoglutarate (3.4 μg/ml), and β-aminopropionic acid (50 μg/ml) were added to the medium. Fibroblasts were cultured for 48 hours, then supernatants were harvested and frozen.

To assess the effect of T cells on collagen production, 2 different sets of experiments were performed. Contact-dependent T cell effects were studied by adding T cell membranes equivalent to 8×10^5 cells/well to fibroblasts. In other experiments, living T cells (2×10^4 cells/well) were added to fibroblasts and activated (or not activated) by CD3 crosslinking in the presence of 1 μg/ml of OKT3. In some experiments TGFβ (10 ng/ml), IL-4 (10 ng/ml), anti-TNF (rsTNFRI; $10^{-8}M$) (25), anti-IFNγ (10 μg/ml) (20), IL-1 receptor antagonist (2 μg/ml) (26), anti-CD54 (10 μg/ml), anti-CD40L (10 μg/ml), irrelevant monoclonal IgG1 (10 μg/ml), or indomethacin (5 μg/ml) was added, together with T cell membranes, with living T cells, or to control wells.

RNA extraction and RNase protection assay (RPA) analysis. Total RNA was isolated with TRIzol reagent (Life Technologies) by lysing T cell clones activated by CD3 crosslinking. The levels of expression of IL-2, IL-4, IL-5, IL-10, IL-14, IL-9, IL-13, IFNγ, and GAPDH mRNA were assessed by RiboQuant RPA, using the hCK-1 multiprobe template set from PharMingen, according to the supplier's instructions.

Northern blot. For Northern blot experiments, 5×10^5 fibroblasts were plated to confluence in 60-mm petri dishes. T cell membranes in concentrations equivalent to 8×10^6 cells were then added to the cultures (typically 200 μl of cell membranes in 2.5 ml), and fibroblasts were further cultured for 14 hours in 1% FCS medium. Total cellular RNA was extracted using TRIzol and analyzed by Northern blot hybridization with ^{32}P-labeled complementary DNA probes specific for proα1(I) (Hf677; a kind gift from Dr. M. B. Goldring, Charlestown, MA), MMP-1 (a kind gift from Dr. H. G. Welgus, St. Louis, MO), and GAPDH (27,28), which was followed by autoradiography. Signal intensity was determined densitometrically using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Determination of type I collagen, MMP-1, and T cell cytokines. To quantify collagen production, the N-terminal type I procollagen propeptide (PINP) was quantified by a competition-based radioimmunoassay (Orion Diagnostica, Espoo, Finland). ProMMP-1 was measured by commercial enzyme-linked immunosorbent assay (ELISA; Binding Site, Birmingham, UK). The sensitivity of both the PINP and the proMMP-1 assays was 2 ng/ml. Production of IFNγ, IL-4, and TNFα in T cell supernatants was assessed by ELISA. The

Table 1. Phenotype characteristics of SSc skin–derived T cell lines*

Patient, fragment	T cell subset					
	TCRα/β+	TCRγ/δ+	CD4+	CD8+	CD4+,CD8+	CD4−,CD8−
A						
1	100	0	79	12	4	5
2	100	0	16	72	11	1
3	100	0	18	72	10	0
4	100	0	14	53	33	0
B						
1	99	1	16	78	4	2
2	100	0	36	55	5	4
C						
1	100	0	19	73	7	1
2	100	0	28	60	11	1
3†	90	0	3	95	1	1
4†	100	0	9	80	6	5
5†	100	0	18	80	2	1
6†	99	1	38	56	5	1
7	100	0	83	9	7	1
8	100	0	17	61	20	2
D						
1	96	4	33	54	8	5
2	100	0	31	60	7	1
E						
1	100	0	20	69	10	1
2	100	0	15	71	12	2
3	100	0	73	23	3	0
4	100	0	100	0	0	0
Mean ± SD	99.00 ± 2.52	0.27 ± 0.88	33.33 ± 27.01	56.67 ± 25.26	8.32 ± 7.21	1.76 ± 1.56

* Values are the percentage. Skin biopsy fragments were cultured individually, and phenotype analysis was performed by fluorescence-activated cell sorting on approximately day 45 of cultures. Except where indicated otherwise, biopsy specimens were obtained from involved skin of the arm. SSc = systemic sclerosis; TCR = T cell receptor.
† Obtained from involved skin of the leg.

sensitivity threshold for detection of IFNγ, IL-4, and TNFα was 5 pg/ml.

Statistical analysis. Student's *t*-test was used when appropriate with 2-tailed *P* values. Calculations were performed using StatView version 5.0 software for the Macintosh (Abacus Concepts, Berkeley, CA).

RESULTS

Generation and characterization of T cell lines derived from SSc skin. To favor the proliferation of SSc skin–derived T cells activated in vivo, we chose to culture skin fragments in the presence of IL-2. T cell outgrowth was observed in skin fragments from all donors (Table 1) and was microscopically identifiable by day 10 of culture. The large majority of growing cells were α/β TCR+, with only 3 of 20 lines containing TCRγ/δ+ T cells (in very low numbers) (Table 1). Four different subsets were identified in TCRα/β+ SSc skin–derived T cells, in terms of CD4 and CD8 expression. As expected, single-positive CD4 and single-positive CD8 T cells were the most abundant, with heterogeneous proportions within the fragments of individual biopsy spec-

imens. In addition, sizable numbers of CD4+,CD8+ double-positive cells were present in 19 of 20 cell lines, and CD4−,CD8− double-negative cells (in low numbers) were present in 16 of 20 cell lines (Table 1). All of the lines expressed low levels of the skin-homing adhesion molecule CLA (data not shown).

To gain further insight into functional characteristics of SSc skin–derived T cell lines, we assessed at the single-cell level their potential to produce IFNγ and IL-4. Compared with control cultures generated under identical conditions using peripheral blood obtained from normal individuals, IL-4 production by SSc skin–derived T cells was significantly higher ($P < 0.0001$). Of interest, striking differences were observed depending on the particular subset analyzed (Figure 1 and Table 2). Thus, CD4+ TCRα/β+ cells were the highest producers of IFNγ, while CD4+,CD8+ double-positive cells were the highest producers of IL-4 (Table 2). The potential to produce IFNγ and IL-4 was statistically different in the 4 subsets generated from SSc skin (Table 2). Overall, these results indicate that T cells infiltrating the skin of
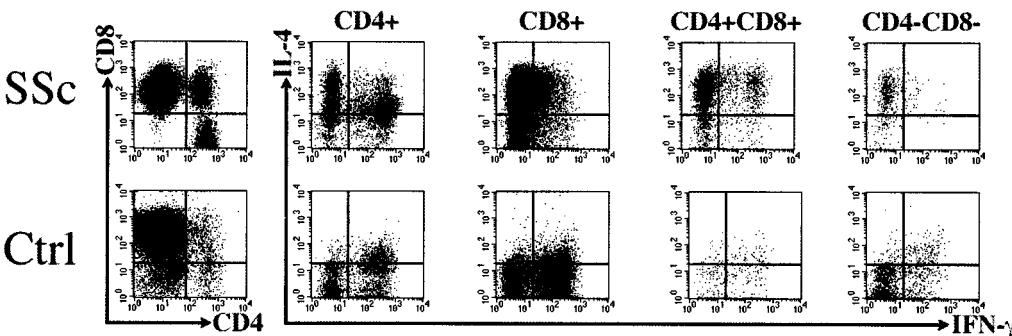
Figure 1. Intracellular production of interferon-γ (IFNγ) and interleukin-4 (IL-4) by a systemic sclerosis (SSc) skin–derived and a control (Ctrl) T cell line. Subsets are defined by surface expression of T cell lineage–specific markers CD4 and CD8. Four-color FACScan analysis was performed as described in Patients and Methods.

patients with early-stage SSc are heterogeneous in terms of CD4 and CD8 expression and are poised for production of high levels of IL-4.

SSc T cell–dependent inhibition of collagen production by both SSc and control fibroblasts. We took advantage of the availability of T cell lines generated from SSc skin to address the question of whether T cells present in the inflammatory infiltrate of patients with early-stage disease could enhance collagen production by SSc or control fibroblasts. In particular, we used plasma cell membranes from skin-derived T cells as effectors to test whether T cells could modulate collagen production by fibroblasts via cell-to-cell contact.

A total of 11 representative SSc skin–derived T cell lines were assayed. All of them inhibited collagen production by both SSc and control fibroblasts. T cell membranes from activated T cells decreased spontaneous production of type I collagen on SSc fibroblasts by a mean (± SD) of 71.6 ± 15.0% and on control fibroblasts by 68.1 ± 19.4%. The T cell lines used in these experiments produced, to various extents, both IFNγ and IL-4 and could not qualify as bona fide Th1- or Th2-like T

cells. To overcome this limitation, we undertook similar experiments using plasma cell membrane preparations from highly polarized cloned CD4+ Th1 or Th2 cells generated from SSc skin or from peripheral blood obtained from healthy individuals (Figure 2). In all circumstances, collagen production was inhibited rather than enhanced.

Of interest, major differences were evident when Th1 and Th2 plasma cell membranes were used as effectors on SSc and control fibroblasts. Both activated Th1 cell membranes and activated Th2 cell membranes were inhibitory on control fibroblasts, with Th1 inhibiting collagen production to a significantly greater degree compared with Th2 ($P = 0.008$) (Figure 3). However, activated Th1 but not Th2 membranes inhibited collagen production by SSc fibroblasts ($P = 0.004$). Nonetheless, when compared with control fibroblasts, SSc fibroblasts were resistant to inhibition by Th1 cells ($P = 0.017$) (Figure 3). Overall, these results indicate that T cells, regardless of their functional phenotype (Th1 and Th2), deliver inhibitory signals to dermal fibroblasts via direct

Table 2. IFNγ and IL-4 production in T cell lines generated from SSc skin*

| Cell type | n | IFNγ/IL-4 production index | | | | |
		All subsets	CD4+	CD8+	CD4+,CD8+	CD4−,CD8−
SSc TCRα/β+	20	46.6 (19.4)†	53.9 (18.4)	38.4 (21.4)	30.1 (15.7)	34.3 (24.1)
Control TCRα/β+	6	84.8 (5.4)	81.3 (9.5)	88.7 (5.4)	82.3 (14.7)	81.2 (11.4)

* The potential for interferon-γ (IFNγ) and interleukin-4 (IL-4) production was evaluated at the single-cell level by intracellular staining upon T cell activation. The IFNγ/IL-4 production index was computed according to the following formula: (mean fluorescence intensity [MFI] IFNγ/[MFI IFNγ + MFI IL-4]) × 100. With this formula, pure Th1-like cells have an index of 100%, and pure Th2-like cells have an index of 1%. Systemic sclerosis (SSc) TCRα/β+ T cell lines were generated from SSc skin, and control TCRα/β+ T cell lines were derived from peripheral blood obtained from healthy individuals matched for sex and age.
† $P < 0.0001$, versus controls.
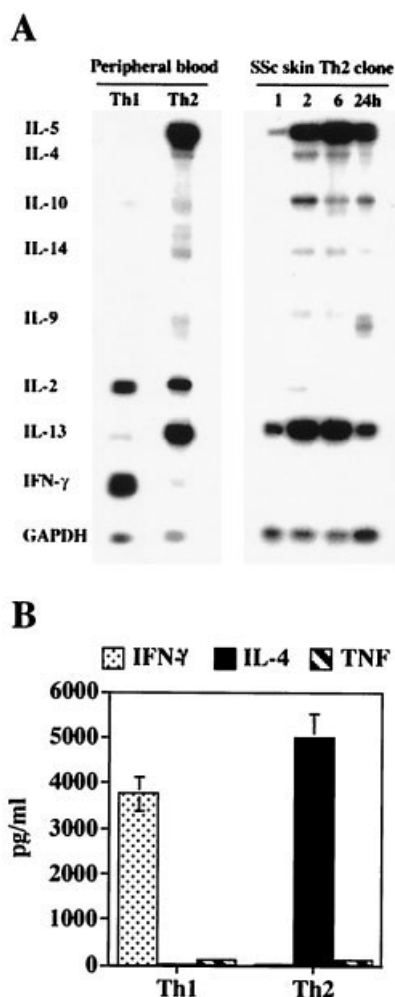
Figure 2. Characterization of SSc skin and peripheral blood T cell clones. **A,** RNase protection assay performed on total RNA extracted from T cell clones activated for 2 hours (or the indicated times for the SSc clone) by CD3 crosslinking in the presence of 5 ng/ml of phorbol myristate acetate. **B,** IFNγ, IL-4, and tumor necrosis factor α (TNFα) were quantified in the supernatants upon activation for 6 hours by CD3 crosslinking of all T cell clones used in this study (Th1, n = 6; Th2, n = 7). Bars show the mean and SEM. See Figure 1 for other definitions.

cell-to-cell interactions, and that SSc fibroblasts are less susceptible to inhibition than are control fibroblasts.
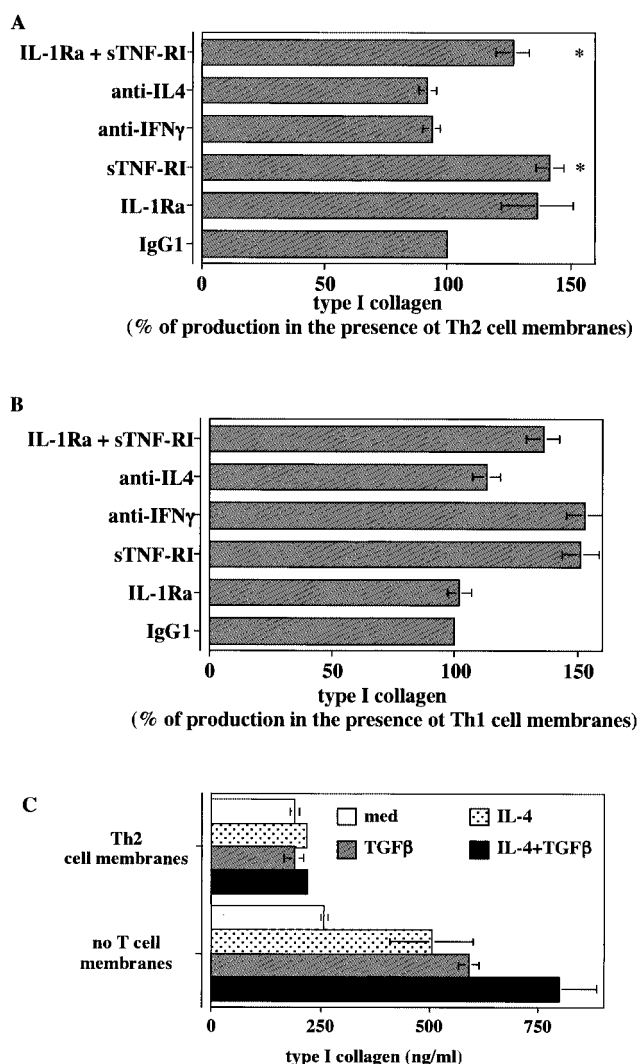
Involvement of membrane-bound TNFα in Th2 cell–contact-dependent inhibition of collagen production by fibroblasts. To identify the mechanisms involved in T cell contact–dependent inhibition of collagen production, we performed experiments in which several cytokines potentially contained in T cell membranes were neutralized. No effects on collagen production were observed when IL-4 and IFNγ were neutralized on

Th2 cell membranes, while the addition of IL-1Ra resulted in variable levels of reduced inhibition (P not significant) (Figure 4A). However, TNFα blockade by sTNFRI consistently resulted in a substantial reduction of the inhibitory activity of Th2 cell membranes (Figure 4A). In addition, when sTNFRI and IL-1Ra were added jointly, no additive effects were observed. Of interest, and consistent with previous findings (20), similar experiments performed with Th1 membranes revealed that, besides TNFα, IFNγ was also involved in contact-dependent inhibition of collagen production by Th1 cells (Figure 4B).

Further experiments were conducted to investigate whether the inhibition induced by Th2 cell membranes was counterbalanced by exogenous profibrotic cytokines. To this end, we cultured fibroblasts in the presence of soluble IL-4 and TGFβ before adding Th2
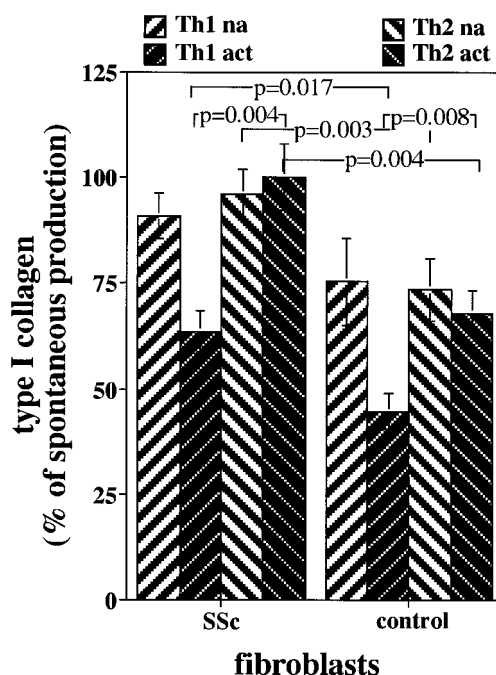
Figure 3. Type I collagen production is inhibited by contact with Th1 and Th2 cells, and systemic sclerosis (SSc) fibroblasts are resistant to inhibition by T cell contact. SSc fibroblasts (n = 3) and control fibroblasts (n = 3), matched for passage as well as for age and sex of the donor, were cultured in the presence of 20 μl of T cell membrane preparations equivalent to 8×10^5 cells from Th1 clones (n = 2) and Th2 clones (n = 3). The N-terminal type I procollagen propeptide was assessed in 48-hour culture supernatants. Bars represent the mean ± SEM of 6 individual experiments with Th1 membranes and 9 individual experiments with Th2 cell membranes. The mean ± SD spontaneous production of type I collagen was 298.88 ± 124.13 ng/ml for SSc fibroblasts and 208.42 ± 34.17 ng/ml for control fibroblasts. na = nonactivated; act = activated.

Figure 4. Th2 cell contact–dependent inhibition of collagen production is mediated by tumor necrosis factor α (TNFα) and is dominant on profibrotic cytokines. Control fibroblasts were cultured in the presence of 20 μl of T cell membrane preparations equivalent to 8 $\times$ 10^5 cells from 3 Th2 clones (**A** and **C**) and from 1 representative Th1 clone (**B**). Blocking reagents (**A** and **B**) or profibrotic cytokines (**C**) were added to fibroblast cultures 30 minutes before the addition of T cell membranes. The N-terminal type I procollagen propeptide was assessed in 48-hour culture supernatants. Bars show the mean $\pm$ SEM (**A** and **C**) and the mean $\pm$ SD (**B**). IL-1Ra = interleukin-1 receptor antagonist; sTNF-RI = soluble TNF receptor I; anti-IFNγ = anti–interferon-γ; med = medium; TGFβ = transforming growth factor β.

cell membranes. As expected, IL-4 and TGFβ potently enhanced collagen production. However, the inhibitory activity of Th2 cell membranes was dominant over the enhancement induced by IL-4, TGFβ, or even IL-4 combined with TGFβ (Figure 4C). Overall, these exper-

iments indicate that contact-dependent inhibition of collagen production by dermal fibroblasts is a powerful characteristic of Th2 cells and is mediated, at least in part, by membrane-bound TNFα.

Inhibition of fibroblast collagen production, by living Th2 cells. Because the results described above were inconsistent with the prediction indicating that Th2 cells, via their production of IL-4 and IL-13, should stimulate fibroblasts to produce collagen, we tested whether intact Th2 cells instead of Th2 cell membranes could have exerted profibrotic activities. Because living, unstimulated Th2 cells (and Th1 cells) cocultured with fibroblasts did not significantly modify collagen production (Figure 5), we tested 2 different experimental systems of T cell activation. To mimic an inflammatory mode of activation we used IL-2, and to mimic an antigen-dependent mode of activation we used CD3 crosslinking. Interestingly, IL-2–dependent activation resulted in significant collagen inhibition by Th1 clones but not by Th2 cell clones. No enhancement was observed (Figure 5).

When T cells were activated by CD3 crosslinking, both Th1 and Th2 cells proved to be powerful inhibitors of collagen production (Figure 5). In the same experimental settings, TNFα blockade resulted in a reversal of inhibition mediated by Th2 cell clones, while IL-4, IFNγ, and IL-1 neutralization was ineffective (Figure 6A). When various reagents, including anti–CD40L, anti–CD54 (ICAM-1), anti-IFNγ, and indomethacin, were
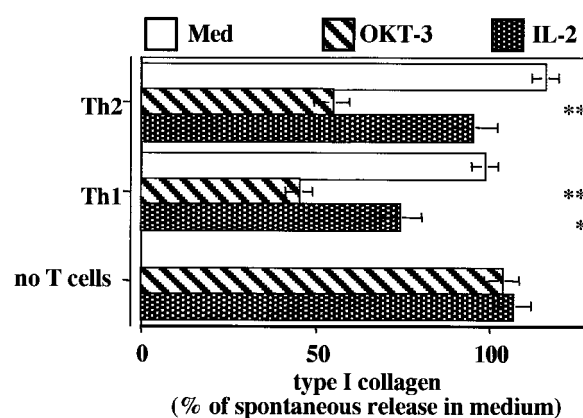
Figure 5. Living Th2 cells activated by CD3 crosslinking inhibit collagen production by cocultured fibroblasts. Fibroblasts were cultured in the presence of 20 $\times$ 10^3 Th cells in medium (med) or with OKT-3 (1 μg/ml) or interleukin-2 (IL-2) (20 units/ml). Th2 clones (n = 2) and Th1 clones (n = 2) were tested in parallel on control (n = 3) and SSc (n = 3) fibroblasts. Bars show the mean $\pm$ SEM. The mean $\pm$ SEM spontaneous production of type I collagen was 245.30 $\pm$ 20.55 ng/ml. $*$ = P = 0.003 versus medium; $**$ = $P < 0.001$ versus medium.

A

B
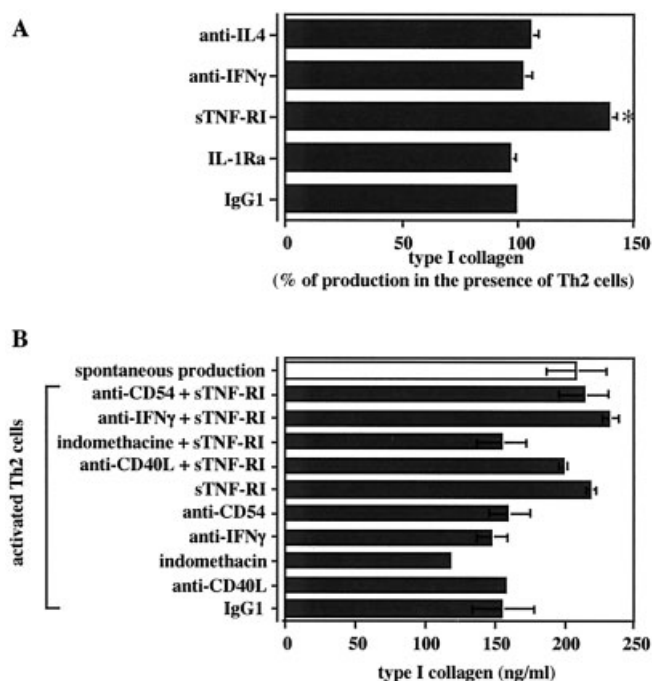
Figure 6. Tumor necrosis factor α (TNFα) neutralization reverses collagen inhibition by live activated Th2 cells. Fibroblasts were cultured in the presence or absence of 20×10^3 Th2 cell clones and OKT3 (1 μg/ml) monoclonal antibody. **A,** Three Th2 clones tested on 2 control fibroblasts. **B,** One Th2 clone tested on normal fibroblasts. Blocking reagents were added to fibroblast cultures 30 minutes before the addition of T cells. N-terminal type I procollagen propeptide was assessed in 48-hour culture supernatants. In **A,** bars show the mean ± SEM of 6 independent experiments. In **B,** bars show the mean ± SD of 1 experiment representative of 3 performed. The open bar represents the spontaneous production of collagen by fibroblasts cultured in the absence of T cells. Anti–IL-4 = anti–interleukin-4; anti-IFNγ = anti–interferon-γ; sTNFRI = soluble TNF receptor I; IL-1Ra = IL-1 receptor antagonist; anti-CD40L = anti–CD40 ligand. ∗ = $P < 0.001$ versus other culture conditions.

tested in conjunction with sTNFRI, no further reversal of inhibition with living Th2 cells was observed (Figure 6B). Thus, upon activation using a mode mimicking antigen-dependent recognition, living Th2 cells (as well as Th1 cells) are capable of inhibiting collagen production by cocultured fibroblasts.

Th2 cell stimulation of interstitial collagenase (MMP-1) production by fibroblasts. To test whether dermal fibroblasts could be stimulated rather than inhibited by T cells, we assessed the levels of interstitial collagenase in the culture supernatants in which collagen was measured. Upon CD3 crosslinking, living Th2 cells (and Th1 cells) substantially enhanced production of MMP-1 by fibroblasts (Figure 7A). Similar observations were made when plasma cell membranes from activated

Th2 cell clones were used as effectors, thus indicating that MMP-1 production depended, at least in part, on T cell fibroblast contact. Of note, when TNFα was neutralized, MMP-1 levels were significantly decreased in the presence of both Th2 and Th1 clones (Figures 7B and C). As expected, IFNγ neutralization resulted in enhanced MMP-1 production only upon induction with Th1 clones (Figure 7C) (29). These results strongly indicate that, under the culture conditions used, dermal fibroblasts were alive and capable of up-regulating their production of MMP-1 when cocultured with activated Th2 cells (and Th1 cells). These results also strengthen the above results on collagen inhibition and indicate that Th2 cells, as well as Th1 cells, specifically inhibit collagen production.

Th2 cell modification of steady-state levels of collagen and MMP-1 in dermal fibroblasts at the mRNA level. Finally, we tested whether the effects of Th2 cells on type I collagen and MMP-1 observed at the protein level were also detectable at the mRNA level. In a contact-dependent model of fibroblast activation, a significant reduction in type I collagen α1 and increased MMP-1 mRNA levels were consistently observed (Figure 8). Thus, Th2 cells affect the steady-state levels of type I collagen and MMP-1 in a manner that reflects protein production.

DISCUSSION

Among the earliest pathologic events documented in the skin of individuals in whom SSc develops, an inflammatory infiltrate rich in T lymphocytes is dominant (2). This observation has led to the hypothesis that T cells may participate in the initiation, or at least the amplification, of the processes leading to fibrosis (1). This hypothesis is even more likely because, according to several authors (8,9), skin-infiltrating T cells are capable of producing IL-4, a cytokine endowed with profibrotic potential (17,19). Results from the present study indicate that T cell lines generated from the skin of patients with early-stage SSc are indeed poised to produce high levels of IL-4; nonetheless, these T cells are capable of specifically suppressing the synthesis of type I collagen by dermal fibroblasts. Thus, this is the first report to show that 1) Th2 T cells exert inhibitory activity on collagen synthesis while inducing interstitial collagenase production at both the mRNA level and the protein level, 2) Th2 cells are antifibrotic mostly via their production of TNFα, 3) the contact between Th2 cells and fibroblasts is relevant to collagen inhibition, and 4) inhibition mediated by Th2 cells dominates the profi-
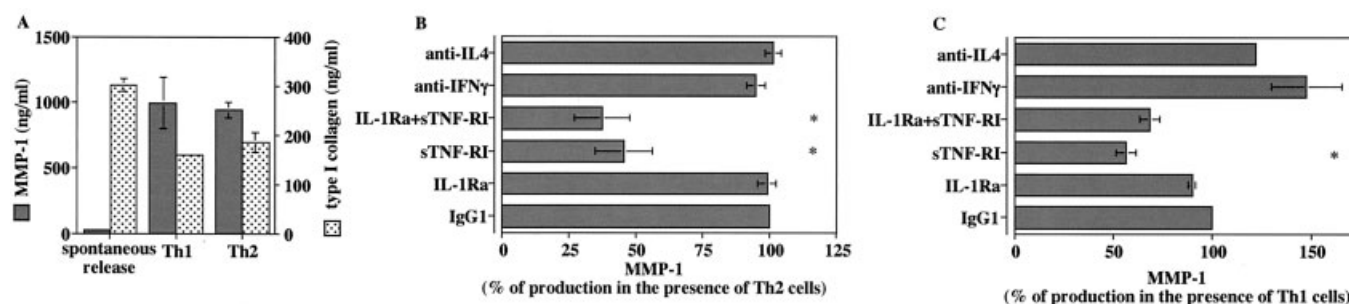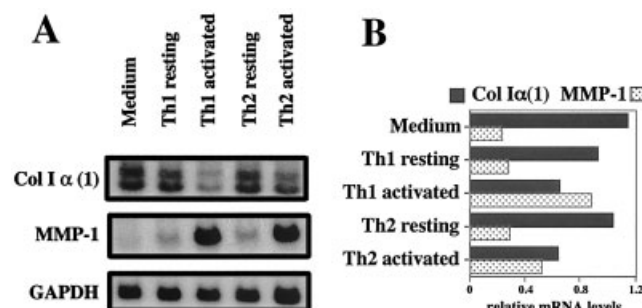
Figure 7. Th2 cells induce collagenase (matrix metalloproteinase 1 [MMP-1]) production by dermal fibroblasts. Fibroblasts were cultured in the presence of 20×10^3 Th2 or Th1 cell clones in medium supplemented with OKT3 (1 μg/ml). ProMMP-1 and type I procollagen were assessed in 48-hour culture supernatants. **A,** One Th2 clone and 1 Th1 clone was tested on control fibroblasts. **B,** Three Th2 clones were tested on 2 control fibroblasts. **C,** One Th1 clone was tested on 2 control fibroblasts. Blocking reagents were added to fibroblast cultures 30 minutes before the addition of T cells. Bars show the mean ± SEM. ∗ = $P < 0.01$ versus culture in the presence of irrelevant IgG1 monoclonal antibody. See Figure 6 for other definitions.

brotic activity of IL-4 and TGFβ. In addition, we confirm previous findings indicating that SSc fibroblasts are resistant to inhibition induced by T cells (20).

Based on our culture system, we generated from SSc skin highly heterogeneous T cell lines containing various proportions of CD4+, CD8+, CD4+,CD8+, CD4−,CD8−, TCRα/β+, and TCRγ/δ+ T cell subsets. The proportions of different subsets recovered after culture may imprecisely reflect the actual proportions of these subsets in the skin. Nonetheless, the fact that we were able to maintain such heterogeneity within replicate cultures of the same biopsy fragments strongly indicates that heterogeneity was indeed present in the original tissue and was not attributable to culture con-

Figure 8. Type I collagen and matrix metalloproteinase 1 (MMP-1) steady-state mRNA levels in fibroblasts activated by Th1 and Th2 cell contact. Fibroblasts were cultured for 14 hours in the presence of 200 μl of T cell membrane preparation equivalent to 8×10^6 cells from 1 Th1 clone and 1 Th2 clone, either nonactivated (resting) or activated by CD3 crosslinking. **A,** Northern blot analysis. **B,** Densitometric values of the ratios of type I collagen α1 (col Iα[1]) and MMP-1 to GAPDH mRNA.

ditions. In addition, the fact that we were able to clone and maintain T cells with extremely polarized phenotypes (pure Th1 or pure Th2 T cells) from SSc skin further indicates that our culture system provided a representative picture of T cells infiltrating SSc skin. Moreover, previous reports indicating that only CD4+ T cell lines can be generated by culturing SSc skin are at odds with the histologically documented presence of CD8+ T cells in addition to CD4+ T cells in SSc skin (3,4,6,8).

The CD8+ T cells we recovered from SSc skin, which had a higher potential to produce IL-4 than did CD4+ T cells, are reminiscent of similar cells observed in BAL fluid obtained from patients with SSc alveolitis (13). In addition, CD8+ T cells with high IL-4 production have also been described in the skin of individuals with human immunodeficiency virus infection who have Job's syndrome–like disease (30). The rare CD4−,CD8− double-negative TCRα/β+ T cell subset was detected in peripheral blood obtained from persons with SSc (31). No reports of CD4+,CD8+ double-positive TCRα/β+ T cells in SSc are available. Similar cells have been described in the skin of patients with atopic dermatitis (32) and are the focus of additional investigations (Parel Y, et al: unpublished observations). Th2 cell generation and function have been particularly well studied in allergic and parasitic disease models. The circumstances that allow T cells (especially CD8+ T cells) to polarize toward high IL-4 production in SSc need to be understood. In particular, it would be important to know whether T cells acquire the Th2 phenotype during antigen priming in the secondary lymphoid or-

gans or during trafficking in the skin undergoing fibrotic changes.

Based on strong evidence indicating that IL-4 and IL-13 mediate profibrotic activities in vitro and in vivo (for review, see ref. 1), we expected Th2 cells to enhance collagen production by dermal fibroblasts. This was not the case in our experimental settings. In all of our experiments, type I collagen production was inhibited when dermal fibroblasts from normal individuals were cultured in the presence of plasma cell membranes from Th2 cells or with living activated Th2 cells. Furthermore, neutralization experiments strongly indicated that TNFα mediated most of the inhibitory activity of Th2 cells. Indeed, TNFα is known to impede, in a dose-dependent manner, collagen production by fibroblasts by inhibiting the transcription of types I and III procollagen mRNA (33–35). In addition, molecular studies have shown that TNFα inhibits TGFβ signaling in human fibroblasts via activator protein 1 activation (36). Consistent with these findings, the inhibitory effect of Th2 cell membranes on collagen production was dominant over the enhancing effect of TGFβ and IL-4 (17,37,38).

The nature of the Th2 cells we used can be questioned. They were cells that produced high levels of IL-4 and little or no IFNγ, by intracellular localization as well as by analysis of culture supernatants upon CD3 crosslinking. The extended phenotype analyzed at the mRNA level by RPA identified IL-4, IL-5, IL-9, IL-13, and IL-14 (but not IFNγ) as prominent bands. In this respect, no differences were demonstrated between SSc skin–derived Th2 cell clones and Th2 cell clones generated from peripheral blood of healthy individuals (24 and Chizzolini et al: unpublished observations). However, based on TNFα protein levels, these Th2 cells could not be distinguished from Th1 cells. This is consistent with data reported in the literature for both human and mouse T helper clones. Indeed, the production of TNFα in Th1 and Th2 cells does not differ (39). The role of lymphotoxin β has not been tested in our experimental system, because lymphotoxin β is known to be expressed by Th1 cells but not by Th2 cells. The plasma cell membranes from activated T cells used in our experiments were prepared after 6 hours of activation by CD3 crosslinking. Thus, it can be argued that their activity on fibroblasts reflected the sum of biologic activities at a given time point unfavorable to collagen synthesis. However, inhibition was also observed when living T cells were cocultured with fibroblasts, allowing a full range of mediators to be expressed for 48 hours.

In addition to CD3 crosslinking that mimics antigen-dependent T cell activation, we tested a cytokine-based mode of T cell activation with IL-2, thought to mimic inflammation-dependent T cell activation (40,41). In these circumstances also, inhibition or no effect rather than enhancement of collagen production was observed. Thus, the bulk of our results strongly point to inhibition as being the net effect of Th2 cells on collagen synthesis by fibroblasts. In this respect, the present data do not support the hypothesis that fibroblasts are driven from a normal to SSc phenotype by Th1 or Th2 cell–dependent signals. On the contrary, based on our data, T cell contact as well as T cell proximity to fibroblasts appear to restrain collagen deposition and simultaneously enhance collagen degradation via the specific induction of MMP-1, a metalloenzyme whose key function is to initiate the digestion of native type I collagen (42).

In accordance with these results, we previously documented that Th2 cells are capable of inducing MMP-1 production in macrophages in an IL-4– and contact-dependent manner (43). How can we reconcile our findings with previously reported data (44,45) indicating that in early SSc skin lesions, collagen synthesis is higher in the proximity of the inflammatory infiltrate? It is possible that T cells may interact with a third type of cell (e.g., monocyte/macrophages or endothelial cells) to deliver profibrotic stimuli. Consistent with this hypothesis is the documented role of TGFβ up-regulation in macrophages followed by enhanced collagen synthesis and lung fibrosis in transgenic mice that express high levels of IL-13 in bronchial epithelial cells (46). Alternatively, T cells may be recruited in response to modifications of primitively activated fibroblasts, and then, according to our results, they may exert essentially inhibitory activities.

Of major interest, SSc fibroblasts were not susceptible to inhibition by Th2 cells when Th2 plasma cell membranes were used as effectors. Th1 cells inhibited SSc fibroblasts, but to a lesser extent than did control fibroblasts. These data confirm and extend previous findings indicating resistance to inhibition of SSc fibroblasts by T cells (20). IFNγ in Th1 cell membranes mediated most of the inhibitory effect of Th1 cells (20), and its absence in Th2 cells may explain the differential effect on SSc fibroblasts of Th2 versus Th1 cells. However, the preferential presence of Th2-polarized cells in SSc skin undergoing fibrosis suggests that, in vivo, TNFα may be involved in inhibition to a much greater degree than is IFNγ. SSc fibroblasts are known to have an intracellular signaling machinery set to maintain high collagen production and to maintain such phenotype in

culture (47,48). This may explain their resistance to inhibition.

In mice transgenic for TNFα production in alveolar epithelium, a pulmonary disease develops that, upon inflammation, results in lung fibrosis (49). A similar model does not exist for dermal fibrosis, but TNFα has been identified in the skin of patients with early-stage SSc (7). To reconcile the role of TNFα as a profibrotic agent in vivo (49) with the documented capacity of TNFα to inhibit collagen production in vitro (35), it has been proposed that TNFα may inactivate collagen receptors expressed on fibroblasts and involved in collagen catabolism (50). Our results, which are in agreement with those from previous studies, indicate that TNFα produced by Th2 cells (and Th1 cells) simultaneously down-regulates collagen production and up-regulates collagenase production by fibroblasts, pointing to a direct antifibrotic effect of TNFα on fibroblasts.

Thus, it could be hypothesized that fibroblasts in inflamed tissues undergoing fibrosis may become insensitive to TNFα. This may be attributable to down-regulation of TNF receptors or to inactivation of transcription factors used in TNF signaling. In this respect, it is interesting to note that in dermal fibroblasts, the c-Jun N-terminal kinase and not the nuclear factor κB kinase pathway is used by TNFα to inhibit collagen gene transcription by preventing Smad-3 binding to cognate DNA sequences or by sequestering the transcriptional coactivator p300 shared with Smad (36,51,52). Therefore, it can be hypothesized that in SSc fibroblasts, the transcriptional machinery is poised to sustain collagen synthesis because intracellular negative regulatory loops attenuating Smad-specific responses are deficient. As such, these results indicate that the use of TNFα-blocking agents to treat patients with fibrotic disease (and in particular those with SSc) may be counterproductive.

Although both Th1 and Th2 cell lines from SSc skin inhibited collagen production in fibroblasts by a contact-dependent mechanism, Th2 cells were less inhibitory than were Th1 cells, supporting a role for Th2 cells in the formation of skin fibrosis. In addition, fibroblasts from patients with SSc were resistant to contact-dependent inhibition, adding target cell resistance to a profibrotic balance in SSc.

ACKNOWLEDGMENT

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