Peripheral T cells from patients with early systemic sclerosis kill autologous fibroblasts in co-culture: is T-cell response aimed to play a protective role?

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Abstract

Objectives. Oligoclonal T-cell infiltrates have been detected in the skin of patients with early dcSSc. Peripheral T cells from patients with early dcSSc co-cultured with autologous fibroblasts have been found to expand the same T-cell clonotypes found in the affected skin. Here, we characterize oligoclonally expanded T lymphocytes and investigate functional changes occurring in early-dcSSc co-cultured T lymphocytes and fibroblasts.

Methods. Peripheral T lymphocytes from five patients with early (<3-year duration) dcSSc were co-cultured with the autologous fibroblasts obtained by punch biopsy of involved skin.

Results. T-cell clonotypes expanded in co-cultures were found to be αβ+ and HLA-DR+, and to promote the apoptosis of autologous fibroblasts. Fibroblasts up-regulated Fas and underwent apoptosis that paired with the expression of Fas ligand (Fasl) on CD4+ T cells. Finally, the addition of a blocking anti-Fas antibody to the co-cultures resulted in a marked reduction of fibroblast apoptosis, suggesting a critical role of Fas/Fasl engagement in mediating apoptosis in co-cultured fibroblasts. In the co-culture supernatants, we found TGF-β, IL-1β, IL-6 and IL-8, cytokines that are known to promote fibrosis in SSc. The same results were registered in each co-culture.

Conclusions. Taken together, these data suggest that T-cell response in SSc may also represent an attempt of the immune system to kill fibroblasts, cells likely expressing (auto)antigens, although the overall outcome of the T-cell response contributes to sustain inflammatory loops leading to fibrosis.

Key words: Systemic sclerosis, T-cell repertoires, Cytokines.

Introduction

SSc is a generalized disorder of connective tissue characterized by microvascular and small artery disease, accumulation of collagen and other constituents of extracellular matrix in the interstitium of skin and internal organs, and autoimmune abnormalities [1, 2]. The aetio-pathogenesis of the disease is unknown; however, endothelial, fibroblast and immune cell activation are thought to play a critical role [3–5].

T-cell–fibroblast interplay is considered a central step leading to activation of fibroblasts and the consequent development of interstitial fibrosis [5]. Indeed, CD4+ T cells having a Th2 phenotype are known to represent a major component of the skin infiltrate in the early steps of this disease [3, 6, 7]. Many studies suggest that these cells are able to activate fibroblasts either by direct contact via CD69 or CD40 ligand or by stimulating overproduction of collagen, releasing IL-4 and other fibroblast-activating cytokines and growth factors as PDGF, IL-8 and TGF-β1 [8–10].

Ultrastructural alterations of endothelial cells with a peculiar perivascular distribution of mononuclear cells represent the earliest pathological lesion detectable in...
the scleroderma skin. Therefore, it has been thought for a long time that, in this disease, inflammatory cells migrate through the endothelial barrier to the interstitial where they contribute to promote fibroblast activation. In the past few years, increasing evidence has revealed a more complex scenario. It has been proved that T cells infiltrate the skin only in the early steps of the disease, indicating that late phases of the disease are not depending on an ongoing T-cell response [5, 11]. Furthermore, T cells are oligoclonally expanded only in the skin, and not in peripheral blood, suggesting that they undergo an antigen-driven expansion in the skin affected by SSc [11, 12].

We have demonstrated that peripheral T cells obtained from patients affected by early diffuse SSc undergo oligoclonal expansion when co-cultured with autologous fibroblasts similar to that detected in the T cells infiltrating the skin of the same patients [12]. These results strongly indicate that fibroblasts are an antigen source in SSc, although it remains to be clarified why fibroblasts become an antigenic trigger and the nature of the antigen(s). On this basis, a better knowledge of T-cell–fibroblast interactions might be crucial for a better understanding of SSc pathophysiology.

**Materials and methods**

**Patients**

Five patients affected by early diffuse SSc and two patients with SLE were enrolled after they gave written informed consent. Table 1 shows the features of SSc patients. Each patient satisfied the ACR classification criteria for his/her disease. All five SSc patients fulfilled the criteria to define SSc patients with early disease according to established guidelines [13–17]; they were not undergoing therapy either at the time of the biopsy and blood collection or before. Punch biopsies, performed for diagnostic purposes, and peripheral blood were obtained from the patients following standard procedures [18]. The Ethics Committee of the Department of Clinical and Experimental Medicine, Second University of Naples, approved the study. All the subjects involved in the study gave written consent according to the Declaration of Helsinki. Two healthy individuals volunteered to give blood and had a skin biopsy to perform control experiments.

**Biopsies**

Biopsies were performed according to standard procedures [12]. Each biopsy was divided into two specimens. The first one was used for spectratyping analysis as detailed elsewhere [12]. The second one was cut into 1–3 pieces, one piece was used to prepare cDNA for spectratyping analysis; the other one or two pieces (depending on size) were used to recover fibroblasts and placed into 6-well flat bottom plate (Costar, Cambridge, MA, USA), and cultured in DMEM (Invitrogen, Milano, Italy) containing 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Samples were kept in an incubator at 37°C, 5% CO₂ atmosphere. After 2–3 weeks of incubation fibroblasts reached confluence, underwent trypsin treatment (3 min at 37°C) and were re-cultured in DMEM. Co-culture experiments were performed on fibroblasts at confluence between passages 3 and 5 [12, 18]. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on Ficoll-Hypaque (Pharmacia LKB Biotechnology, Piscataway, NJ, USA) according to standard procedures, washed, counted and cultured.

**Cultures and FACS analysis**

We prepared co-cultures using both fibroblasts and PBMCs from the same patient at 1 : 10 ratio. Co-cultures were prepared as follows: fibroblasts recovered from biopsies were cultured until they were at confluence then, after 24–36 h, autologous PBMCs were added to the cultures as described below. As control, fibroblasts alone and PBMCs alone plus rIL-2 were cultured for the same time. In the co-cultures, we removed the autologous PBMCs after 10 days and cultured the remaining cells for two more weeks. Co-cultures were performed in RPMI-1640 medium (Invitrogen) supplemented with 5% FCS, 1 mM L-glutamine, 100 mg/ml streptomycin, 100 μg/ml penicillin and 10 or 20 U/ml human recombinant IL-2 (Peprotech, Milan, Italy) in 6-well flat bottom plates (Costar). After 10 days of incubation monolayer status was evaluated and non-adherent cells were washed out, characterized by flow cytometry and used to prepare RNA. Flow cytometry was performed staining the cells with anti-CD3-FITC, anti-CD4-PE, anti-CD8-PerCP, anti-TCR-γδ-PE and anti-TCR-γ6-PE and anti-HLA-DR, anti-Fasl, AnnexinV, anti-CD16, anti-CD56, anti-CD161, anti-Fas (Becton-Dickinson, Milan, Italy) and using a FACScalibur (Becton-Dickinson). The data were analysed using CellQuest software (Becton-Dickinson). Pictures of co-cultures were taken by putting the plates under a Leica DMRB microscope equipped with a MPS32 camera. The culture sets with cells obtained from SLE patients and healthy volunteers were studied in the same way.

**Fas inhibition experiments**

To test the effects of Fas inhibition, we added an anti-Fas neutralizing antibody (500 ng/ml; clone ZB4; Upstate, NY, USA) to the co-cultures. The antibody was added to fibroblasts 1 h before adding autologous PBMCs and every 24 h to the time point of apoptosis analysis. The concentration of antibody was determined in titration experiments.

**Apoptosis analysis**

Apoptosis in the co-cultures was measured at Day 10. The Annexin V-FITC-labelled Apoptosis Detection Kit (Becton-Dickinson) was used to detect and quantify apoptosis by flow cytometry according to the manufacturer’s instructions. Cells were collected by centrifugation for 10 min at 500 g and then re-suspended at a density of 1 × 10⁵ cells/ml in 1 × binding buffer (HEPES buffer, 10 mM, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂,
and 1.8 mM CaCl₂ and stained simultaneously with FITC-labelled Annexin V and propidium iodide (PI). PI was used as a cell viability marker. Cells were analysed using a FACScalibur flow cytometer. CD3⁺ cells were not included in the analysis.

**T-cell repertoire analysis**

The TCR repertoire was studied by CDR3 length analysis, a well-established PCR-based technique we refer to as spectratyping or immunoscope, a technique that we have previously detailed [12, 19–22]. First, RNA preparation was done using the guanidium hydrochloride-containing Trizol Reagent (Life Technologies, GIBCO-BRL, Gaithersburg, MD, USA) following the manufacturer’s instructions. First-strand cDNA synthesis was performed using oligo(dT) as a primer for reverse transcription (RT) of 1 μg total RNA using Moloney murine leukaemia virus-RT (MMLV-RT; Life Technologies) as reported before [12]. Then, cDNAs recovered from the different samples and culture conditions described were amplified under non-saturating PCR conditions with TCR-BV family-specific primers amplifying for two different TCR families in each PCR reaction. All the reactions also contained a β-actin-specific primer pair producing a 6-FAM-labelled 230-bp product as an internal control. The amount of template cDNA for each sample was assessed by preliminary titration amplifying for total TCR message. After amplification, each different PCR product was run in a fluorescence-based DNA sequencer (Applied Biosystem 377 model) in the presence of Rox-labelled size markers. The data were analysed by means of Applied Biosystems Genescan software that allows us to assign size and peak areas to the different PCR products.

Generation of spectratyping

Each different PCR product (0.5 μl) was diluted 1:1 with distilled water, then the sample was boiled and loaded on a sequencing gel and then run in a fluorescence-based DNA sequencer (Applied Biosystem 377 model) in the presence of Rox-labelled size markers. The data were analysed by means of Applied Biosystems Genescan software that allows us to assign size and peak areas to the different PCR products. The data for each TCR-V family were then visualized as chromatograms. TCR spectratyping of a healthy PBMC repertoire typically results in a banding pattern composed of between seven and eight bands at 3-nt base intervals, reflecting the correct ‘in-frame’ nature of functionally rearranged chain TCR gene products. The limited number of PCR cycles used, 30, leads to the generation of PCR products with a distribution representative of the starting material, i.e. a Gaussian distribution [12, 19, 21]. Each alteration in distribution and/or in intensity of the single bands represents a perturbation of the specific TCR family. Since only one TCR-β chain is productively rearranged, with the analysis of β TCR repertoire we may follow the dynamics of T-cell clonotypes in vivo and in vitro. The analysis of CDR3 length also provides information concerning the region that is critical for antigen recognition, thus allowing us to follow the overall dynamics of T-cell responses [12, 19–22]. Each band in a spectratype, while uniform in size, is heterogeneous with respect to the nucleotide sequences it contains, as would be expected, given the high degree of diversity required of TCR CDR3 regions.

**Cytokine analysis**

Culture supernatants were collected at Days 0, 5 and 10 and then analysed by FlowCytomix (Bender...
Medsystem, San Diego, CA, USA). Human Th1/Th2 11plex sample kits (Bender MedSystems) are intended for quantitative detection of multiple biomarkers by flow cytometry including IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, TNF-α and TNF-β. TGF-β was measured by using the same technique in a single plex assay. The results were normalized by a standard curve and analysed using FlowCytomixPro software. Each experiment reported was performed in triplicate at three independent times.

**Results**

Dynamics of fibroblast growth in co-cultures

Autologous PBMCs were added to SSc fibroblasts at confluence, at a 1:10 (fibroblasts: PBMCs) ratio and the co-cultures were controlled daily. In these cultures, the confluence of fibroblasts was progressively disrupted by adding autologous PBMCs. Intriguingly, SSc fibroblasts went to confluence again after removal of non-adherent cells at Day 10. Figure 1A shows the picture of co-culture of one SSc patient with fibroblasts at confluence (Fig. 1Aa), at Day 0, after adding PBMCs (Fig. 1Ab), after 10 days of co-culturing (Fig. 1Ac) and at Day 25, after the removal (at Day 10) of non-adherent cells (Fig. 1Ad). There was an extensive rupture of the fibroblast layer after 10 days of co-culture (Fig. 1Ac), while SSc fibroblasts went to confluence again upon removal of non-adherent cells (Fig. 1Ad). Similar results were observed in all the co-cultures of the five different SSc patients. The non-adherent cells recovered from the co-culture at Day 10 were analysed by FACS analysis and were found to be mainly CD3+β+HLA-DR+ cells [CD3+β+DR+ = 65 (23)%; CD4+β+DR+ = 42 (28)%]. We did not detect ruptures of the fibroblast layer in the co-cultures with cells from SLE patients or healthy controls (supplementary figure 2S).

**Fig. 1** Co-cultures and T-cell repertoires. (A) A representative picture of co-cultures performed with SSc PBMCs and autologous fibroblasts: (a) confluence of fibroblasts; (b) Day 0, fibroblasts plus autologous PBMCs; (c) Day 10, before removal of PBMCs; and (d) Day 25, 15 days after removal of fibroblasts (the arrow is pointing to the expanded image). Similar results were observed in all the co-cultures, each one performed in triplicate. (B) A synopsis of T-cell repertoire found at Day 10, corresponding to the time where we observed the rupture of fibroblast layer. Each box indicates a specific TCR-BV family. Open boxes with letter A indicate absence of message for that family; open boxes, a normal TCR family; dashed boxes, skewing; and closed boxes, a single dominant peak. The analysis showed similar oligoclonal T-cell expansions observed in the biopsy (B) and in the co-culture (C), while the peripheral blood displays a normal T-cell repertoire (P). This is a representative experiment, similar data were observed in all the co-cultures. Supplementary figures 1S.1–1S.4 (available as supplementary data at Rheumatology Online) show the actual spectratyping profiles observed for the experiment shown here with the same labelling (P, B and C); supplementary figure 1S.5 (available as supplementary data at Rheumatology Online) shows the synopsis of TCR analysis for the co-cultures prepared with the cells obtained from the other four subjects.
available as supplementary data at *Rheumatology* Online). As expected, all co-cultures prepared using non-autologous settings (i.e. T cells vs non-autologous fibroblasts), from either patients or controls, were characterized by anti-fibroblast activity. This finding was not surprising since, in this case, we were looking at an alloresponse driven by a different HLA that is the most powerful ‘antigen’ known (data not shown).

**Rupture of fibroblast layer in co-cultures is paired with T-cell oligoclonal expansions**

Non-adherent cells were mainly T cells; therefore, we assessed the repertoire by CDR3 length analysis at Day 10 on the cells recovered by the co-cultures. Figure 1B shows an example of the TCR repertoire analysis at this stage in one representative co-culture. Here, the T cells undergo oligoclonal expansions and, more importantly, these expansions have similar spectratyping profiles in the biopsy (B) and in the co-culture (C) performed with fibroblasts and PBMCs of the same patient; a normal T-cell repertoire was found in the periphery (P). Similar results were found in all co-cultures prepared from SSc patients, indicating that a specific antigen-driven T-cell expansion is pairing the rupture of the fibroblast layer (supplementary figure 1S5, available as supplementary data at *Rheumatology* Online), as we found in other previous experiments [12]. We did not find T-cell expansion or the rupture of the fibroblast layer in co-culture sets with cells obtained from SLE patients and healthy controls (data not shown), indicating that T-cell response to fibroblast characterizes SSc.

**Rupture of fibroblast layer in co-culture is due to increased apoptosis of fibroblasts**

To better characterize the phenomenon observed at Day 10 in co-cultures, we examined apoptosis in co-cultures, analysing the expression of Annexin V in PI-negative fibroblasts. Figure 2 shows that co-cultures at Day 10 presented a marked increase in Annexin V cells, thus indicating that rupture of the fibroblast layer was due to increased apoptosis of fibroblasts in the presence of autologous PBMCs. In Fig. 2A, the FACS dot/plot profile of one representative co-culture is shown. Figure 2B reports the values of Annexin V-positive fibroblasts in

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**Fig. 2** Analysis of apoptosis in co-cultures. (A) The measurement of the expression of Annexin V done by flow cytometry gating PI-negative cells. While (A) shows representative experiments, detailed data found in the co-cultures performed with the cells of the five SSc patients are expressed in (B). Values are expressed as mean (s.d.). Significant differences, *P* < 0.05; **P* < 0.001 after ANOVA test. Each experiment reported was performed in triplicate at three independent times. Supplementary figure 2S (available as supplementary data at *Rheumatology* Online) shows a representative experiment performed in the same setting on one healthy donor and one SLE patient. No differences were observed in the level of Annexin V measured in the co-cultured in comparison with fibroblast alone. Similar results were obtained on the other control.
all the co-cultures performed. Each co-culture, using PBMCs and fibroblasts of one patient, was performed in triplicate. Co-cultures performed on samples derived from SLE patients and healthy controls did not show increased apoptosis of fibroblasts (supplementary figure 2S, available as supplementary data at Rheumatology Online).

Fibroblast-T-cell co-cultures are characterized by T-cell activation and involvement of the Fas/Fas ligand pathway

To characterize the mechanisms underlying the increased apoptosis of fibroblasts in the co-cultures, we studied the expression of Fas on fibroblasts in co-cultures. As expected [5], we found that SSc fibroblasts express constitutively Fas but they up-regulated Fas in co-culture. The expression of Fas in one representative co-culture is shown in Fig. 3A. Similar results were obtained in all the co-cultures. Conversely, we analysed the expression of Fas ligand (Fasl) on CD3+ cells in co-culture, performing the analysis at different times due to the kinetics of Fasl expression on activated T cells. T cells in co-cultures expressed Fasl on the surface (Fig. 3B) between Day 3 and Day 5, indicating that T cells were involved in mediating fibroblast apoptosis in co-culture and suggesting that specifically activated autologous T cells kill SSc fibroblasts through the Fas/Fasl pathway.

Blocking of Fas/Fasl pathways by adding anti-Fas antibody inhibits fibroblast apoptosis

The experiments we performed so far suggested that an expansion of antigen-specific T cells expressing Fasl, was inducing fibroblast apoptosis in co-cultures. In order to further assess the role of the Fas/Fasl pathway in these experiments, we performed co-culture adding a blocking anti-Fas antibody. Strikingly, the use of this antibody in culture markedly reduced apoptosis of fibroblasts (Fig. 4A), indicating that the Fas/Fasl pathway was accounting for the killing we observed in co-cultures. We observed similar results in all the experiments (Fig. 4B).

Supernatants of co-cultures contain Th2, pro-fibrotic cytokines

Finally, we measured the cytokines in the supernatants of co-cultures looking at several cytokines. We found that TGF-β, IL-1β, IL-6 and IL-8 were increased in the co-cultures of SSc fibroblasts with autologous T cells (Fig. 5), although we also observed a modulation of their secretion in co-cultures. These data suggested that interactions between T cells and fibroblasts led to the production of cytokines known to have pro-fibrotic properties and reported to be increased in scleroderma [5, 24–29].

Discussion

The findings reported point out four main aspects: (i) SSc PBMCs co-cultured with autologous skin fibroblasts undergo activation and oligoclonal expansion similar to those found in the affected skin; (ii) skin fibroblasts co-cultured with autologous PBMCs up-regulate Fas and undergo apoptosis, while T cells up-regulate Fasl; (iii) in co-cultures, apoptosis of fibroblasts is reduced by an anti-Fas antibody, fibroblasts re-grow and come again to confluence upon PBMC removal; and (iv) interactions between T cells and fibroblasts lead to the production of cytokines that play a role in promoting the phenomena inducing clinical features of SSc.

Interactions between T cells and fibroblasts may be a key point in the pathogenesis of SSc. Several studies showed...
that oligoclonal, antigen-driven, T-cell expansion characterizes the early phase of SSc [4, 5, 12, 23, 30, 31]. We showed that co-cultures of PBMCs and autologous fibroblasts from SSc patients induce the expansion of T cells similar to that seen in T cells derived from skin biopsies [12]. Therefore, the co-culture of SSc fibroblasts and autologous PBMCs seems to resemble the in vivo cell/cell dynamics and can be used as a tool to study interactions between T cell and fibroblast in SSc.

In these experiments, using this in vitro system, we showed that oligoclonal expansions of T cells in the co-culture were paired by increased apoptosis of fibroblasts. These findings prompted us to further characterize co-cultures performed with SSc fibroblasts and autologous PBMCs. These data were indeed surprising since, as mentioned, it has been widely reported that SSc fibroblasts are resistant to apoptosis.

The removal of non-adherent cells, mainly activated T cells, paired with a re-growth of SSc fibroblast. These data together with the data of the Fas blocking, suggest that here T cells were needed to induce apoptosis through activation of the Fas/Fasl pathway. Overall, these results support the hypothesis that, in this in vitro system, T lymphocyte response is triggered by antigens expressed on the surface of fibroblasts.

Alterations in the Fas/Fasl pathway seem to be tightly connected to the development of SSc and other autoimmune diseases [5, 32–34], probably due to an impairment in the homoeostasis of cell regulation in the immune system.

Different reasons seem to account for the resistance of SSc fibroblasts to apoptosis in vivo [5] including a particular polymorphism of the Fas promoter gene found in SSc patients. It has been recently reported that this polymorphism affects responses to Fas-mediated apoptosis, acting directly by producing a protein unable to generate an efficient signalling, or indirectly by increasing the amount of soluble Fas (sFas) and interfering with the ligand [32].

Thus, the absence in our system of sFas, whose levels are increased in vivo in SSc patients, may in part explain the detection of apoptosis in vitro. On the other hand, in the co-cultures T-cell–fibroblast interactions led to an up-regulation of Fas on SSc fibroblasts in comparison with Fas expression on fibroblasts kept alone in culture. The increased expression of Fas is likely allowing more

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**Fig. 4** Anti-Fas antibody reduces apoptosis in co-cultures. (A) A representative experiment with the adding of anti-Fas antibody reducing apoptosis in co-cultures. Detailed data found in the co-cultures performed with the cells of five SSc patients are expressed (B). Values are expressed as mean (S.D.). Significant differences, *P < 0.05; **P < 0.001 after ANOVA test. All the experiments were performed in triplicate at three independent times.
efficient interaction and better pro-apoptotic signalling, further explaining why we could detect apoptosis in our experiments.

Recently, it has been reported that γδ T cells, oligoclonally expanded in SSc, are able to secrete cytokines and induce death of fibroblasts in a contact-dependent manner when activated by specific antigens [35]. Moreover, it has been shown that fibroblasts, particularly those from patients in the early stages of SSc, exhibited markedly increased expression of Types 1 and 3 procollagen, and inhibition of collagen synthesis was observed when fibroblasts were co-cultured with activated, but not with unstimulated, T cells [36, 37]. Altogether, these data may shed new light on the T-cell response in the early phase of SSc, suggesting that in vivo response may be aimed to stop fibroblast growth. We may therefore hypothesize that, in the early phase of SSc, an ongoing triggering of the immune response by an altered fibroblast in SSc could activate T-cell response and contribute to induce all the cascade of events responsible for production of several auto-antibodies and other soluble factors that may further amplify a loop inducing fibroblast growth receptor. In this line, this view is strengthened by our data on the cytokines measured in co-culture supernatants.

In fact, we observed in the co-culture supernatants the presence of cytokines promoting the pro-fibrotic activity of fibroblasts (IL-1β, TGF-β, IL-6, IL-8) and able to down-regulate T-cell activation (TGF-β). The changes in cytokine production found in these cultures could also be implicated in modulating resistance to apoptosis of SSc fibroblasts in vivo. The observation that IL-1β increase paired with IL-6 decrease in the co-cultures could be of particular interest due to the pleiotropic effects of these two cytokines on cell growth and apoptosis, and considering the proposed role for IL-1β in SSc. On the other hand, it has to be remembered that production of several factors over time by primarily altered fibroblasts, such as TGF-β and IL-10, may per se impair the immune response. These same factors promote the growth of fibroblasts establishing a loop that exits into the well-known modifications characterizing SSc. Further experiments aiming to characterize cytokine production in fibroblasts and T cells in these settings will be needed to further clarify this issue. It is also to be considered that while T-cell response can be able to control fibroblast growth in vitro, the same T response could not be efficient in vivo due to the complexity of the mechanisms underlying autoimmune disease [38].

Experiments ongoing in our laboratory are aiming to enlarge the number of patients studied and to characterize in detail the phenotype of T-cell in co-cultures to better characterize this response and to assess the extension of the meaning of these data. For instance, it is of interest to note that CD4 and CD8 T cells seem to be able to expand differentially in co-cultures depending on several factors.

**Fig. 5** Levels of cytokines measured in the co-culture supernatants. Values obtained in the control cultures (fibroblasts alone and PBMCs alone) have been subtracted from the values measured in the corresponding co-cultures and reported here. Values are expressed as mean (s.d.). All the experiments were performed in triplicate.
due to differences between individuals and to culture conditions, such as the amount of rIL-2 used and the lasting time of the co-cultures.

It is also worth noting that we found an increased number of CD4+CD161+ T cells, corresponding to Th17 cells [22, 39–41] that seem to play a critical role in SSc (data not shown).

Although obtained on a small number of patients, the experiments reported here suggest that fibroblasts may be a source of auto-antigen in SSc and, in the early phase of the disease they may contribute to the immune activation that helps to establish the loop of mechanisms finally leading to the disease.

Finally, molecular mechanism and stimuli that affect fibroblast and contribute to induce SSc remain to be elucidated, as well as the characterization of the antigen(s) triggering the immune response needs further studies.

### Rheumatology key messages

- SSc fibroblasts stimulate specific T-cell expansion.
- T cells from SSc patients kill autologous fibroblasts in co-culture.
- This study indicates new mechanisms in the development of SSc.

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### Supplementary data

Supplementary data are available at Rheumatology Online.

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from patients with heparin-induced thrombocytopenia/


