

Frequency of Th1, Th2 and Th17 producing T lymphocytes in bronchoalveolar lavage of patients with systemic sclerosis

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Abstract

Objective

The pathophysiology of the lung fibrotic process in systemic sclerosis (SSc) is not fully elucidated. Since this pattern represents the leading cause of death in SSc, the knowledge of its actual pathophysiology is basic to prevent and stage pulmonary damage. In this study, we aimed to further investigate the relationship between the functional profiles of bronchoalveolar lavage (BAL) T cells and the pulmonary manifestation of the disease.

Methods

With this aim, we assessed the frequency of Th1, Th2 and Th17 producing T-lymphocytes and their effector cytokines in BAL of SSc patients without signs or symptoms of lung interstitial involvement (SScFib-) and with interstitial lung fibrosis (SScFib+). We also study as control groups: patients with usual interstitial pneumonia (UIP), patients with sarcoidosis and 9 healthy controls (NHCs).

Results

SScFib- showed an increase in BAL Th1/Th2 balance compared to NHCs, which was even higher than that observed in sarcoidosis. SScFib+ showed a shift towards a lower Th1/Th2 ratio as compared to SScFib-. The frequency of Th17 BAL T cells did not change among study groups.

Conclusion

Our data confirm the Th1/Th2 imbalance hypothesis on the pathogenesis of interstitial fibrosis in SSc patients, and suggest a possible utility in the assessment of BAL Th1/Th2 ratio.

Key words

SSc_{Fib-}, SSc_{Fib+}, UIP, sarcoidosis, BAL, Th1, Th2 and Th17 response, ELISPOT.

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Introduction

Systemic sclerosis (SSc) is characterized by the excessive deposition of collagen and other connective tissue macromolecules in skin and multiple internal organs and alterations in the microvasculature, humoral and cellular immunologic abnormalities (1-4). Parenchymal lung involvement is observed in nearly half of patients, and is characterized by interstitial fibrosis (ILD), that eventually progresses to severe restrictive lung disease in 15-20% of cases. Since this pattern is the leading cause of death in SSc, knowledge of its exact pathophysiology is fundamental to prevent end stage lung damage. The pathologic pathways underlying the presence or progression of ILD are still poorly clarified (5). From a clinical point of view, patients are stratified on the basis of HRCT and lung function (6-8). The utility of bronchoalveolar lavage (BAL) in the diagnosis and/or the characterization of SSc patients has been recently revisited and reappraised by several studies (9-11). BAL neutrophilia and/or eosinophilia per se do not predict disease progression or response to therapy, but rather merely reflect the extension of lung fibrosis. Consequently, in spite of the current understanding of the pathogenesis of SSc and the discovery of surrogate markers of lung involvement (12, 13), clinicians caring for SSc patients lack reliable biological markers stratifying disease activity or predicting clinical outcomes. While the role of inflammation as the main driving force behind progressive idiopathic lung fibrosis has still to be proven, there are several clinical features that support this causal relationship in SSc. Several experimental studies have focused on the type of inflammatory reaction associated with lung fibrogenesis. T cells have been shown to be required for the initiation of bleomycin induced pulmonary fibrosis. Indeed, the disease cannot be induced in T deficient mice (14), while it can be induced by the passive transfer of T cells (15). Moreover, the induction of fibrosis in animal has been associated with an expansion of type 2 cytokines inducing a fibroblast proliferation and extracellular matrix deposition. On the

contrary, type 1 cytokines fail to induce significant fibrotic changes (16). Similar results have been obtained in humans. Several studies indicate that the pathogenesis of idiopathic lung fibrosis is linked to an increased local production of Th2-related factors, including IL-4, IL-5 and IL-13, whereas less fibrogenic granulomatoses, such as sarcoidosis, seem to be associated with the expansion of a type-1 response and the production of the anti-fibrotic Th1-related cytokines IFN- γ and IL-12 (17-19). By analogy, these mechanisms are thought to be present in secondary ILD diseases such as that associated with SSc (2, 20, 21). Activated CD8⁺ T lymphocytes in BAL from SSc patients with alveolitis show higher Th2 cytokine (IL-4 and IL-5) mRNA expression, suggesting that in SSc a specific population of activated T cells exhibiting a profibrotic Th2/Tc2-polarized phenotype may be potentially relevant in mediating tissue fibrosis (22, 23). High levels of IL-4 have been demonstrated in exhaled breath condensates of SSc patients with pulmonary fibrosis as compared to NHCs (24). The presence of a peripheral polarized Th2 response in SSc patients with manifestation of lung disease has been demonstrated in two recent studies. Boin and collaborators showed that SSc-related lung fibrosis disease strongly correlated with a Th2/Tc2-polarized immune response of circulating T cells, as measured by the CCR5/CRTH2 T cell ratio (25), whereas Antonelli and collaborators demonstrated in their longitudinal study that serum levels of the Th1 chemokine CXCL10 declined during follow-up, as opposed to those of the Th2-related chemokine CCL2 which remained unmodified, giving a significant increase in the CCL2/CXCL10 ratio in the fifth year (26). Although all findings suggest that in SSc a specific population of activated T cells exhibiting a profibrotic Th2/Tc2-polarized phenotype may be potentially relevant in mediating tissue fibrosis, a clear association between a type 2 cell activation pattern and a higher functional decline remains to be demonstrated. Finally, evidence has been provided regarding the involvement of peripheral Th17 cells or IL-17 protein in the

Competing interests: none declared.

pathogenesis of scleroderma (27-30). Th17 cells represents the only additional effector CD4 T cell arm to be evidence since the original discovery of Th1 and Th2 two decades ago (31). To our knowledge, no data has been published thus far on the frequency of Th17 cells in BAL from SSc patients, nor on the relationship between this cell subset and SSc-associated pulmonary fibrosis. In this study, we thought to further investigate the relationship between the functional BAL T cell profile in SSc patients and the pulmonary manifestation of the disease. With this aim in mind, we studied the frequency of Th1-, Th2- and Th17-secreting cells and levels of the cytokines expressing the activation of each lymphocyte subset in BAL from SSc patients with and without evidence of ILD. Results were compared to those obtained in 3 control groups: normal healthy subjects (NHCs), patients with usual interstitial pneumonia (UIP) and patients with sarcoidosis.

Patients and methods

Study groups

We included in this study five groups of individuals: SSc_{Fib-}: 10 SSc patients with no signs or symptoms of ILD; SSc_{Fib+}: 21 SSc patients with signs and symptoms of ILD; UIP: 16 patients with UIP-type idiopathic pulmonary fibrosis; sarcoidosis: 15 patients with stage II disease; and NHCs: 9 healthy control subjects. Informed written consent was obtained from all participants. Demographic, clinical and functional characteristics of patient and control groups are shown in Table I.

All SSc patients were outpatients at the Rheumatology Unit of the Policlinico San Matteo Foundation, Pavia, and satisfied the preliminary ACR criteria for disease classification (32). None were being treated with prednisone or other immunosuppressive agents at the time of enrollment. According to more recent guidelines (32), the diagnosis of SSc-associated ILD was based on clinical, functional and CT data. Since histology is not considered a tool to assess the severity of lung disease (32, 33), none of our SSc patients underwent surgical lung biopsy. Respiratory

involvement was defined as being of at least a grade one severity according to the disease severity scale for SSc. This was determined in all cases by carbon monoxide transfer (TLCO), forced vital capacity (FVC), chest radiography, and high resolution computed tomography (HRCT). The "Katzerooni" score for fibrosis was calculated as previously stated (34) by the same expert radiologist in all cases. Pulmonary function tests were performed using the ATS guidelines (35) and expressed as a percentage of predicted values. From a clinical point of view, SSc patients were divided into pre-Sclerodermia (pre-SSc), limited (lcSSc) and diffuse (dcSSc) cutaneous disease. Antinuclear antibodies (ANA) were detected by indirect immunofluorescence on HEp-2 cells and anticentromere antibodies (ACA) were identified from ANA patterns. Anti-topoisomerase I (anti-Scl70) and other antibodies to extractable nuclear antigens were evaluated by ELISA (Diamedix Co, Miami Florida, USA).

No evidence of lung involvement was found in SSc_{Fib-} patients either during lung function tests or on HRCT scan

(Katzerooni fibrosis score: 0). They included: 9 (90%) lcSSc; 1 (10%) dcSSc. In SSc_{Fib+} patients ILD was defined as a >20% reduction from the expected TLCO or FVC values, associated with the presence of significant fibrosis on HRCT (grade ≥ 1 fibrosis score). This group included 9 patients (42.8%) with lcSSc and 12 (57.2%) with dcSSc. All SSc patients tested positive for ANA, 5 SSc_{Fib-} and 3 SSc_{Fib+} tested positive for ACA, and 8 SSc_{Fib+} had anti-Scl70 auto-antibodies (Table II).

Sarcoidosis patients were enrolled following their first clinical evaluation and had not received previous treatment. Diagnosis was made on the basis of the clinical picture, the presence of non-caseating granulomas in trans-bronchial biopsy (8 cases), lymph-nodal or lung thoracoscopic biopsy (7 cases) and the exclusion of other similar diseases, as previously stated (36). On the basis of radiographic evidence all patients were classified as being into stage II of the disease.

All UIP patients met recently stated diagnostic criteria (37) and were submitted to open lung or video-assisted

Table I. Demographic characteristics and respiratory functional tests in study groups.

Characteristic	NHCs	SSc _{Fib-}	SSc _{Fib+}	Sarcoidosis	UIP
Number	9	10	21	15	16
Age	42.7 \pm 12.1	51.6 \pm 8.5	59.6 \pm 11.6	31.4 \pm 7.8	56.0 \pm 16.8
Male/female	5/4	1/9	2/19	6/9	11/5
Smoke: ex/yes	0/3	5/0	6/1	0/0	9/2
*Pulmonary Function					
FVC	107.9 \pm 9.7	119.6 \pm 12.6	69.9 \pm 10.6	89.7 \pm 5.1	68.7 \pm 14.2
FEV ₁	98.5 \pm 5.1	126.9 \pm 14.5	90.3 \pm 12.9	87.2 \pm 8.2	78.4 \pm 11.9
TL _{CO}	115.6 \pm 12.7	71.6 \pm 14.9	50.1 \pm 15.0	71.3 \pm 7.1	46.6 \pm 14.8

*Lung function tests are expressed as % predicted. FVC: forced vital capacity; FEV₁: forced expired volume in 1 second; TL_{CO}: carbon monoxide transfer; NHCs: normal healthy controls; SSc: systemic sclerosis; SSc_{Fib-}: SSc patients with no signs and symptoms of lung involvement; SSc_{Fib+}: SSc patients with functional and HRCT signs of lung fibrosis; Sarcoidosis: patients with stage II disease; UIP: patients with usual interstitial pneumonia.

Table II. Skin involvement and auto-antibody profiles of SSc patients.

Patients	lcSSc/dcSSc	ANA	ACA	Scl70
		positive/negative	positive/negative	positive/negative
SSc _{Fib-} (n, 10)	9/1	10/0	5/5	0/10
SSc _{Fib+} (n, 21)	9/12	21/0	3/18	8/13

SSc: systemic sclerosis; SSc_{Fib-}: SSc patients with no signs and symptoms of lung involvement; SSc_{Fib+}: SSc patients with functional and HRCT signs of lung fibrosis; lcSSc: limited cutaneous disease; dcSSc: diffuse cutaneous disease; ANA: antinuclear antibodies; ACA: anticentromere antibodies; Scl70: anti-topoisomerase I antibodies.

thoracoscopic biopsy which showed a typical UIP pattern. None of them had been previously treated with steroids or other immuno-suppressive drugs.

NHCs included 3 patients undergoing bronchoscopy for occasional upper respiratory bleeding, and 6 volunteers. No clinical or microbiological evidence of infection was found in any of the patient groups.

Broncho-alveolar lavage

BAL was performed as previously described (38). Briefly, the distal tip of the bronchoscope was wedged into the middle lobe or lingular bronchus, and 150 ml of warm sterile saline solution were instilled in a 30-ml bolus and then serially recovered by gentle aspiration. The first aliquot collected was used for microbiological analysis, including examinations for common bacteria and fungi, direct acid fast bacilli smears (Kinyoun method) and cultures for mycobacteria, microscopic examination for *Pneumocystis carinii* (Gomorri Grocott silver stain). Total and differential cell counts on the remaining aliquots were performed using standard hemocytometer and May-Grümwald-Giemsa + Papanicolaou stainings.

Enzyme linked immuno-spot (ELISPOT) assay

BAL T lymphocytes were purified by positive selection using the CD3 T cell Isolation Kit (Miltenyi Biotec, Germany), following the manufacturer's instructions. ELISPOT was performed using 96-well plates (Polyfiltronics, Rockland MA) coated with 100 µl of specific capture antibodies (clone 2G1,

5µg/ml and clone TRFK5, 5µg/ml for IFN-γ and IL-5, respectively; PharMingen, San Diego CA) in PBS overnight at 4°C. The plates were then washed and blocked for 2 hours at room temperature with PBS+10% FCS. A total of 5 x 10⁴ CD3+ T cells were added to each well in 100 µl of complete RPMI medium. To detect all potentially producing effectors (maximal stimulation), BAL T cells were stimulated with phytohemagglutinin A (PhA, Sigma; 15 µg/ml). Control wells contained only CD3+ cells in complete medium. After an incubation period of 24-48 hours at 37°C, the plates were washed and 100 µl of biotinylated detection antibodies (IFN-γ: clone B133.5, 1 µg/ml; IL-5: clone JES1-5A10, 2 µg/ml; PharMingen) were added for 2 hours at 37°C. After washing, 100 µl of HRP-Conjugated Streptavidin (Endogen) were added for 30 min at room temperature. The plates were washed again and the spots, developed using 3-amino-9-ethylcarbazole (Sigma), were counted using an Automated Elisa-Spot Assay Video Analysis System (A-EL-VIS, Hannover, Germany). IL-17-producing cells were assessed using a commercially available IL-17 ELISPOT assay (Biosource), following the manufacturer's instructions. Results were expressed as the number of cytokine-producing cells/10⁵ BAL T lymphocytes. Spots obtained in control wells were always subtracted from mitogen-stimulated wells.

Th-1, Th-2 and Th-17 proteins

A customized RayBio™ Human Cytokine Array (Raybiotech) was used for the simultaneous detection of Th-1

(IFN-γ and IL-12p40p70) and Th-2 (IL-4 and IL-5) cytokines. BAL-f samples were processed according to the manufacturer's instructions. Membranes were exposed to Kodak x-omat film at room temperature. The signal intensity of individual spots was detected and quantified by using a chemiluminescence imaging device. By subtracting the background staining and normalizing to the positive controls on the same membrane, we obtained relative protein concentrations.

The quantitative sandwich enzyme immunoassay technique (Quantikine IL-17 Immunoassay; R & D System Inc.) was used to quantify IL-17 protein in BAL-f, according to the manufacturer's instructions. The detection limit was 15 pg/ml.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Differences among groups were analysed with the Student' t test, since all analysed variables passed the normality test. The accepted level of significance was 5%.

Results

BAL cytology

Results of cytology, performed on BAL in the five study groups are detailed in Table III. A significant increase in the percentages of lymphocytes, neutrophils and eosinophils was detected in SSc_{Fib+} and UIP compared to NHCs. In SSc_{Fib-} and sarcoidosis, BAL cytology showed a significant increase only of the lymphocyte percentage. As expected, in SSc_{Fib+} there was a significant increase in BAL neutrophils and eosinophils compared to SSc_{Fib-}. As for the

Table III. BAL cytology in the study groups.

BAL cytology	NHCs	SScFib-	SScFib+	UIP	Sarcoidosis
Total cells (10 ⁵ /ml)	0.25 ± 0.15	0.17 ± 0.11	0.18 ± 0.10	0.20 ± 0.23	0.33 ± 0.19
Macrophages (%)	93.89 ± 3.10	90.40 ± 4.65	84.86 ± 8.33*	80.00 ± 11.47*	76.67 ± 13.23*
Lymphocytes (%)	4.56 ± 2.07	7.80 ± 3.77*	9.10 ± 5.60*	8.06 ± 12.19*	20.00 ± 10.12*
Neutrophils (%)	1.56 ± 1.42	1.60 ± 1.17	4.00 ± 2.35* #	6.88 ± 3.69*	2.47 ± 2.20
Eosinophils (%)	0.00 ± 0.00	0.20 ± 0.42	2.05 ± 3.22* #	5.13 ± 3.20*	0.87 ± 1.85
CD4+/CD8+ ratio	2.4 ± 0.71	2.62 ± 1.27	3.1 8 ± 3.81	1.70 ± 1.23	5.51 ± 3.22*

NHCs: normal healthy controls; SSc: systemic sclerosis; SSc_{Fib-}: SSc patients with no signs and symptoms of lung involvement; SSc_{Fib+}: SSc patients with functional and HRCT signs of lung fibrosis; Sarcoidosis: patients with stage II disease; UIP: patients with usual interstitial pneumonia.

*p<0.05 compared to NHCs; #p<0.01 compared to SSc_{Fib-}.

CD4/CD8 ratio in BAL lymphocytes, while no difference was observed in SSc_{Fib-}, SSc_{Fib+} and UIP patients compared to NHCs, as expected a significant increase was found in sarcoidosis.

Frequency of cytokine-producing BAL T lymphocytes

The ELISPOT assay was performed for three different cytokines: IFN- γ , IL-5 and IL-17, with both unstimulated BAL T lymphocytes (basal condition) and after stimulation with PhA (maximal stimulation). As shown in table IV, in basal conditions SSc_{Fib-} and SSc_{Fib+} patients did not display a significant variation in the frequency of IFN- γ producing BAL lymphocytes compared to NHCs, while a significant decrease ($p < 0.01$) was present in UIP patients. Moreover, results obtained in both groups of SSc subjects were significantly higher than those measured in the UIP group ($p < 0.01$). Unexpectedly, sarcoidosis patients did not present any increase in the basal frequency of IFN- γ producing cells compared to controls.

As far as the frequency of IL-5-producing clones, SSc_{Fib-} and sarcoidosis patients had significantly reduced counts compared to controls ($p < 0.01$), while no difference was observed in SSc_{Fib+} or UIP. Finally, no variation in Th17 clones was observed in each patient group compared to NHCs ($p > 0.05$).

Results concerning the frequency of cytokine-producing BAL T cells under maximal stimulation are illustrated in Fig. 1. While the mean counts of IFN- γ producing BAL lymphocytes showed a significant change among different patient groups, the frequency of IL-5 or IL-17 producing clones did not change significantly. A marked and significant increase in IFN- γ producing clones/ 10^5 BAL lymphocytes was found in SSc_{Fib-}, sarcoidosis and SSc_{Fib+} patients ($p < 0.01$) as compared to NHCs (Fig. 1a). In UIP patients the difference was not significant (> 0.05). On the contrary, the frequency of IL-5 (Fig. 1b) or IL-17 (Fig. 1c) producing clones did not change significantly among study groups, even though an upward trend was observed in UIP patients and SSc_{Fib+} for IL-5.

Moreover, no significant differences were observed in Th1, Th2 and Th17

Table IV. Frequency of cytokine-producing BAL T lymphocytes in basal conditions, as assessed by ELISPOT.

Study groups	IFN- γ	IL-5	IL-17
NHCs	6.1 \pm 2.1	4.5 \pm 1.9	3.1 \pm 1.5
SSc _{Fib-}	9.1 \pm 5.1 [§]	1.3 \pm 0.9*	5.6 \pm 2.4
SSc _{Fib+}	4.7 \pm 1.8 [§]	2.9 \pm 1.3	4.8 \pm 1.8
Sarcoidosis	4.0 \pm 3.9	1.8 \pm 1.5*	2.6 \pm 1.5
UIP	1.2 \pm 1.1*	3.3 \pm 2.5	4.1 \pm 1.7

Results represent the mean spot number of duplicate cultures/ 10^5 T lymphocytes \pm SD.

NHCs: normal healthy controls; SSc: systemic fibrosis; SSc_{Fib-}: SSc patients with no signs and symptoms of lung involvement; SSc_{Fib+}: SSc patients with functional and HRCT signs of lung fibrosis; Sarcoidosis: patients with stage II disease; UIP: patients with usual interstitial pneumonia.

* $p < 0.01$ compared to NHCs; [§] $p < 0.01$ compared to UIP

clones when SSc_{Fib+} patients were stratified according to the auto-antibody profile in SCL70-positive and SCL70-negative (data not shown).

Th1/Th2 balance

Differences between groups were more evident when calculating the ratios between IFN- γ and IL-5. (Fig 2). We found a significant increase in the ratio between mean spontaneous IFN- γ and IL-5 producing clones in BAL lymphocytes of SSc_{Fib-} (6.51 \pm 2.01) and sarcoidosis patients (2.60 \pm 0.51) compared to NHCs (1.13 \pm 0.45) (Fig. 2a). SSc_{Fib+} and UIP patients did not differ appreciably from NHCs, but a significant decrease in Th1/Th2 ratio was observed in UIP patients as compared to sarcoidosis and SSc_{Fib-} study groups.

Maximal IFN- γ /IL-5 ratio in NHCs was 14.14 \pm 5.1 (Fig 2b). This ratio increased markedly and significantly in SSc_{Fib-} and sarcoidosis patients as compared to NHCs, most likely as a consequence of the increase in IFN- γ counts. A lower but significant increase was detectable in SSc_{Fib+} compared to NHCs, whereas no variation was found in UIP patients. Comparing patients groups, we found that maximal Th1/Th2 ratio in SSc_{Fib+} decreased significantly as compared to SSc_{Fib-}, and that in UIP was significantly lower than that measured in both SSc_{Fib-} and sarcoidosis patients.

Th-1, Th-2 and Th-17 proteins

The concentrations of type 1 (IFN- γ and IL-12p40p70) and type 2 (IL-4 and IL-5) cytokines, and IL-17 were measured in BAL-f of SSc patients by means of a

human protein array format and a quantitative sandwich enzyme immunoassay technique, respectively. Levels of IFN- γ and IL-12p40p70 were significantly lower in SSc_{Fib+} than in SSc_{Fib-}, while IL-4 and IL-5 values were significantly elevated in SSc_{Fib+} as compared with SSc_{Fib-} (Fig. 3). Levels of IL-17 were below the detection limit in the majority of SSc patients (data not shown).

Discussion

On the basis of the present results the following conclusions can be drawn: 1) The ELISPOT is a feasible method for the assessment of T cell functional response also in BAL; 2) Maximal T cell response in BAL in physiologic conditions is predominantly Th1; 3) SSc_{Fib-} patients with no clinical, functional or radiological signs of lung involvement have a clear expansion of the Th1 functional response in BAL; 4) Expansion of Th1 response is present also in BAL of SSc_{Fib+} but the entity of Th1 subset and their effector cytokines, as well as the ratio Th1/Th2 are markedly decreased compared to SSc_{Fib-}; 5) The frequency of BAL Th17 clones does not vary among study groups, suggesting that this cell subset has no clear role in the pathogenesis of ILD; 6) Finally, confirming the Th1/Th2 imbalance hypothesis, UIP patients have a significantly lower IFN- γ /IL-5 ratio compared to sarcoidosis patients. Our results concerning ELISPOT are in accordance with those of previous studies (39, 40), suggesting that this method can be useful to assess the Th1/Th2 balance in BAL. We found that IFN- γ /IL-5 ratio in BAL of NHCs was

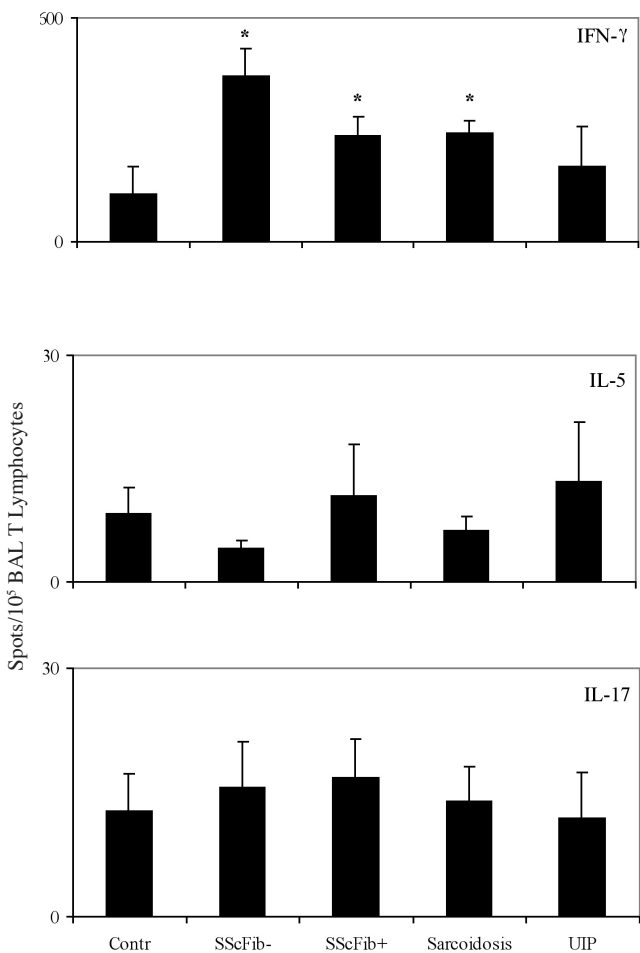


Fig. 1. Frequency of Th1 (IFN- γ), Th2 (IL-5) and Th17 producing BAL T lymphocytes under maximal stimulation with phytohemagglutinin A. All samples have been processed in duplicate cultures. Spots obtained in basal condition were always subtracted. Results represent the mean number of cytokine-producing cells/10⁵ BAL T lymphocytes. NHCs: normal healthy controls; SSc: systemic sclerosis; SSc_{Fib-}: SSc patients with no signs and symptoms of lung involvement; SSc_{Fib+}: SSc patients with functional and HRCT signs of lung fibrosis; Sarcoidosis: patients with stage II disease; UIP: patients with usual interstitial pneumonia. * $p < 0.01$ compared to NHCs.

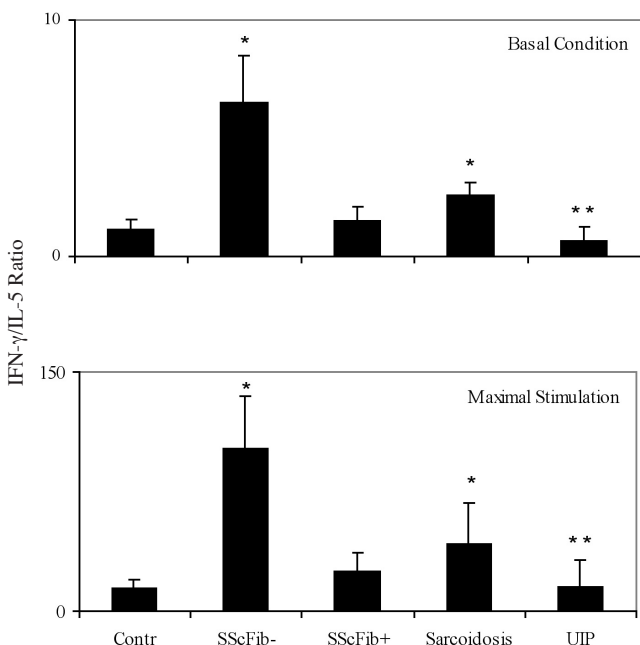


Fig. 2. Ratio between Th1 and Th2 (IFN- γ /IL-5) producing BAL T clones in basal condition and under maximal stimulation. NHCs: normal healthy controls; SSc: systemic sclerosis; SSc_{Fib-}: SSc patients with no signs and symptoms of lung involvement; SSc_{Fib+}: SSc patients with functional and HRCT signs of lung fibrosis; sarcoidosis: patients with stage II disease; UIP: patients with usual interstitial pneumonia. * $p < 0.01$ compared to NHCs; ** $p < 0.01$ compared to SSc_{Fib-} and sarcoidosis.

nearly 1 when cells were not stimulated, suggesting that this *in vitro* conditions probably better represents the actual

in vivo pattern of response in a normal non-inflamed lung. On the contrary, when we assessed the number of all po-

tentially responding Th1 or Th2 clones in NHCs, the balance shifted markedly towards a predominantly Th1 pattern of response, similar to that observed in the peripheral blood of normal healthy subjects (41). This is a reasonable finding, since it is well known that a type 1 response, through the high expression of IFN- γ and the subsequent activation of phagocytes and antigen-presenting cells, is the most efficient first line defence against foreign agents. Although the number of subjects included in this group was small, no significant differences were observed with respect to smoking habits.

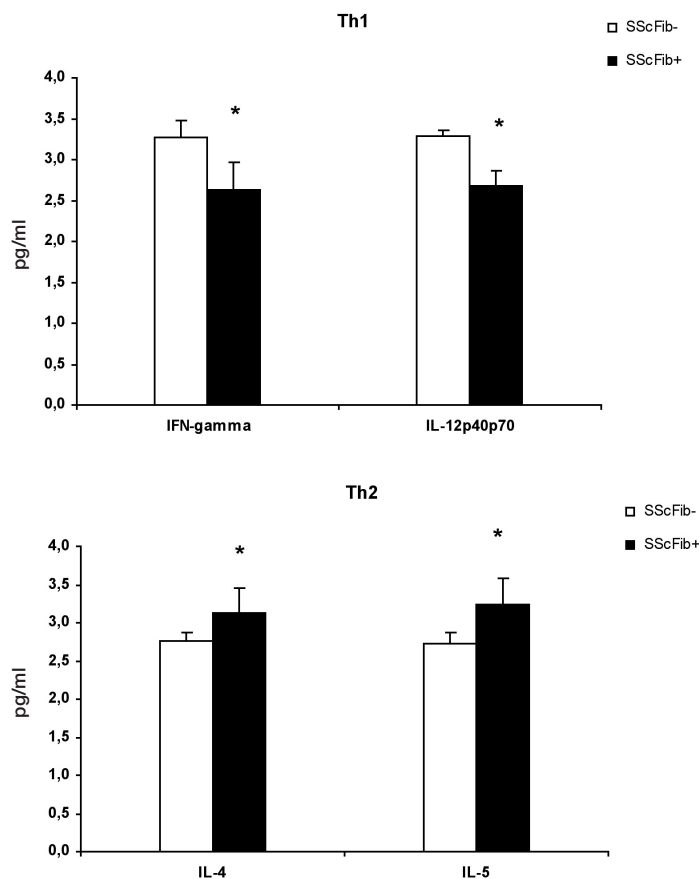
In order to better understand the pathophysiology of the lung fibrotic process in SSc, we assessed the Th1/Th2 balance in a selected group of SSc patients with and without evidence of lung fibrosis, on the basis of HRCT and lung function. In SSc_{Fib-} a clear shift towards a Th1 response was present, whose entity was significantly higher than that found in NHCs. Such a pattern was detectable in basal conditions and became even more evident when cells were maximally stimulated *in vitro* with mitogens. The Th1 shift in SSc_{Fib-} was even higher to that observed in patients with stage II sarcoidosis. Results from animal studies demonstrate that the expansion of the Th1 profile is generally associated with little or no fibrogenesis and, therefore, with a better clinical outcome (42, 43). Thus, our results indicate that SSc patients with no functional or HRCT sign of interstitial lung involvement are characterized by a consistent degree of inflammation, markedly Th1 oriented. Lung inflammation might occur in the very early stages of the disease process predating interstitial fibrogenesis, that eventually can occur together with a non-inflammatory, fibrogenic Th2-like shift. In fact, our SSc_{Fib+} patients who showed a clear and overt fibrotic lung damage had lower Th1 response (less IFN- γ producing clones and lower levels of type 1 cytokines), a shift towards a more pronounced Th2 response (more IL-5 producing clones and higher levels of type 2 cytokines), and consequently lower Th1/Th2 ratio as compared to SSc_{Fib-} patients. These

Fig. 3. Levels of Th1 and Th2 cytokines in BAL-f of systemic sclerosis patients

Type 1 (IFN- γ and IL-12p40p70) and type 2 (IL-4 and IL-5) cytokine levels were simultaneously measured in BAL-f samples of SSc patients by means of a customized protein array. Results relative to positive controls were expressed in pg/ml.

□, SSc_{Fib-} (SSc patients with no signs and symptoms of lung involvement); ■, SSc_{Fib+} (SSc patients with functional and HRCT signs of lung fibrosis)

* $p < 0.05$



observations support a wide series of previous experimental data, corroborate the emerging hypothesis for the pathogenesis of fibrotic disorders, and offer clear confirmation to the hypothesis of a Th2-polarized phenotype in SSc-associated ILD, in analogy to what has been already described for fibrotic skin involvement in scleroderma (44, 45).

Furthermore, we observed differences in T cell functional responses between SSc_{Fib+} and UIP-like IPF patients, with a higher Th1/Th2 ratio in the former group. According to recent studies, the prevalent lung histology in SSc is a NSIP pattern (46). Moreover, evidence has been provided on difference with regard to cytokine and chemokine gene expression, and protein levels between idiopathic NSIP and UIP, being the degree of IFN- γ and CCL5/RANTES higher in the former (47-49). This more "Th1" oriented cytokine and chemokine profile was thought to be related to the better clinical outcome and lower degree of fibrotic evolution of NSIP patients.

Finally, we did not observe significant variation in Th17 BAL cells among

study groups, even if a slight but not significant increase in the number of IL-17-producing BAL cells was found in SSc patients compared to the other study groups. Th17 cells represent a unique CD4⁺ T cell subset characterized by the production of IL-17, a highly inflammatory cytokine with robust effects on stromal cells in many tissues (31). Th17 cells play an important role in the pathogenesis of a diverse group of human diseases, among which is inflammatory dermatosis (27, 50). Increased expression of IL-17 mRNA in lymphocytes from skin and lung of SSc patients or enhanced serum IL-17 secretion has been demonstrated in most patients with SSc regardless of the disease subset, suggesting that the protein may be involved in a common pathway of SSc development (28, 29). We found no variation in the frequency of Th17 BAL cells between SSc_{Fib-} and SSc_{Fib+}, suggesting that the induction of this cell subset has no clear role in the development of SSc-related ILD. Unfortunately, levels of IL-17 protein in BAL-f were below or around the de-

tection limits in nearly all SSc patients. In all, our results suggest that the Th17 response has no clear role in SSc-related ILD, different from that observed in skin involvement.

In conclusion, our data confirm that the ELISPOT assay can be a useful tool in assessing the Th1/Th2 balance of BAL lymphocytes in SSc patients and also raise the question of whether the routine and longitudinal assessment of the Th1/Th2 ratio in SSc could be of clinical help in defining those patients with a higher risk of fibrotic evolution. However, this study has several limitations, such as the sample size of SSc patient groups, which was relatively small, and above all its cross-sectional nature. Further longitudinal studies with a large patient cohort are necessary in order to assess whether a shift from Th1 to Th2 response may be considered predictive of clinical outcome and progression of pulmonary fibrosis.

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