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B cell depletion treatment decreases CD4+IL4+ and CD4+CD40L+ T cells in patients with systemic sclerosis

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Abstract

Recent data suggests that rituximab may favorably affect skin fibrosis and lung function in patients with systemic sclerosis. Based on experimental data suggesting a key role of B and T cells in scleroderma we aimed to explore the effect(s) of rituximab treatment on T cell subpopulations. Fifteen patients with scleroderma who received rituximab treatment and six who received standard treatment alone were recruited. Peripheral CD4+IL4+, CD4+INFγ+, CD4+IL17+ and CD4+CD40L+ T cells were assessed using flow cytometry. Using ELISA, serum levels of IL4 were assessed. Skin CD4+IL4+ T cells were assessed with confocal microscopy from skin biopsies. Following rituximab treatment skin CD4+IL4+ T cells obviously decreased as seen with confocal microscopy. Moreover, peripheral CD4+IL4+ T cells decreased significantly compared to those from patients who received standard treatment alone: median (IQR): 14.9 (22.63–12.88) vs 7.87 (12.81–4.9)%, $p=0.005$ and 9.43 (19.53–7.50)% vs 14.86 (21.96–6.75)%, $p=$NS at baseline and 6 months later respectively, whereas there was no difference in serum IL4 levels. Peripheral CD4+CD40L+ T cells also decreased significantly following rituximab treatment compared to those from patients who received standard treatment alone: median (IQR): 17.78 (25.64–14.44)% vs 8.15 (22.85–3.08)%, $p=0.04$ and 22.13 (58.77–8.20)% vs 72.11 (73.05–20.45)%, $p=$NS at baseline and 6 months later respectively. Furthermore, peripheral CD4+INFγ+ and CD4+IL17+ T cells revealed no differences following rituximab treatment. Our study demonstrates a link between rituximab treatment and CD4+IL4+ T cell decrease both in the skin and peripheral blood of patients with SSc.

Keywords Scleroderma · Rituximab · Lymphocytes · B cells · T cells

Introduction

Systemic sclerosis (SSc) is a rheumatic disease with a complex pathogenesis comprising of autoimmune phenomena, vascular damage and fibrosis. Hence a considerable amount of research has focused on a better understanding of the pathogenic mechanisms that govern the disease process. Rituximab (RTX) is a chimeric monoclonal antibody which targets and depletes B cells. It has been used successfully in the therapy of patients with lymphoma [1] and other autoimmune diseases including rheumatoid arthritis, ANCA-associated vasculitis, systemic lupus erythematosus, immune thrombocytopenic purpura and Graft Versus Host Disease (GVHD).

GVHD has several similarities with SSc, including scleroderma-like skin lesions. Moreover several patients suffering from chronic GVHD exhibit circulating autoantibodies and frequently suffer from autoimmune manifestations. As a result some authorities consider GVHD a systemic autoimmune disease [2]. RTX has been used for the management of these patients with encouraging results [3]. Several centers including ours have used RTX in the treatment of resistant SSc with beneficial results [4-10].

The potential role of T cells in the pathogenesis of SSc has been shown in previous studies [11, 12]. Circulating Th2 and Th17 cells as well as IL4 and IL17 serum cytokine

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levels of patients with SSc have been found increased compared to healthy subjects [13-15].

Based on the above we further sought to investigate the potential effect(s) of B cell depletion therapy on the percentages of circulating T cells of patients with SSc to shed light on the mechanism of action of RTX. We assessed T helper cell subsets. T cells express either CD8 glycoprotein or CD4 glycoprotein T cell receptor (TCR) and are characterized as cytotoxic or helper T cells, respectively. According to the cytokine they produce T helper cells are divided into different subsets. Th1 cells produce INFγ and TNF, Th2 cells produce IL4, IL5, and IL13 and Th17 cells produce IL17, IL21, IL22, IL25, and IL26 [16]. More specifically, we aimed at assessing peripheral CD4+IL4+, CD4+INFγ+, and CD4+IL17+ T cells, the expression of CD4+IL4+ T cells in the skin as well as circulating levels of IL4 in patients with SSc prior to and following B cell depletion therapy. Moreover, we assessed CD4+CD40L+ T cells in the periphery of patients with SSc prior to and following RTX treatment. The CD40L molecule is a member of the TNF superfamily and is mainly expressed from T helper cells shortly following their activation [17]. The interaction between CD40, a molecule mainly expressed on B cells, macrophages and dendritic cells, and its ligand CD40L plays an important role in the immune system orchestrating several processes [18, 19].

We report herein that peripheral blood CD4+CD40L+ T cells and both peripheral and skin CD4+IL4+cells decrease significantly in patients with SSc following B cell depletion treatment.

Materials and methods

Patients and controls

Twenty one patients (19 females, 2 males) with SSc were recruited. All study subjects were diagnosed with SSc according to the 1980 American College of Rheumatology criteria. All of them were Caucasian. Baseline demographic and clinical characteristics as well as standard treatment of patients are presented in Table 1.

Table 1 Demographics and clinical parameters of the cohort

Patient no/sex	Age in years	Disease duration in years	FVC	DLCO	Disease subset	AutoAb	Concurrent treatment
Treatment group							
1/F	40	1	120	70	Limited	–	–
2/F	77	2	84	32	Diffuse	Sclero 70	Cs
3/F	55	8	106	74	Diffuse	Sclero 70	MMF, Bos
4/F	56	5	68	38	Diffuse	Sclero 70	MMF, Cs, Bos
5/F	56	1	62	62	Diffuse	–	–
6/F	64	7	61	47	Diffuse	Sclero 70	MMF, Cs, Bos
7/F	55	4	96	83	Diffuse	–	MTX
8/F	56	6	70	33	Diffuse	Sclero 70	Cs, Bos
9/M	42	13	46	54	Limited	–	MMF, Cs, Bos
10/F	56	7	90	67	Diffuse	Sclero 70	MMF
11/M	39	13	30	14	Diffuse	Sclero 70	MMF, Cs, Bos
12/F	47	6	85	67	Diffuse	Sclero 70	D-Penicillamine, Cs, Bos
13/F	36	2	96	82	Diffuse	Sclero 70	Cs
14/F	76	2	88	65	Diffuse	Sclero 70	Cs, Bos
15/F	56	1	94	45	Limited	CenpB	–
Control group							
1/F	86	12	53	15	Diffuse	Sclero 70	Cs
2/F	73	15	83	77	Diffuse	Sclero 70	Cs
3/F	57	9	101	79	Limited	–	–
4/F	46	3	89	62	Diffuse	Sclero 70	CYC, Bos
5/F	61	12	86	52	Diffuse	–	MMF
6/F	81	5	76		Limited	–	HCQ, Cs

All patients were ANA-positive

Cs low-dose corticosteroids, *MMF* mycophenolate mofetil, *Bos* Bosentan, *CYC* Cyclophosphamide, MTX Methotrexate, HCQ Hydroxychloroquine

Fifteen patients received either two or four cycles of RTX according to the rheumatoid arthritis or the NHL protocol, respectively (scleroderma RTX group). Six out of these 15 patients received two cycles of RTX 1gr each 15 days apart. Nine out of these 15 patients received 4-weekly cycles of RTX at a dose of 375 mg/m^2. Six patients received standard treatment alone (scleroderma control group). All patients were evaluated at baseline and 6 months later. During the 6-month period of the study patients' treatment remained unchanged. All study patients gave written informed consent and an ethics committee based at Patras University Hospital in Greece approved the study protocol which fulfilled the Declaration of Helsinki ethical standards.

Cells and antibodies

Previous studies have suggested that B cell depletion treatment alters peripheral T cell subpopulations as well [20]. To address this issue in RTX-treated SSc patients we evaluated for potential alterations in the percentages of circulating Th2 cells. We performed flow cytometry on PBMCs of 12 out of 15 patients with SSc (10 with diffuse and 2 with limited SSc) treated with RTX on top of standard treatment at time points 0 and 6 months. In addition, we analyzed 6 patients with scleroderma (4 with diffuse and 2 with limited) receiving standard treatment alone (at 2 different time points, 6 months apart). 20 ml of heparinized peripheral venous blood was drawn from all patients. Using standard methods we isolated Peripheral Blood Mononuclear Cells (PBMCs). PBMCs were enriched in CD4+ T cells using a human CD4 isolation kit purchased from Miltenyi (Auburn, CA) according to the manufacturer's instructions using a negative selection strategy. Percentages of CD4+ T cells in the obtained population in all cases were > 92% as determined by flow cytometer (Beckman Coulter, Brea, CA, USA). Cells were rested overnight and stained with intracellular or surface antibodies using standard methods described previously. When intracellular staining was used, PBMCs enriched in CD4+ T cells were stimulated with PMA, Iomycin and Brefeldin A for 6 h according to time–response experiments (data not shown) whereas for surface staining cells were stimulated with PMA and Iomycin for 4 h according to time–response curves (data non shown). Murine anti-human monoclonal antibodies used were: anti-IL4 PE (R&D systems, Minneapolis, MN, USA), anti-INFγ FITC (BD Biosciences, San Jose, CA, USA), anti-IL17 APC (eBiosciences, San Diego, CA, USA) and surface anti-CD4 ECD (Beckman Coulter, Brea, CA, USA), anti-CD40L PE (R&D systems, Minneapolis, MN, USA).

Flow cytometry

Flow cytometry was performed using a Beckman Coulter cytometer. Results were analyzed using the CXP Cytometer software version 2.2 (Beckman Coulter, Brea, CA, USA). One million PBMCs from 12 patients belonging to the scleroderma RTX group and 6 belonging to the scleroderma control group highly enriched in CD4+ T cells (> 92%) were stimulated in vitro with PMA and ionomycin and then stained with either anti-IL4, anti-INFγ or anti-IL17 mAb according to the methods described previously. In addition, one million PBMCs from 8 patients belonging to the scleroderma RTX group and 3 belonging to the scleroderma control group highly enriched in CD4+ T cells (> 92%) were stimulated in vitro with PMA and ionomycin and then stained with anti-CD40L mAb according to the methods described previously. Th1, Th2, and Th17 cells were defined as PBMCs enriched in CD4 cells if stained positive for intracellular INFγ, IL4 and IL17, respectively.

ELISA

Serum from all study subjects was obtained by centrifugation of 10 ml of venous blood at 1700 rpm for 10 min. Serum IL4 levels were determined with a qualitative sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, MN, USA).

Skin biopsies immunofluorescence

Skin biopsies (of 5 mm diameter each) were obtained from four patients belonging to the scleroderma RTX group prior to and 6 months following RTX administration. All biopsies were obtained from skin lesions of the patient's forearm; the second biopsy was obtained from skin lesions next to (distance < 2 cm) the first biopsy. The same procedure was also performed in 2 SSc patients belonging to the scleroderma control group. 10% neutral buffered formalin was used for the fixation of skin tissue sections; subsequently all skin biopsies were embedded in paraffin. These tissue sections (4 μm-thick) were immunohistochemically assessed. After deparaffinization, skin tissues were rehydrated using serially decreased ethanol concentration. Subsequently, immunofluorescence experiments were performed as previously described [21]. The following primary antibodies were used: rabbit anti-IL4 (1:1000, Acris Antibodies, Cat. No AM20353PU-N) and mouse anti-CD4 (1:100, Acris Antibodies, Cat. No AP20210PU-N). Subsequently secondary antibodies at a dilution of 1:1000 were used; the

goat anti-rabbit Alexa Fluor 488 and the goat anti-mouse Alexa Fluor 568 (Molecular Probes). Nuclei DNA was visualized with Draq5 (Biostatus), while slides were mounted in Mowiol 4-88 (Calbiochem, Gibbstown, NJ, USA). Confocal fluorescence microscopy (Leica TCS SP5 with a Leica DMI6000B microscope) was used for sections' analysis. We then processed the digital images with Adobe Photoshop, Adobe Illustrator and ImageJ software.

Statistical analysis

The statistical analysis of our data was made using the paired Wilcoxon test (GraphPad Prism software version 5, La Jolla, CA, USA). Results were expressed as median and interquantile range (IQR). Values of $p \leq 0.05$ were considered as statistically significant.

Results

Peripheral CD4+IL4+cells from patients with SSc decrease significantly following B cell depletion treatment

The percentages of peripheral CD4+IL4+ T cells decreased at 6 months following RTX infusion: median (IQR): 14.9 (22.63–12.88)% vs 7.87 (12.81–4.90)% at time points 0 and 6 months following RTX treatment, respectively ($p = 0.005$) as shown in Fig. 1a along with a representative experiment (Fig. 2a, b). In contrast, in the scleroderma control group no statistically significant differences were found in the percentages of circulating CD4+IL4+ T cells [median (IQR): 9.43 (19.53–7.50)% vs 14.86 (21.96–6.75)% at baseline and 6 months later, respectively, $p =$ NS]. Results are shown in Fig. 1b along with a representative experiment (Fig. 2c, d). A separate analysis of the subgroup of RTX-treated patients with diffuse SSc ($n = 10$) disclosed similar results. More specifically, the percentages of peripheral CD4+IL4+ T cells decreased: [median (IQR): 17.25 (23.37–12.45)% vs

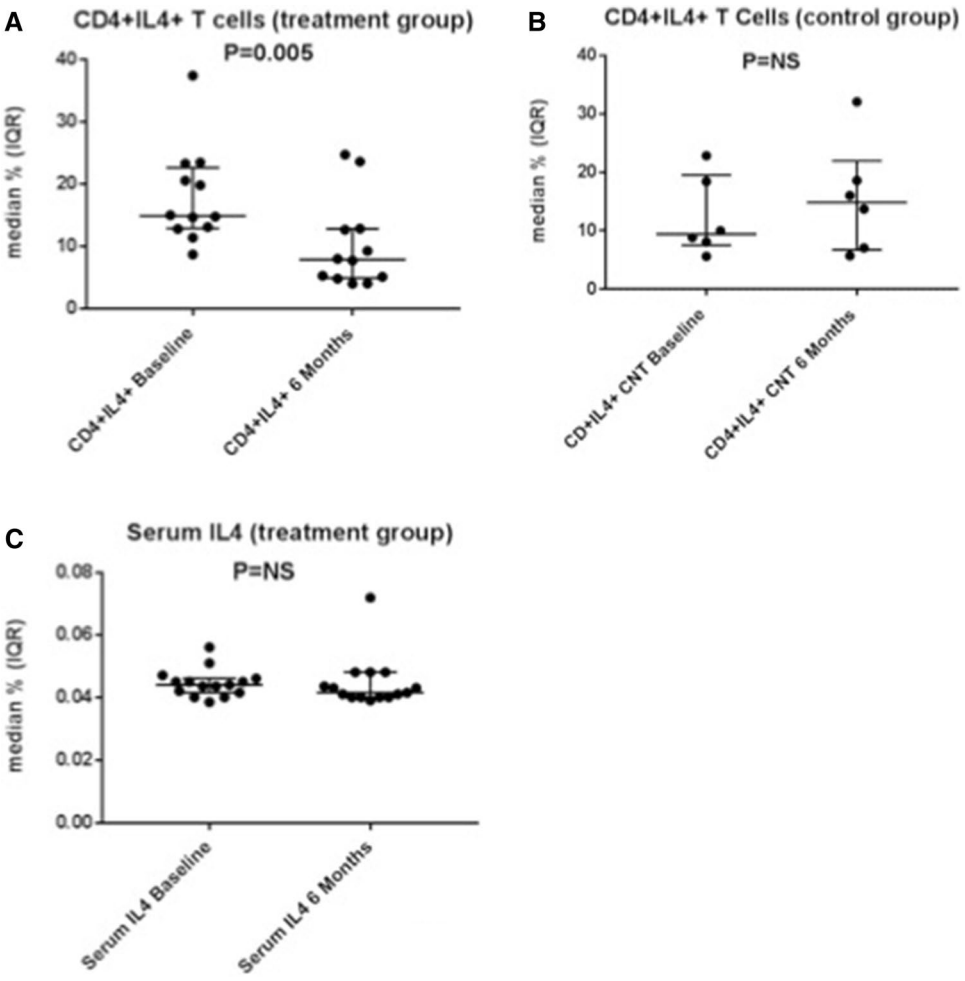

Fig. 1 Peripheral CD4+IL4+ T cells decrease significantly in SSc patients receiving RTX treatment (**a**) while no differences are seen in the scleroderma control group at baseline and 6 months later (**b**). The mean Optical Density of serum IL4 in patients with SSc treated with RTX reveals no differences at baseline and 6 months later (**c**)

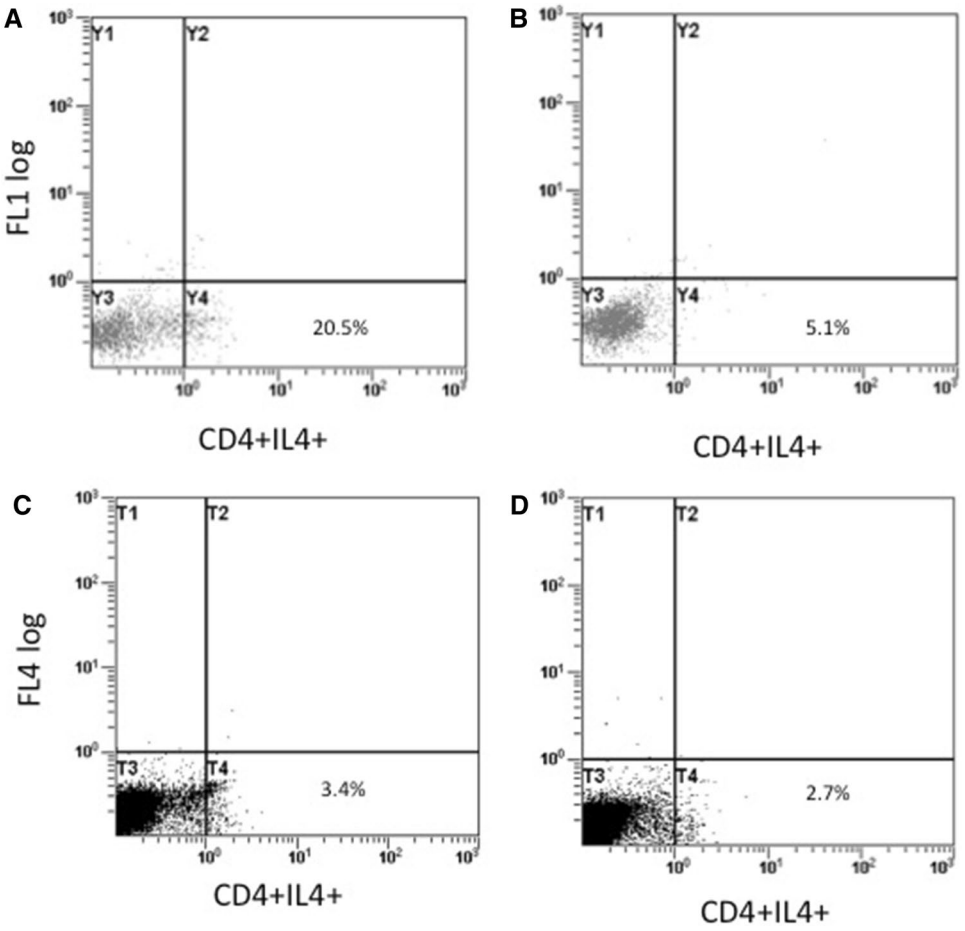

Fig. 2 A representative experiment in a patient with SSc showing flow cytometry analysis of peripheral PBMCs highly enriched in CD4+ T cells single-stained with anti-IL4 mAb at baseline (**a**) and following RTX treatment (**b**). CD4+IL4+ T cells significantly decrease following treatment. A representative experiment showing flow cytometry analysis of peripheral PBMCs highly enriched in CD4+ T cells single-stained with anti-IL4 mAb in a patient with SSc who did not receive RTX treatment. CD4+IL4+ T cells reveal no differences at baseline (**c**) and 6 months later (**d**)

6.51 (15.41–4.63)% prior to and 6 months following RTX treatment, respectively ($p = 0.019$)]. Analyzing the 4 control patients with diffuse SSc disclosed that percentages of peripheral CD4+IL4+ T cells were similar at the 2 different time points (0 and 6 months) [median (IQR): 9.06 (16.32–6.26)% vs 11.55 (28.04–6.09)% at baseline and 6 months later, respectively, $p = $ NS].

Based on the data presented above we suggest that B cell depletion treatment when added on top of standard treatment but not standard treatment alone correlates with a significant reduction of peripheral CD4+IL4+ T cells.

Peripheral CD4+INFγ+ T cells from patients with SSc are not affected by RTX treatment

Percentages of CD4+INFγ+ T cells did not differ significantly prior to and following RTX treatment [median (IQR): 2,95 (5.32–1.00)% and 1.57 (2.79–0.97)% at baseline and 6 months later, $p = $ NS]. Furthermore, no differences were found in CD4+INFγ+ T cells in the scleroderma control group [median (IQR): 1.38 (2.81–0.31)% and 1.58 (2.93–0.35)% at baseline and 6 months later, $p = $ NS].

Peripheral CD4+IL17+ T cells from patients with SSc are not affected following RTX treatment

CD4+IL17+ T cells prior to and following RTX administration did not differ significantly [median (IQR): 0.19 (0.36–0.14)% and 0.18 (0.26–0.09)% at baseline and 6 months later, $p = $ NS]. No differences were found in CD4+IL17+ T cells in patients of the scleroderma control group as well [median (IQR): 0.36 (0.51–0.18)% and 0.35 (0.58–0.09)% at baseline and 6 months later, $p = $ NS].

Serum IL4 levels remain similar in patients with scleroderma prior to and following RTX treatment

Having found that CD4+IL4+ cells in the peripheral blood from patients with scleroderma decrease following RTX treatment we pursued to investigate whether IL4 levels in the sera of such patients show any difference following RTX treatment. To this end we conducted a qualitative sandwich ELISA measuring the Optical Densities (ODs) of IL4 prior to and following RTX treatment. The assay was performed in triplicates according to the manufacturer's recommendations

and the mean value was calculated. We found no differences in IL4 levels prior to and following RTX treatment [median (IQR): 0.044 (0.046–0.041)% and 0.041 (0.048–0.040)% at baseline and 6 months later, $p = \text{NS}$]. Results are shown in Fig. 1c.

CD4+CD40L+ T cells significantly decrease following RTX treatment in patients with scleroderma

Increased percentages of activated circulating T cells have been previously reported in patients with SSc [22]. Therefore, we pursued to investigate whether RTX treatment in SSc patients has any effects on the activation of CD4+ T cells. For this reason we measured the expression of CD40L on the cell surface of CD4+ T cells of 8 out of 15 patients with scleroderma prior to and following RTX treatment. In addition, we analyzed 3 patients with scleroderma receiving standard treatment alone (at 2 different time points, 6 months apart). CD4+CD40L+ T cells significantly decreased after RTX treatment as shown in Fig. 3a [median (IQR): 17.78 (25.64–14.44)% vs. 8.15 (22.85–3.08)% at baseline and 6 months later, $p = 0.04$) along with a representative experiment (Fig. 3b, c). In contrast, in the scleroderma control group no statistically significant differences were found in the percentages of circulating CD4+CD40L+ T cells: median (IQR): 22.13 (58.77–8.20)% vs 72.11 (73.05–20.45)% at baseline and 6 months later, respectively, $p = \text{NS}$ (data not shown). Interestingly, there was an increase in the percentage of circulating CD4+CD40L+ T cells which did not reach statistical significance though.

CD4+IL4+ T cells in the skin of scleroderma patients treated with RTX obviously decrease

To investigate whether the decrease in CD4+IL4+ T cells in the peripheral blood was concomitant with a decrease in IL4-expressing cells in the skin of scleroderma patients after RTX treatment we performed immunofluorescence in skin biopsies obtained from four patients of the scleroderma RTX group and two patients of the scleroderma control group. Skin biopsies were immunostained with antibodies against CD4 and IL4 as described in Methods. A marked decrease in CD4+IL4+ T cells expression in the skin was observed in three out of four patients following RTX treatment while no differences were found in CD4+IL4+ T cell expression in the skin of controls. One representative experiment from a patient in the scleroderma RTX group at baseline (Fig. 4a)

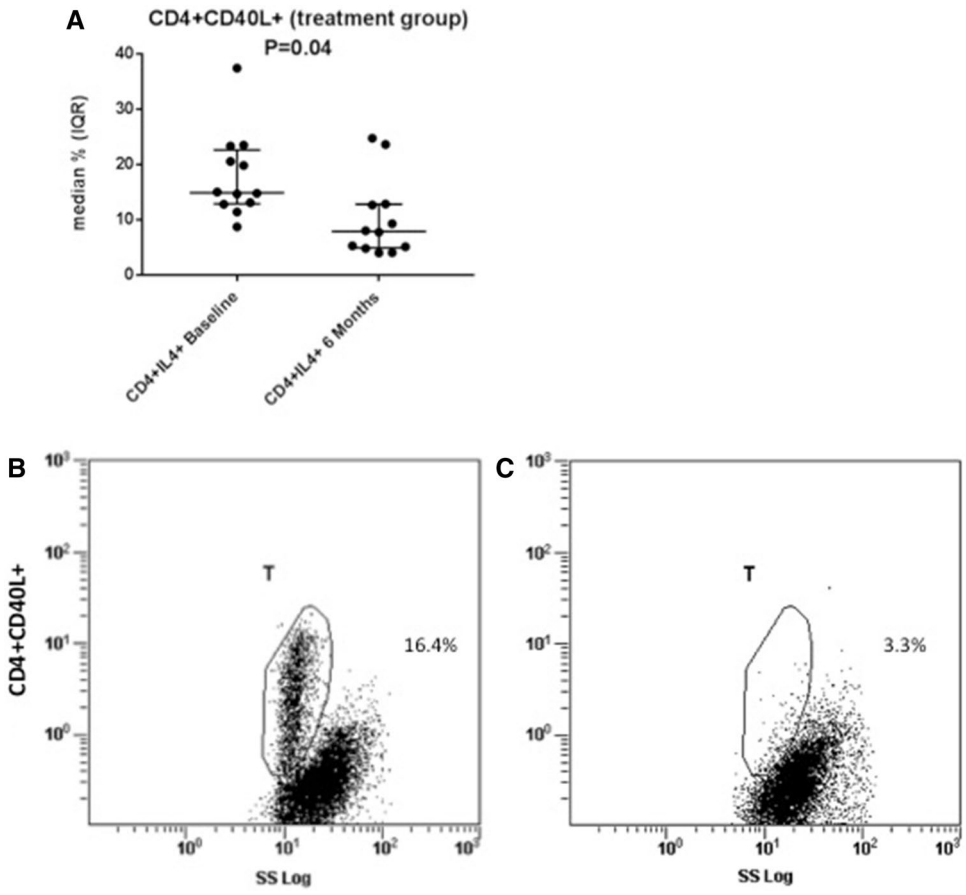

Fig. 3 Peripheral CD4+CD40L+ T cells decrease significantly following RTX treatment in patients with SSc (**a**). A representative experiment in a patient with SSc showing flow cytometry analysis of peripheral PBMCs highly enriched in CD4+ T cells single-stained with anti-CD40L mAb at baseline (**b**) and following RTX treatment (**c**). CD4+CD40L+ T cells significantly decrease following treatment

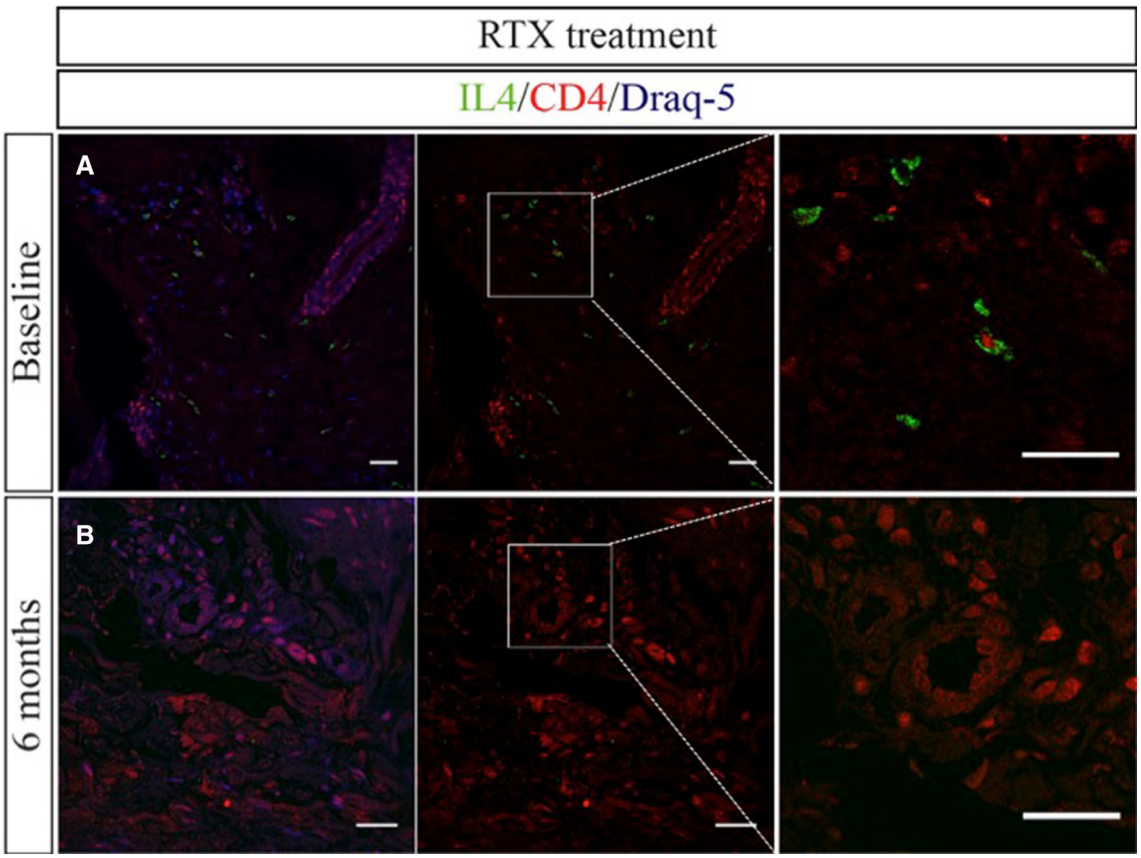

Fig. 4 Immunofluorescence experiments indicating the number of CD4+ and IL4+ T cells in skin biopsies derived from scleroderma patients. Immunofluorescence experiments on skin biopsies were performed using antibodies against CD4 and IL4. Representative confocal images indicating single and double-positive T cells for CD4 and IL4 expression in the skin of a patient with SSc before (**a**) and following RTX treatment (**b**). DNA was stained with Draq-5. Scale bars 30 μm

and following B cell depletion treatment (Fig. 4b) is depicted in Fig. 4. Figure 5a, b shows double CD4+IL4+ stained skin cells of a patient in the scleroderma control group at baseline and at 6 months, respectively. Based on the data presented above we suggest that the decrease in CD4+IL4+ T cells in the peripheral blood of patients with SSc following B cell depletion treatment correlates with a decrease of CD4+IL4+ expressing cells in the skin.

Discussion

Clinical research over the past years indicates that RTX may have disease-modifying properties in SSc. Our group has reported that long-term treatment with RTX associates with improvement of skin fibrosis and stabilization/improvement of pulmonary function tests compared to standard treatment [23]. Despite the fact that a randomized controlled trial exploring the effect of RTX in SSc has not yet been performed, all open label trials performed up until now, have provided clinical and/or histological evidence favouring RTX. Two large-scale multicenter studies, including one from the EUSTAR group have shown beneficial results [6-10]. Based on these, RTX could be considered as a promising option for patients suffering from resistant to standard treatment ILD associated with SSc.

The mechanism of action of RTX in SSc remains largely unknown. We have previously reported that B cell depletion treatment significantly downregulates platelet-derived growth factor receptor expression and activation in scleroderma skin [24]. Moreover, RTX treatment restores the expression of the Wnt pathway inhibitor, Dkk-1, in skin fibroblasts only in the subset of patients who respond to treatment but not in those that do not respond [25]. Even though both of these findings may have pathogenetic implications they cannot elucidate how B cell depletion mediates its beneficial effects in SSc. It has long been recognized that RTX directly targets and depletes B cells. However this treatment affects the immune system broader by indirectly affecting other cells as well, such as T cells [26]. A lot of experimental data point to the direction that T cells may be pathogenetically involved in SSc. Scleroderma skin from

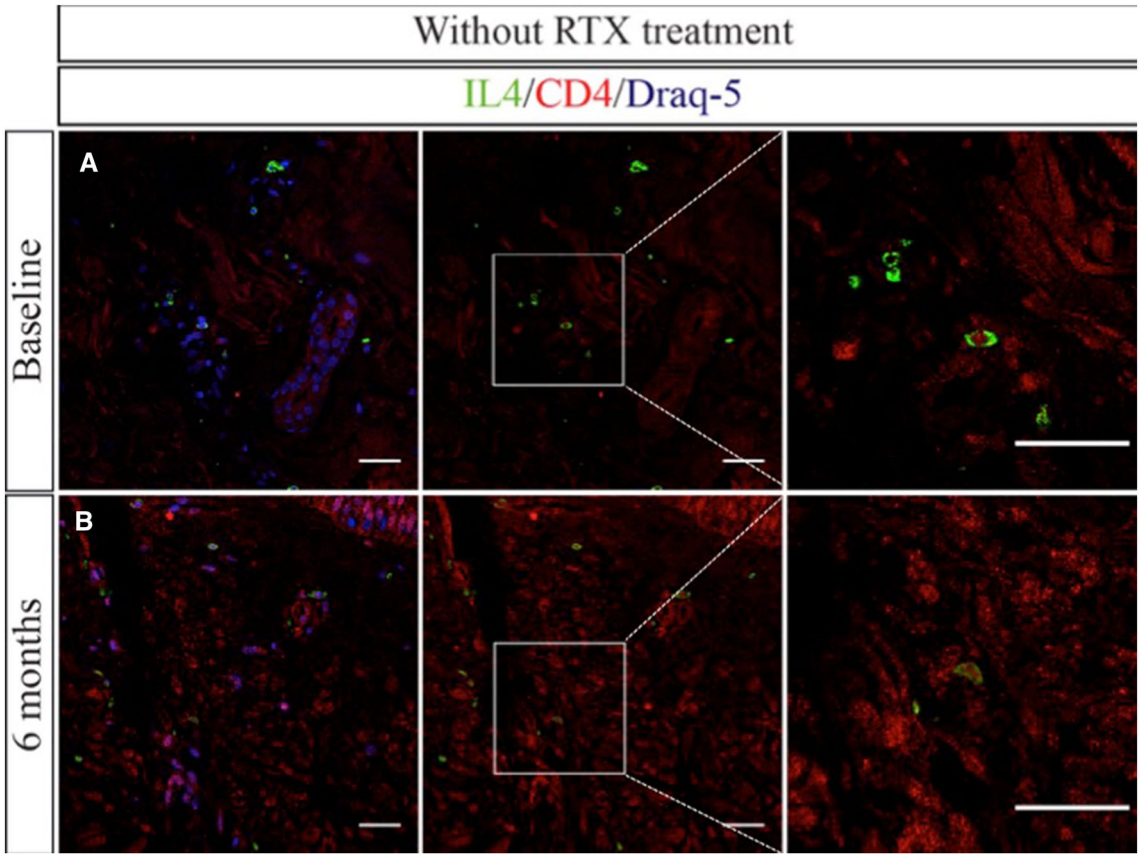

Fig. 5 CD4+IL4+ T cells are observed in the scleroderma control group. Skin biopsies of a patient with SSc that did not receive RTX treatment and were immunostained with anti-CD4 and anti-IL4 mAbs at baseline and 6 months later. A representative confocal image indicating double CD4+IL4+-stained skin T cells at baseline (**a**) and 6 months later (**b**) is shown. Cell nuclei were visualized with Draq-5. Scale bars 30 µm

patients with early disease is infiltrated by T cells which are oligoclonally expanded suggesting that an antigen-driven process may activate and expand T cells in scleroderma skin early in the disease course. These data indicate that T cells may be centrally involved in the pathogenesis of SSc [27]. Based on these, we pursued to investigate the effect of RTX on CD4+ T cells in patients with SSc. We focused on the Th2 subset since the literature supports a profibrotic role for these cells. We found that RTX treatment reduces circulating as well as skin CD4+IL4+ T cells in patients with SSc but has no effect on serum IL4. Moreover RTX treatment downregulates T cell activation as assessed by the surface expression of CD40L in these patients.

The effect of rituximab on CD4+IL4+ T cells is noteworthy because both CD4+IL4+ T cells and IL4 have been reported to contribute to the fibrosis of the heart, liver, kidneys, and skin. For example, Kanellakis et al. showed that IL-4 contributes significantly to cardiac fibrosis [28]. The major amount of IL-4 is produced by mast cells when the heart pressure exceeds normal limits. The researchers constricted the aorta between the two carotid arteries and induced left ventricular fibrosis together with IL-4 production. When they used antibodies targeting IL-4 they found that fibrosis decreased together with interstitial fibroblasts. Furthermore, Aoudjehane et al. showed in vitro that IL4 activates and increases collagen production by human-cultured intrahepatic fibroblasts (hIHFs) isolated from normal liver tissue via STAT-6 pathway [29]. They examined this effect using activation markers and found that their expression was reduced by serum deprivation and came back to normal by adding IL-4. Moreover, hIHFs expressed the IL-4 receptor and the STAT-6 pathway was activated during incubation with IL-4. When antibodies targeting IL-4 were added this activation was inhibited. Moreover, mRNA expression of collagens I, II, and IV as well as collagen levels in supernatants of cell cultures increased after incubation of hIHFs with IL-4.

The profibrotic effects of IL4 both in patients and animal models of systemic sclerosis are well known. IL-4 is a cytokine with multiple actions. Cell growth and immune system function are closely related to IL-4. Moreover IL-4 downregulates T-helper 1 cell activity and differentiates

T-cells towards a Th2 phenotype. IL-4 has been found to be increased in the skin and the serum of patients with systemic sclerosis when compared to healthy controls [30]. Furthermore, IL-4 can stimulate fibroblast collagen gene expression and has mitogenic effect on these cells [31-34]. Interestingly, Ong et al. found that when antibodies against IL-4 were administered early to Tsk/+ mice, a murine model of scleroderma, dermal fibrosis did not develop [35]. In our study we have found that B cell depletion therapy reduces circulating CD4+IL4+ cells but has no effect on soluble IL4. This may mean that other cells apart from Th2 may produce IL4 or, alternatively, that tissue expression of IL4 may be more important than serum levels.

In an effort to further explore the pathogenic mechanisms we pursued to investigate the effect of rituximab on the activation of CD4+ T cells. It has been found that skin and periphery of patients with SSc exhibit activated T cells, expressing CD69 or CD40L. Kalogerou et al. studied skin biopsies from patients with SSc and found increased mononuclear T cells and macrophages in lesional skin and areas around vessels. Expression of CD69 was found increased in these mononuclear cells which mostly contained T cells and macrophages [36]. Recently, Valentini et al. documented increased expression of CD40L on circulating activated T cells from subjects with SSc [22]. In this study we have found a significant decrease on circulating CD4+CD40L+ T cells following RTX treatment which shows that B cell depletion has an incompletely understood but clear effect on T cells by suppressing their activation, a finding with potential pathogenetic implications.

We further sought to explore whether the decrease in the peripheral CD4+IL4+ T cells following B cell depletion treatment is reflected at skin level. We found a significant decrease of CD4+IL4+ T cells in the skin of patients with SSc following RTX administration. Our group used the MRSS tool to assess skin thickening in patients with SSc and found that it significantly decreases following RTX treatment. Taking into account the profibrotic properties of Th2 cells we propose that the beneficial effect of RTX on scleroderma skin may be at least partially mediated by depletion of CD4+IL4+ T cells. However the mechanisms implicated need to be further elucidated.

The primary goal of our research was to investigate the potential mechanism of action of RTX in patients with SSc and provide new insights into potential pathogenetic mechanisms of B cell depletion treatment. However our study has limitations. The number of patients studied was relatively small and the population studied was heterogeneous including patients with both early and late SSc. However, this study provides experimental evidence that could directly or indirectly indicate that B cell depletion treatment in patients with SSc may have a further than B cell depletion role in a target organ such as the skin by altering non-B cell lymphocytic subpopulations.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the "Patras University Hospital (Greece)" ethics committee (ISRCTN99672071) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Written informed consent was obtained from all individual participants included in the study.

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