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Abstract

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Interleukin-17A+ Cell Counts Are Increased in Systemic Sclerosis Skin and Their Number Is Inversely Correlated With the Extent of Skin Involvement

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Objective. Levels of interleukin-17A (IL-17A) have been found to be increased in synovial fluid from individuals with systemic sclerosis (SSc). This study was undertaken to investigate whether IL-17A–producing cells are present in affected SSc skin, and whether IL-17A exerts a role in the transdifferentiation of myofibroblasts.

Methods. Skin biopsy samples were obtained from the involved skin of 8 SSc patients and from 8 healthy control donors undergoing plastic surgery. Immunohistochemistry and multicolor immunofluorescence techniques were used to identify and quantify the cell subsets in vivo, including IL-17A+, IL-4+, CD3+, tryptase-positive, α-smooth muscle actin (α-SMA)–positive, myeloperoxidase-positive, and CD1a+ cells. Dermal fibroblast cell lines were generated from all skin biopsy samples, and quantitative polymerase chain re-

action, Western blotting, and solid-phase assays were used to quantify α-SMA, type I collagen, and matrix metalloproteinase 1 (MMP-1) production by the cultured fibroblasts.

Results. IL-17A+ cells were significantly more numerous in SSc skin than in healthy control skin (P = 0.0019) and were observed to be present in both the superficial and deep dermis. Involvement of both T cells and tryptase-positive mast cells in the production of IL-17A was observed. Fibroblasts positive for α-SMA were found adjacent to IL-17A+ cells, but not IL-4+ cells. However, IL-17A did not induce α-SMA expression in cultured fibroblasts. In the presence of IL-17A, the α-SMA expression induced in response to transforming growth factor β was decreased, while MMP-1 production was directly enhanced. Furthermore, the frequency of IL-17A+ cells was higher in the skin of SSc patients with greater severity of skin fibrosis (lower global skin thickness score).

Conclusion. IL-17A+ cells belonging to the innate and adaptive immune system are numerous in SSc skin. IL-17A participates in inflammation while exerting an inhibitory activity on myofibroblast transdifferentiation. These findings are consistent with the notion that IL-17A has a direct negative-regulatory role in the development of dermal fibrosis in humans.

Systemic sclerosis (SSc) is an autoimmune disease that results in fibrosis of the skin and internal organs. Immuno-inflammatory events are characteristi-
cally present in SSc and thought to participate in disease development (1,2). Fibrosis in SSc can be partly attributed to the activation of tissue-resident fibroblasts and their transdifferentiation into myofibroblasts. In SSc skin, fibroblasts are known to express α-smooth muscle actin (α-SMA), actively synthesize type I collagen and
other extracellular matrix (ECM) components, and display increased numbers in the affected skin, particularly in the deep dermis (3,4).

T cell abnormalities have been documented in patients with SSc, with several reports indicating that Th2-like cells producing interleukin-4 (IL-4) and IL-13 are preferentially increased in the peripheral blood and target organs of patients with SSc (5–8). Since IL-4 and IL-13 have direct profibrotic properties that enhance ECM deposition (9,10), Th2-like cells are thought to have pathogenic potential in SSc. Recently, we and other investigators have documented an increase in the number of IL-17+ and Th17 cells in the peripheral blood and target organs of patients with SSc (11–17). Moreover, IL-17 signaling–related genes were found to be preferentially expressed in the lung tissue of patients with pulmonary arterial hypertension (18).

In rodents, IL-17A is thought to have a profibrotic effect on fibroblasts, favoring collagen deposition in vitro and participating in the development of bleomycin-induced lung and skin fibrosis (19–21). Conversely, in humans, although IL-17 induces fibroblast proliferation (11) and the production of IL-6 and IL-8 (22,23), it seems to not directly induce collagen synthesis (11), but may actually restrain type I collagen production (24). In addition, in a model assessing human colonic subepithelial myofibroblasts, IL-17A was shown to induce proinflammatory responses, by increasing the secretion of cytokines and matrix metalloproteinases (MMPs) (25,26). Similarly, IL-17 was shown to induce MMP production in human fibroblast-like synoviocytes from rheumatoid arthritis patients (27,28).

Mast cells are powerful producers of inflammatory mediators, including cytokines (29). IL-17A–producing mast cells have been identified in the affected skin of patients with psoriatic arthritis, as well as in the synovium of patients with rheumatoid arthritis or spondyloarthritis (30–32). Increased numbers of mast cells have been observed in affected SSc skin, although this appears to be dependent on the stage of the disease (33), and these cells have been shown to degranulate in vivo (34,35). Mast cells have been proposed to participate in the development of fibrosis; however, results have been inconclusive and do not clearly support a profibrotic potential of these cells, particularly in SSc (29).

Since only limited information exists on the presence and cell origin of IL-17 in SSc and since a better understanding of the role of IL-17 in the development of fibrosis in humans is needed, we addressed whether IL-17+ cells could be found in the affected skin of SSc patients. Our results show that cells producing IL-17A are largely more numerous in SSc skin than in normal skin, and comprise both Th17 cells and IL-17A+ mast cells. Of major importance, we observed a nonrandom proximity between IL-17A+ cells and myofibroblasts in SSc skin. However, IL-17A did not induce α-SMA expression in dermal fibroblasts, but rather induced the production of MMP-1. Furthermore, the number of IL-17A+ cells was negatively correlated with the extent of skin fibrosis as assessed by the modified Rodnan skin thickness score (MRSS). These findings provide new evidence to support a role for IL-17A as a negative regulator of the dermal fibrotic process in SSc.

PATIENTS AND METHODS

Subjects. Eight patients with SSc (mean age 56.8 years, range 29–72 years) presenting at the Rheumatology Unit of the Gaetano Pini Hospital in Milan, Italy over a 2-week period were prospectively included. All patients met the American College of Rheumatology diagnostic criteria for SSc (36), with subsets defined according to the classification criteria of LeRoy et al (37). None of the patients had received immunosuppressive therapy, apart from a low dose of glucocorticoids (≤6 mg per day), at the time of sampling (details on the clinical characteristics of the patient population are available from the corresponding author upon request). Skin biopsies were performed on the affected skin of all SSc patients. The control group consisted of 8 age- and sex-matched healthy donors who had undergone corrective breast or abdomen surgery at the Department of Plastic Surgery of Clinique de La Tour in Geneva, Switzerland. None of the healthy individuals had dermatologic disorders and none had received immunosuppressive agents or glucocorticoids.

Skin biopsy samples were divided into 2 fragments. One of the fragments was embedded in paraffin for immunohistochemical processing, and the other was used for ex vivo fibroblast generation. This study was approved by the ethics committee of the institutions involved and was conducted according to the Declaration of Helsinki. Written informed consent was obtained from each individual.

Reagents. Polyclonal goat anti-human IL-4, polyclonal goat anti-human IL-17A, recombinant human IL-17A, recombinant human transforming growth factor β (TGFβ), and the MMP-1 enzyme-linked immunosorbent assay (ELISA) Duoset kit were from R&D Systems. Dulbecco’s modified Eagle’s medium (DMEM), phosphate buffered saline (PBS), glutamine, penicillin, streptomycin, trypsin, and fetal calf serum (FCS) were from Gibco. Type I collagenase, tyramine, and 3,3’-diaminobenzidine (DAB) were from Sigma. The human Vectastain ABC kit was from Vector, UK. Monoclonal mouse anti-human mast cell tryptase (clone AA1) and biotinylated goat and mouse immunoglobulins were from Dako, while Alexa 488–conjugated donkey anti-goat and Alexa 568–conjugated donkey anti-mouse or anti-rabbit antibodies were from Invitrogen. Fluoprep mounting medium was from BioMerieux. The N-terminal propeptide of type I procollagen (PINP) assay was from Orion Diagnostica. Anti–α-SMA (clone 1A4), anti-CD68 (clone KP1), and anti-CD1a (clone O10)
were from Immunotech. Monoclonal mouse anti-human anti-CD3 (clone PS1) and polyclonal rabbit anti-human myeloperoxidase (MPO) were from Novocastra.

**Fibroblast cultures.** Fibroblast cell lines were generated from the skin biopsy samples after 0.1% type I collagenase digestion of skin tissue sections at 37°C for 2 hours. Adherent cells were grown in DMEM containing 1% non-essential amino acids, 1% l-glutamine, 1% sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% FCS. Fibroblasts were used between the third and sixth passages.

**Immunohistochemistry.** After being deparaffinized in xylene and absolute ethanol, tissue sections were placed in a jar filled with citrate buffer, pH 7.5, and heated in a microwave at 600W for 20 minutes. Endogenous peroxidase activity was inhibited by treatment with 0.3% hydrogen peroxide in 50% methanol. The tissue sections were blocked in PBS containing 4% bovine serum albumin (BSA) for 1 hour at room temperature, incubated for 1 hour with primary antibodies diluted in PBS–4% BSA, washed, and incubated for 30 minutes with a biotinylated secondary antibody. Immune reactivity was detected using the Vectastain ABC kit, using DAB as substrate.

**Quantification of immunohistochemical and indirect immunofluorescence results.** Immunohistochemical images were acquired by scanning of the whole tissue sections using a Mirax scan. At least 2 sections, having a mean area of 11.9 mm², were analyzed per individual. A semiautomated method to quantify the number of IL-17A+ cells in each region of the skin was developed using Metamorph/MetaXpress software. A threshold for positivity was defined for each color channel. DAPI staining (blue) was used to determine a size limit for subsequent analysis. This mask was applied to pictures obtained with the green and red channels. The resulting images were semiautomatically processed with the Integrated Morphometry Analysis tool, which measures the number of positive cells and the area parameters. At least 10 fields of 0.044 mm², as defined by the microscope, were analyzed. Results are expressed as the number of positive cells per surface and per field.

To assess the proximity between α-SMA–positive cells (red) and IL-17A– or IL-4–positive cells (green), the entire tissue sections were manually scanned by the operator. An image for every α-SMA–positive cell having the morphologic features of a myofibroblast was captured and analyzed for the presence of adjacent green-staining cells. Morphologic discrimination between α-SMA–positive pericytes and myofibroblasts was manually performed by the operator. To verify the coherence of the results, representative images were independently reviewed by 2 operators (MET and PL).

**Real-time quantitative polymerase chain reaction (PCR).** Total RNA was extracted from trypsinized fibroblasts using the RNEasy Micro kit (Qiagen), and complementary DNA was synthesized from 0.25 μg of total RNA using random hexamers and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. SYBR Green assays were performed on a 7900HT sequence detection system (SDS) (Applied Biosystems). Each reaction was performed in triplicate. Raw threshold cycle values obtained with SDS version 2.2.2 software (Applied Biosystems) were analyzed as described previously (38), and the more stable housekeeping genes (GAPDH and EEF1A1) were selected for normalization. Oligonucleotides were obtained from Invitrogen (Hs_α-SMA and Hs_GAPDH) and Qiagen (Hs_EEF1A1_2_5G, catalog no. QT01669934).

**Western blotting.** Fibroblasts were lysed for 10 minutes on ice in prechilled radioimmunoprecipitation assay buffer supplemented with 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 100 mM okadic acid, 1× Complete protease inhibitor cocktail (Roche), and 0.2 mM phenylmethylsulfonyl fluoride. Protein extracts were clarified by centrifugation and stored at −20°C until used. For Western blotting, 15 μg of total protein extract was separated in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, under reducing conditions, and electroblotted onto nitrocellulose membranes (Amersham Hybond enhanced chemiluminescence [ECL]; GE Healthcare Zurich). Blots were incubated with mouse anti–α-SMA (Abcam) or anti–β-tubulin (Sigma) antibodies, followed by incubation with peroxidase-conjugated mouse antiserum. An ECL blotting analysis system (GE Healthcare Life Sciences) was used for antigen–antibody detection. Quantification analysis was performed with ImageJ software (http://rsbweb.nih.gov/ij) and values were normalized to those for β-tubulin.

**MMP-1 and collagen assays.** MMP-1 was quantified in culture supernatants by ELISA (R&D System), and collagen production was assessed by radioimmunoassay quantification of PINP (Orion Diagnostica) according to the manufacturer’s instructions.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism software version 4.0. Nonparametric data were used to compare populations. The Pearson’s test was used for correlation studies. P values less than 0.05 were considered statistically significant. Box plots were automatically generated, in which the box represents the 25th and 75th percentile values, the line within the box represents the median (50th percentile), and the whiskers extend above and below the box to show the 5th and 95th percentiles.
RESULTS

Overexpression of IL-17A and increased numbers of Th17 cells in SSc skin. We first aimed at determining the presence and topographic location of IL-17A+ cells in healthy and affected SSc skin. IL-17A+ cells were detected in all SSc and control skin biopsy samples examined (Figures 1A and B). However, IL-17A+ cells were significantly more numerous in the 8 clinically involved SSc skin samples, whereas only a few positive cells were found in the 8 skin samples from healthy individuals (Figure 1C). IL-17A+ cells were evenly distributed in the superficial (papillary) and deep (reticular) dermis (Figure 1D). These findings were specific, since use of the negative control antibody resulted in very little or no background staining (results not shown). No differences were observed between patients with limited cutaneous SSc and those with diffuse cutaneous SSc or between patients with early SSc and those with late SSc (results not shown).

We then assessed whether the IL-17A–expressing cells were T cells, by costaining the tissue sections with anti-CD3 monoclonal antibodies (Figure 2A). Within the T cell population, those cells expressing IL-17A, namely Th17 cells, were observed in both SSc and normal skin. Using a semiautomated and unbiased analysis of 10 fields for each section (2 skin sections per sample), we found a greater number of T cells per field in SSc skin than in healthy skin (Figure 2B). More interestingly, Th17 cells were more numerous, when assessed as numbers per field, numbers per normalized area, and as a percentage of T cells, in SSc skin compared to healthy skin (Figure 2C). The fact that the number of Th17 cells was higher in SSc skin than in control skin, taking into account the surface as well as the total number of cells, indicates that this finding was robust, irrespective of the density of the inflammatory infiltrate and the thickness of the dermis.

Figure 1. Overrepresentation of interleukin-17A+ (IL-17A+) cells in systemic sclerosis (SSc) skin compared to healthy control skin. A and B, Immunohistochemical analysis was used to detect IL-17A in healthy skin (A) and lesional SSc skin (B). The arrow indicates IL-17A+ (brown staining) cells. Results shown are representative of 8 healthy donor and 8 SSc skin biopsy samples. Original magnification × 20. Insets, Higher-magnification views (original magnification × 40). C and D, The frequency of IL-17A–producing cells was determined by histologic examination of the skin tissue samples (entire dermis [C] or superficial and deep dermis [D]) from healthy donors (HD) and SSc patients. Each symbol represents an individual subject; horizontal lines show the mean. Significance was assessed by Mann-Whitney test. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.37860/abstract.

Figure 2. Increased numbers of Th17 cells in SSc skin compared to healthy control skin. A, Immunofluorescence confocal laser scanning microscopy was used to assess expression of IL-17A (green), in combination with staining for CD3 (red) and DAPI staining of nuclei (blue), in one representative skin section. Original magnification × 40. B and C, The number of T cells (CD3+ cells per field) (B) or Th17 cells (CD3+IL-17A+ cells per field, per area, and as a percentage of T cells) (C) was determined in 8 healthy donor and 8 SSc skin samples. Values are shown as box plots, in which the boxes represent the 25th and 75th percentiles, the lines within the boxes represent the median, and the whiskers show the 5th and 95th percentiles. Significance was assessed by Mann-Whitney test. See Figure 1 for definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.37860/abstract.
identifying mast cells, with CD68 for macrophages, with MPO for neutrophils, and with CD1a for Langerhans' cells. We found that both in SSc skin and in healthy skin, IL-17A mainly colocalized with tryptase (Figure 3A, yellow arrow), thus identifying mast cells as major producers of IL-17A.

However, not all mast cells were IL-17A positive. The number of tryptase-positive mast cells tended to be higher in SSc skin compared to healthy skin ($P = 0.06$) (Figure 3B). More impressively, by using colocalization, we found that the number of IL-17A+ mast cells was significantly higher per field ($P = 0.03$) and as a percentage of all mast cells ($P = 0.02$) in SSc skin compared to control skin (Figures 3C and D). In addition to mast cells and T cells, neutrophils and macrophages accounted for 10–20% of all IL-17A+ cells in SSc skin. We were unable to identify IL-17A+CD1a+ cells in any of the skin samples (results not shown). Overall, these findings strongly indicate that IL-17A–producing cells belonging to both the innate (mast cells) and adaptive

![Figure 3](image)

**Figure 3.** Increased numbers of IL-17A+ mast cells in SSc skin compared to healthy control skin. A, Immunofluorescence analysis was used to assess expression of IL-17A (green), in combination with staining for tryptase (red) and DAPI staining of nuclei (blue), in one representative skin section. Original magnification × 40. The arrows indicate tryptase-positive IL-17A+ mast cells. The broken line indicates the dermo–epidermal junction. B–D, The number of tryptase-positive cells (B) or tryptase-positive IL-17A+ cells (number per field [C] and as a percentage of all mast cells [D]) was determined in 8 healthy donor and 8 SSc skin samples. Values are shown as box plots, in which the boxes represent the 25th and 75th percentiles, the lines within the boxes represent the median, and the whiskers show the 5th and 95th percentiles. Significance was assessed by Mann-Whitney test. See Figure 1 for other definitions.

![Figure 4](image)

**Figure 4.** Preferential proximity of myofibroblasts to IL-17A+ cells in SSc skin. A and B, Indirect immunofluorescence analysis was used to assess expression of IL-17A (A) or IL-4 (B) (green) and α-smooth muscle actin (α-SMA) (red), in combination with DAPI staining of nuclei (blue), in the superficial dermis (i) and deep dermis (ii) of SSc skin. Note that in B (panel i), the α-SMA–positive cells have the morphologic appearance of pericytes. Original magnification × 40 in i, × 63 in ii. C, The number of IL-17A+ or IL-4+ (cytokine+) cells and α-SMA–positive cells (myofibroblasts) per field was evaluated by semiautomated counting. Proximity was defined as a distance of ≤10 μm between α-SMA-positive myofibroblasts and IL-17A+ or IL-4+ cells. Values are shown as box plots, in which the boxes represent the 25th and 75th percentiles, the lines within the boxes represent the median, and the whiskers show the 5th and 95th percentiles. Solid circles are outliers. Significance was assessed by Mann-Whitney test. See Figure 1 for other definitions.

(T cells) immune response are increased in the skin of patients with SSc.
Proximity of IL-17A cells and α-SMA–positive fibroblasts in vivo. Fibroblasts expressing α-SMA, namely myofibroblasts, are endowed with high synthetic capacity and are characteristically present in SSc skin (4). To address whether there may be a topographic relationship between IL-17A cells and myofibroblasts, we assessed whether α-SMA–positive cells could be found in close proximity to IL-17A cells. Consistent with previous reports (4), in normal skin, we observed α-SMA–positive pericytes and follicular smooth muscle cells, but we were not able to detect myofibroblasts (39). In SSc skin, however, α-SMA–positive myofibroblasts were easily found (Figure 4A, panel i) and were localized mostly in the deep dermis (Figure 4A, panel ii). Intriguingly, at least one IL-17A+ cell was found in close proximity (within 10 μm) to every α-SMA–positive myofibroblast (Figure 4A, panels i and ii). This pattern was not observed for IL-4+ cells (most likely, Th2 cells) (40), which were mostly found in the superficial dermis in a perivascular distribution (Figure 4B, panel i), and in no case were they found in proximity to α-SMA–positive myofibroblasts (Figure 4B, panel ii), confirming the specificity of our findings.

When assessed by semiautomated counting, we found significantly higher numbers of IL-17A+ cells, compared to IL-4+ cells, in close proximity to skin myofibroblasts (Figure 4C). These results identify a topographic relationship between IL-17A+ cells and α-SMA–positive myofibroblasts.

Inhibitory role of IL-17A against α-SMA expression induced by TGFβ in dermal fibroblasts. Since IL-17A+ cells were found in close proximity to myofibroblasts, we next addressed whether IL-17A could be directly involved in α-SMA–positive myofibroblast generation. When SSc and healthy control dermal fibroblasts were cultured in vitro with IL-17A, there was no observable increase in α-SMA levels, neither at the steady-state messenger RNA (mRNA) level as detected by quantitative PCR nor at the protein level as detected by Western blotting (Figures 5A and B). In contrast, TGFβ, used as a positive control, induced a powerful increase in α-SMA expression, at both the mRNA and protein level (Figures 5A and B).

We then investigated whether IL-17A could modulate the capacity of TGFβ to induce α-SMA in fibroblasts. In the presence of IL-17A, the α-SMA synthesis...
driven by TGFβ was partially, but significantly, inhibited in both SSc and healthy dermal fibroblasts (Figure 5B). This effect of IL-17A appeared to be specific for α-SMA expression, since IL-17A did not directly induce/reduce nor modulate the type I collagen production induced by TGFβ (Figure 5C). Most interestingly, under the same culture conditions, IL-17A induced a dramatic and specific (since it was inhibited by IL-17A blockade) increase in MMP-1 protein production in SSc and healthy dermal fibroblasts (Figure 5D).

**Inverse relationship between the frequency of IL-17A+ cells and the extent of skin thickness.** We then sought to determine whether the number of IL-17A+ cells present in the skin might be correlated with meaningful clinical parameters. To this aim, we assessed the overall extent of skin thickness using the MRSS scoring system, which largely reflects the fibrotic process in SSc skin (41). When the frequency of IL-17A+ cells or the number of IL-17A+ cells per area was plotted against the MRSS value for each of the 8 SSc skin samples, we found a strong negative correlation (Pearson’s r = −0.8374, P = 0.0095 and Pearson’s r = −0.735, P = 0.0458, respectively) (Figure 6). In contrast, no association was found between the frequency of IL-17A+ cells and either age, limited versus diffuse cutaneous SSc, disease duration, diffusing capacity of the lung for carbon monoxide, presence of digital ulcers, or the autoantibody status. Furthermore, the past use of immunosuppressive agents (>6 months prior to biopsy) in 2 of the individuals assessed (open circles in Figure 6) was not systematically associated with the lowest numbers of IL-17A+ cells, thus arguing against an effect of previous therapy in our results. Within the limit of the small size of our study population, the reverse relationship between IL-17A+ cells and the MRSS suggests that IL-17A–producing cells may oppose, rather than favor, the development of skin fibrosis.

**DISCUSSION**

In the present study, we demonstrate that IL-17A+ producing cells are present in the skin and that their numbers are higher in SSc affected skin compared to the skin of healthy donors. Surprisingly, the frequency of IL-17A+ cells was found to be correlated inversely with the extent of skin fibrosis as assessed by the MRSS. Moreover, in vitro, IL-17A did not favor type I collagen production, but rather decreased the differentiation of fibroblasts into myofibroblasts in response to TGFβ and directly enhanced MMP-1 production by dermal fibroblasts. Taken together, our findings support a role for IL-17A+ cells in controlling, rather than enhancing, skin fibrosis in SSc.

Our findings confirm and extend those from a previous report, mostly based on the quantification of IL-17A mRNA, indicating that IL-17A expression is enhanced in SSc skin compared to control skin (24). By careful histologic analysis, we observed, for the first time, that IL-17A+ cells were present in both the

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Inverse relationship between the frequency of interleukin-17A+ (IL-17A+) cells and the extent of skin thickness. The modified Rodnan skin thickness score (MRSS) was assessed by 2 independent clinicians at the time of skin biopsy. IL-17A+ cells were identified by immunohistochemistry. Each solid circle represents a single individual. Open circles indicate individual patients who received immunosuppressive agents more than 6 months before the biopsy. The upper panel shows the percentage of IL-17A+ cells of all cells examined. The lower panel shows the absolute number of IL-17+ cells per unit of dermis surface examined. Significance was assessed by Pearson’s correlation test.
superficial and deep dermis. This was in sharp contrast with that observed for IL-4+ cells, which were located essentially in the superficial dermis, mostly with a perivascular location. The interest of this observation relies on the fact that myofibroblasts (α-SMA–positive fibroblasts), as has been reported previously (4), were found to be essentially present in the deep dermis of SSc skin. Thus, IL-17A+ cells, rather than IL-4+ cells, were in close topographic proximity to biosynthetically active fibroblasts, an observation that argues in favor of a nonrandom association. For this reason, we studied the effect of IL-17A on dermal fibroblasts in vitro. Much to our surprise, IL-17A did not induce the expression of α-SMA in SSc and healthy control dermal fibroblasts, either at the mRNA or protein level, but rather reversed the increased expression of α-SMA induced in response to TGFβ.

Furthermore, IL-17A did not directly favor type I collagen production and did not enhance type I collagen production induced by TGFβ. However, consistent with previous results obtained with fibroblast-like synoviocytes (22), IL-17A induced MMP-1 production by dermal fibroblasts. Furthermore, in partial agreement with recent data indicating that IL-17A decreases type I collagen production specifically in normal, but not in SSc, fibroblasts (24), we found that IL-17A inhibited α-SMA expression induced by TGFβ in both SSc and healthy fibroblasts, whereas we did not observe any detectable effect on type I collagen production. Moreover, we observed that Th17 cells potently inhibited collagen production in healthy and SSc fibroblasts (results not shown). Therefore, taken together, our results and those from previous studies support a model that could be specifically applicable to human cells, and possibly different from murine models, in which IL-17A+ cells might be specifically attracted by myofibroblasts and may actually participate in inflammation by not necessarily favoring, but rather inhibiting, fibrosis (9,24).

Many cell types are known to contribute to the production of IL-17A in the skin. It has been previously shown that mast cells are abundant in SSc skin, although data have been controversial with regard to their relative numbers, possibly varying according to disease subtype and disease duration (29). However, in involved skin, mast cells appear to actively degranulate (34) and may represent a major source of TGFβ (35). By using tryptase to identify mast cells in the skin (42), we found that the mast cell numbers tended to be increased in SSc skin compared to healthy skin; however, those cells coexpressing IL-17A and tryptase were dramatically more numerous in SSc skin. In psoriatic skin, mast cells represent the major source of IL-17A and have been proposed to be central to the pathogenesis of the disease in association with IL-17A+ neutrophils, via degranulation and extracellular trap formation induced by IL-1 and IL-23 (31). To the best of our knowledge, the expression of IL-17A by mast cells has been described in psoriasis, and IL-17A+ mast cells have been observed in the inflamed joints of patients with rheumatoid arthritis, patients with psoriatic arthritis, patients with ankylosing spondylitis, and patients with osteoarthritis (30,32). Remarkably, at variance with SSc, all these conditions are characterized by tissue inflammation and/or destruction, rather than fibrosis.

The increased numbers of IL-17A+ cells were observed in all skin biopsy samples obtained from the affected SSc skin of prospectively recruited inpatients with active disease. These included individuals with either limited or diffuse cutaneous SSc, with various lengths of disease duration and various medication histories. Further studies should address whether the IL-17A+ cell numbers are increased in clinically uninvolved skin, and whether they appear early in the disease course and are still present late in the atrophic phase of the disease. Although, in vitro, IL-17A exerts mostly an antifibrotic activity when directly acting on fibroblasts, the high levels of IL-17A in the skin of SSc patients do not completely prevent fibrosis. It should be considered that SSc fibroblasts, when embedded in tissue matrix, are under the influence of several mediators and may be intrinsically more resistant to inhibition than their healthy counterparts (1). Nevertheless, we found that the frequency of IL-17A+ cells was inversely correlated with the MRSS, thus supporting the notion that, consistent with the in vitro data, IL-17A–producing cells have a protective role in fibrosis.

The reason that IL-17A is overexpressed in SSc skin is not known. Tumor necrosis factor (TNF) levels are increased in patients with SSc (43,44), and TNF has been shown to induce IL-17A expression in mast cells in a retinoic acid receptor–related orphan nuclear receptor–dependent manner (30). In addition, it is very likely that IL-23 could play a role, since, in addition to displaying increased levels in the sera of SSc patients (14,45), it, in conjunction with inflammatory cytokines, is among the factors allowing Th17 cell differentiation and expansion in humans (46). Furthermore IL-23 participates in the activation of mast cells (31). In this respect, we have recently shown that prostaglandin I2 (PGI2) analogs increase Th17 cell responses in patients with SSc by favoring IL-23 production by monocytes (47). Whether PGI2 analogs could also affect the
proportion of IL-17A+ cells in the skin remains to be established; however, none of the 8 patients with SSc who donated skin samples in our study had received iloprost in the previous 6 months.

A further unanswered question is why the levels of IL-23 are increased in SSc. In analogy with the recent demonstration that binding of interferon regulatory factor 3 to the IL-23p19 promoter correlated with the levels of IL-23 in patients with systemic lupus erythematosus (SLE) (48), it could be hypothesized that similar mechanisms may be instrumental in patients with SSc to induce IL-23 and, thereafter, IL-17 production. This is in keeping with the observed commonalities across autoimmune systemic disorders, in particular between SLE and SSc. Indeed, although their phenotypic expression may differ, SSc and SLE share abnormalities at the level of soluble mediators thought to be pathogenetically relevant (49). In this respect, it should be stressed that IL-17 participates in the generation of germinal centers and autoantibody production in both mice (50) and humans (51).

In conclusion, we have shown that the levels of IL-17A, produced mainly by mast cells and T cells, are increased in the affected skin of patients with SSc. The bulk of our in vivo and in vitro data, in conjunction with the findings recently presented by other investigators, indicate that IL-17A, when participating in the local inflammatory response, may preferentially restrain the profibrotic phenotype of SSc fibroblasts, in particular by balancing the effects of TGFβ. This opens new perspectives on our understanding of the fine regulation of processes leading to fibrosis and may provide the basis for new therapeutic strategies.

**AUTHOR CONTRIBUTIONS**

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. 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 conception and design.** Truchetet, Brembilla, Montanari, Zeni, Meroni, Chizzolini.

**Acquisition of data.** Truchetet, Brembilla, Montanari, Lonati, Raschi, Zeni, Fontao, Meroni.

**Analysis and interpretation of data.** Truchetet, Brembilla, Montanari, Lonati, Fontao, Meroni, Chizzolini.

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