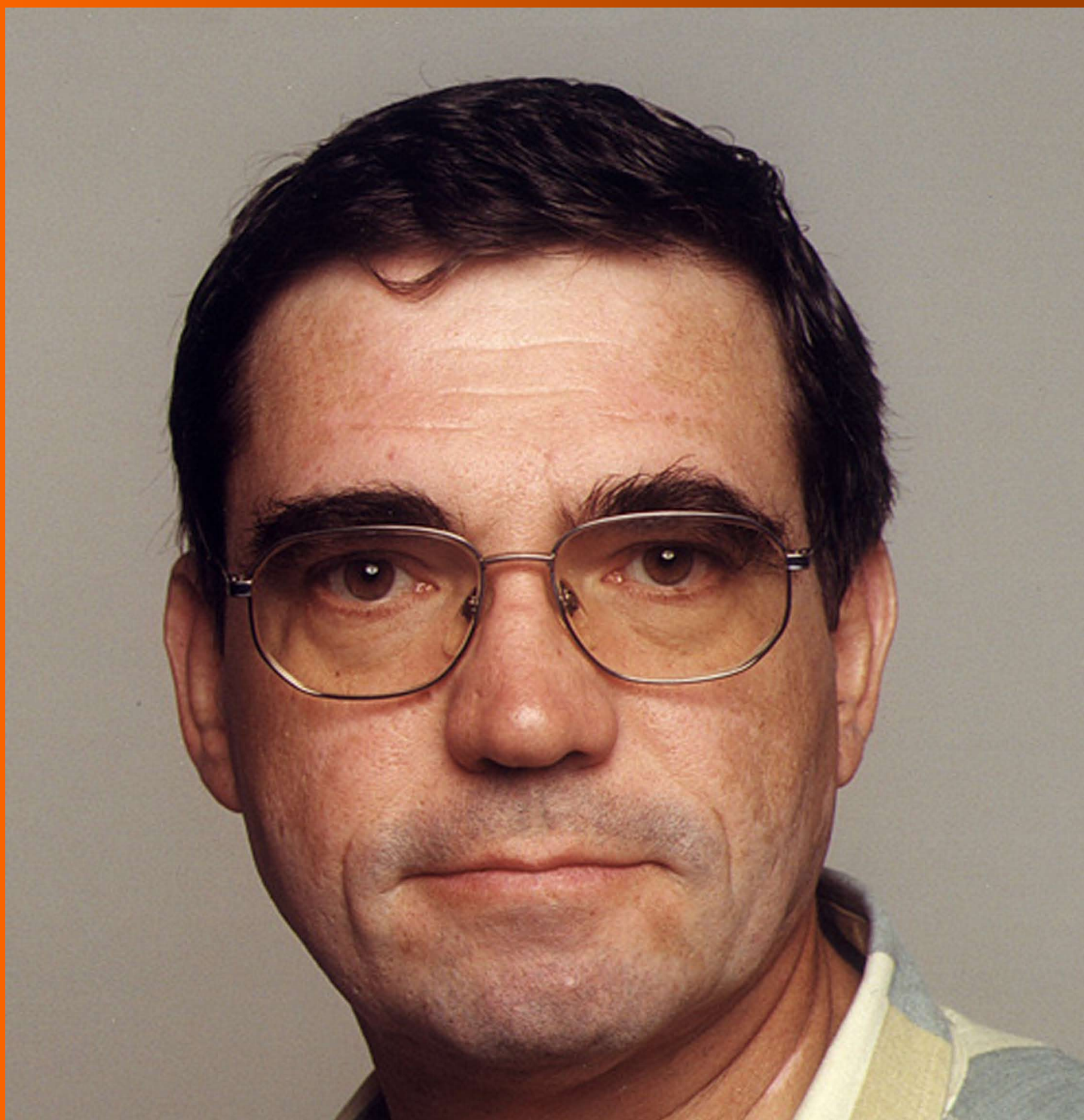


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World Journal of Experimental Medicine is now indexed in PubMed, PubMed Central.

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I-IV Editorial Board

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NAME OF JOURNAL
World Journal of Experimental Medicine

ISSN
ISSN 2220-315X (online)

LAUNCH DATE
December 20, 2011

FREQUENCY
Quarterly

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PUBLICATION DATE
August 20, 2017

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Basic Study

Treg/Th17 cell balance and phytohaemagglutinin activation of T lymphocytes in peripheral blood of systemic sclerosis patients

Ekaterina Krasimirova, Tsvetelina Velikova, Ekaterina Ivanova-Todorova, Kalina Tumangelova-Yuzeir, Desislava Kalinova, Vladimira Boyadzhieva, Nikolay Stoilov, Tsvetelina Yoneva, Rasho Rashkov, Dobroslav Kyurkchiev

Ekaterina Krasimirova, Tsvetelina Velikova, Ekaterina Ivanova-Todorova, Kalina Tumangelova-Yuzeir, Dobroslav Kyurkchiev, Laboratory of Clinical Immunology, University Hospital "St. Ivan Rilski", Department of Clinical Laboratory and Clinical Immunology, Medical University of Sofia, 1431 Sofia, Bulgaria

Desislava Kalinova, Vladimira Boyadzhieva, Nikolay Stoilov, Tsvetelina Yoneva, Rasho Rashkov, Clinic of Rheumatology, University Hospital "St. Ivan Rilski", Department of Internal Medicine, Medical University of Sofia, 1431 Sofia, Bulgaria

Author contributions: All authors contributed equally to this work; Krasimirova E, Velikova T, Ivanova-Todorova E, Tumangelova-Yuzeir K and Kyurkchiev D performed the research; Kalinova D, Boyadzhieva V, Stoilov N, Yoneva T and Rashkov R contributed patient assessment and clinical materials; Krasimirova E and Velikova T analysed the data; Krasimirova E, Ivanova-Todorova E and Kyurkchiev D wrote the paper; Kyurkchiev D approved the final version of the article to be published.

Institutional review board statement: All peripheral blood samples were taken from patients and healthy control subjects after informed written consent and ethical permission was obtained for participation in this study. The study was reviewed and approved by the Institutional Review Board of University Hospital Saint Ivan Rilski, Sofia, Bulgaria.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Data sharing statement: No additional data are available.

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Manuscript source: Invited manuscript

Correspondence to: Dobroslav Kyurkchiev, MD, PhD, DSc, Associate Professor, Laboratory of Clinical Immunology, University Hospital "St. Ivan Rilski", Department of Clinical Laboratory and Clinical Immunology, Medical University of Sofia, Ivan Geshov Str. 15, 1431 Sofia, Bulgaria. dkyurkchiev@medfac.mu-sofia.bg
Telephone: +359-2-8524957

Received: January 26, 2017

Peer-review started: February 8, 2017

First decision: May 10, 2017

Revised: May 26, 2017

Accepted: June 30, 2017

Article in press: July 3, 2017

Published online: August 20, 2017

Abstract

AIM

To investigate T-cell activation, the percentage of peripheral T regulatory cells (Tregs), Th17 cells and the circulating cytokine profile in systemic sclerosis (SSc).

METHODS

We enrolled a total of 24 SSc patients and 16 healthy controls in the study and divided the patients as having diffuse cutaneous SSc (dcSSc, $n = 13$) or limited cutaneous SSc (lcSSc, $n = 11$). We performed a further subdivision of the patients regarding the stage of the disease - early, intermediate or late. Peripheral venous blood samples were collected from all subjects. We performed flow cytometric analysis of the activation

capacity of T-lymphocytes upon stimulation with PHA-M and of the percentage of peripheral Tregs and Th17 cells in both patients and healthy controls. We used ELISA to quantitate serum levels of human interleukin (IL)-6, IL-10, tissue growth factor- β 1 (TGF- β 1), and IL-17A.

RESULTS

We identified a decreased percentage of CD3+CD69+ cells in PHA-stimulated samples from SSc patients in comparison with healthy controls ($13.35\% \pm 2.90\%$ vs $37.03\% \pm 2.33\%$, $P < 0.001$). However, we did not establish a correlation between the down-regulated CD3+CD69+ cells and the clinical subset, nor regarding the stage of the disease. The activated CD4+CD25+ peripheral lymphocytes were represented in decreased percentage in patients when compared to controls ($6.30\% \pm 0.68\%$ vs $9.36\% \pm 1.08\%$, $P = 0.016$). Regarding the forms of the disease, dcSSc patients demonstrated lower frequency of CD4+CD25+ T cells against healthy subjects ($5.95\% \pm 0.89\%$ vs $9.36\% \pm 1.08\%$, $P = 0.025$). With regard to Th17 cells, our patients demonstrated increased percentage in comparison with controls ($18.13\% \pm 1.55\%$ vs $13.73\% \pm 1.21\%$, $P = 0.031$). We detected up-regulated Th17 cells within the lcSSc subset against controls ($20.46\% \pm 2.41\%$ vs $13.73\% \pm 1.21\%$, $P = 0.025$), nevertheless no difference was found between dcSSc and lcSSc patients. Flow cytometric analysis revealed an increased percentage of CD4+CD25-Foxp3+ in dcSSc patients compared to controls ($10.94\% \pm 1.65\%$ vs $6.88\% \pm 0.91\%$, $P = 0.032$). Regarding the peripheral cytokine profile, we detected raised levels of IL-6 [2.10 (1.05 - 4.60) pg/mL vs 0.00 pg/mL, $P < 0.001$], TGF- β 1 (19.94 ± 3.35 ng/mL vs 10.03 ± 2.25 ng/mL, $P = 0.02$), IL-10 (2.83 ± 0.44 pg/mL vs 0.68 ± 0.51 pg/mL, $P = 0.008$), and IL-17A [6.30 (2.50 - 15.60) pg/mL vs 0 (0.00 - 0.05) pg/mL, $P < 0.001$] in patients when compared to healthy controls. Furthermore, we found increased circulating IL-10, TGF- β , IL-6 and IL-17A in the lcSSc subset vs control subjects, as it follows: IL-10 (3.32 ± 0.59 pg/mL vs 0.68 ± 0.51 pg/mL, $P = 0.003$), TGF- β 1 (22.82 ± 4.99 ng/mL vs 10.03 ± 2.25 ng/mL, $P = 0.031$), IL-6 [2.08 (1.51 - 4.69) pg/mL vs 0.00 pg/mL, $P < 0.001$], and IL-17A [14.50 (8.55 - 41.65) pg/mL vs 0.00 (0.00 - 0.05) pg/mL, $P < 0.001$]. Furthermore, circulating IL-17A was higher in lcSSc as opposed to dcSSc subset (31.99 ± 13.29 pg/mL vs 7.14 ± 3.01 pg/mL, $P = 0.008$). Within the dcSSc subset, raised levels of IL-17A and IL-6 were detected vs healthy controls: IL-17A [2.60 (0.45 - 9.80) pg/mL vs 0.00 (0.00 - 0.05) pg/mL, $P < 0.001$], IL-6 [2.80 (1.03 - 7.23) pg/mL vs 0.00 pg/mL, $P < 0.001$]. Regarding the stages of the disease, TGF- β 1 serum levels were increased in early stage against late stage, independently from the SSc phenotype (30.03 ± 4.59 ng/mL vs 13.08 ± 4.50 ng/mL, $P = 0.017$).

CONCLUSION

It is likely that the altered percentage of Th17 and CD4+CD25-FoxP3+ cells along with the peripheral cytokine profile in patients with SSc may play a key role in the pathogenesis of the disease.

Key words: Systemic sclerosis; T-cell activation; Th17; Tregs; CD4+CD25-Foxp3+ cells; Interleukin-17; Tissue

growth factor- β ; Interleukin-10; Interleukin-6

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Core tip: Systemic sclerosis (SSc) is a devastating autoimmune disorder, which can be subclassified into limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc) based on the skin manifestations. One of the original contributions of our study has demonstrated a decreased capacity for PHA-induced peripheral T-cell activation in patients with SSc. For the first time, our research group has identified an up-regulated percentage of CD4+CD25-FoxP3+ cells in the dcSSc subset. Regarding the peripheral cytokine profile in SSc, the serum levels of interleukin (IL)-17A have been increased in lcSSc as opposed to the dcSSc subset. The rest of our data, concerning the elevated circulating IL-6, IL-10, and TGF- β in SSc patients, has confirmed literature-based results.

Krasimirova E, Velikova T, Ivanova-Todorova E, Tumangelova-Yuzeir K, Kalinova D, Boyadzhieva V, Stoilov N, Yoneva T, Rashkov R, Kyurkchiev D. Treg/Th17 cell balance and phytohaemagglutinin activation of T lymphocytes in peripheral blood of systemic sclerosis patients. *World J Exp Med* 2017; 7(3): 84-96 Available from: URL: <http://www.wjgnet.com/2220-315X/full/v7/i3/84.htm> DOI: <http://dx.doi.org/10.5493/wjem.v7.i3.84>

INTRODUCTION

Systemic sclerosis (SSc) is a generalized debilitating connective tissue disease affecting the skin and internal organs characterized by vasculopathy, fibrosis, and autoimmune alterations^[1]. SSc is subclassified into two major clinical subsets, namely diffuse cutaneous (dcSSc) and limited cutaneous (lcSSc) form depending on the spread of the skin sclerosis^[2]. Each of these subtypes has three stages - early, intermediate and late^[2,3]. The dcSSc form distinguishes by rapidly progressive fibrosis of the skin and internal organs, which is a major cause of morbidity and mortality of the patients^[4]. The lcSSc form is marked by vascular injury with milder skin and visceral fibrosis and generally, has a low progression rate^[2,3].

The autoimmune dysregulation in SSc comprises lymphocyte activation that leads to the generation of autoantibodies, abnormal production of cytokines and chemokines, and impairment of the innate immunity^[5-7]. Over the last decade, the accumulating data has shown the central role of T lymphocytes in the pathogenesis of SSc^[8,9].

It is thought that the cytokine production by T cells influences the function of fibroblasts and endothelial cells, thereby playing a central role in vascular disease and fibrosis development^[1,5]. Therefore, many efforts have been made to identify the T cell derived cytokine patterns in SSc and the subsets of T helpers involved. Most studies performed in SSc patients have examined

the characteristics of T cells isolated from peripheral blood.

There is a strong evidence in literature for altered T-cell activation^[10-12] and T helper cells abnormalities in SSc^[8,9]. Several authors have reported higher frequency of Th17 lymphocytes in the peripheral blood of SSc patients and have pointed out the role of these cells as a factor engaged in the pathogenesis of the disease^[13-15]. Th17 cells, firstly described in 2005, produce interleukin (IL)-17A, IL-17F, IL-21, IL-22, and IL-26 and play a key role in host defense against extracellular bacteria and fungi^[16]. Recent data has revealed their implication in the pathogenesis of several inflammatory and autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis, investigating their animal models - experimental autoimmune encephalomyelitis and collagen-induced arthritis^[17]. IL-17 is an inducer of the surface expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) by endothelial cells, and foreskin fibroblasts and induces the production of IL-1 and IL-6^[18-20]. IL-17 also increases the production of pro-inflammatory cytokines such as chemokine (C-C motif) ligand 2 (CCL2), IL-6, IL-8 by synoviocytes and fibroblasts from both human skin and lungs^[19,21]. Regarding the fibrotic process in SSc, IL-17 inhibits type I and type III collagen deposition^[18,22] and reduces the connective tissue growth factor (CTGF) production *via* up-regulation of miR-129-5p in dermal fibroblasts^[23]. Animal models of SSc have demonstrated the involvement of IL-17 in the bleomycin-induced lung and skin fibrosis^[24-26]. Meanwhile, human studies have reported inverse correlation between the number of IL-17+ cells in the skin of SSc patients and the extent of skin sclerosis^[27].

Not only Th17 cells, but also Tregs (CD4+FoxP3+) are involved in pathogenesis of SSc and there is a controversial data concerning their functional and numerical alterations. Some authors have found markedly up-regulated Tregs in all SSc phenotypes^[10,28] particularly in active and severe disease^[29]. Tregs from SSc patients demonstrated a diminished ability to control CD4 effector T cells and this defective function seemed to correlate with lower expression of CD69 and tissue growth factor- β (TGF- β) levels^[10]. One study did not found Treg alterations in SSc patients compared to control groups^[15]. Finally, several studies demonstrated a decreased frequency/impaired function of Tregs in SSc^[30-32].

The CD4+Foxp3+ T cells produce anti-inflammatory cytokines including TGF- β and IL-10 and Tregs are mandatory to establish immune tolerance. TGF- β is a master regulator of the fibrotic process and alterations in TGF- β signaling are well described in SSc^[11]. TGF- β promotes the fibrosis by both stimulating the synthesis, and suppressing the degradation of extracellular matrix^[11]. TGF- β is involved in the generation of peripheral Tregs as well^[33]. Accordingly, the same cytokine, TGF- β , is implicated in the generation of two functionally opposite T cell subsets, effectors - Th17 and Tregs, and

the co-presence or not of pro-inflammatory cytokines, such as IL-6 and IL-1, determines the fate of TGF- β -exposed T cells^[30]. Thus, the concomitance of TGF- β and IL-6 in SSc skin infiltrates could favor the generation of effector Th17 cells at the expense of Tregs, leading to complete alteration of the homeostatic equilibrium. Regarding IL-10, it has been reported to be increased in the serum of SSc patients^[34]. Moreover, one paper has revealed that the raised serum levels of IL-10, and IL-6 correlated positively with the interstitial lung disease and the modified Rodnan skin score (MRSS) of patients^[35].

Based on all the aforementioned data, we decided to evaluate the activation capacity of T cells in the peripheral blood of SSc patients and healthy controls, using phytohemagglutinin (PHA-M). Our next aim was to determine both the percentage of the effector (Th17) and regulatory (Treg) cell subsets in the peripheral blood of the patients and the controls. We also investigated the serum levels of the peripheral cytokine milieu in both SSc patients and controls, *scilicet*, IL-6, IL-10, TGF- β 1 and IL-17A.

It is laborious to obtain reliable incidence and prevalence estimates of SSc since the disease rarely occurs. Up to now, no studies have been carried out on the SSc incidence and prevalence in Bulgarian population. However, several epidemiological studies have been performed in Southeastern Europe. For instance, the incidence of SSc in Greece (North West) was 11 cases/million per year and the prevalence - 7.7 cases/million (1981-2002^[36]). Respectively, the estimated prevalence of SSc in Croatia (Split-Dalmatia) based on 2008 data was 15 cases/million^[37]. Although our study included a relatively small cohort of SSc patients, it could be assessed quite representative for our population if compared to the existing epidemiological data.

MATERIALS AND METHODS

Ethical committee statement

Informed written consent was obtained from all the subjects, enrolled in our study after approval of the Ethics Committee at the University Hospital St. Ivan Rilski, Sofia. All experiments carried out complied with the Declaration of Helsinki.

Population studied

Twenty-four patients, who attended the Clinic of Rheumatology of Department of Internal Medicine, Medical University of Sofia, were enrolled in this study. The mean age of the patients (male - 1, female - 23) was 47.1 ± 13.2 years. All the patients fulfilled the 2013 ACR/EULAR Criteria for the classification of SSc^[38] and were divided as having dcSSc or lcSSc depending on the extent of skin sclerosis^[2]. A further subdivision of the patients was performed in the groups based on the years from diagnosis^[3]. Patients with dcSSc were divided in three groups: Early dcSSc (< 3 years' duration), intermediate (3-6 years) and late (6+ years). In the

Table 1 Clinical data of patients with systemic sclerosis, enrolled in the study

Patient No.	Gender	Age	Form	Stage	Active SSc	Visceral damage	Autoantibodies ¹	Treatment regimen
1	M	50	dcSSc	Intermediate	Yes	No	Speckled	PMP
2	F	49	dcSSc	Late	No	E	Anti-Scl70	MTX
3	F	55	dcSSc	Intermediate	No	E	Speckled	MP
4	F	58	dcSSc	Late	Yes	PF	Anti-Scl70	PMP, PCYP
5	F	44	dcSSc	Early	Yes	PF	Anti-Scl70	DPA, MP
6	F	27	lcSSc	Early	Yes	No	Anti-Scl70	DPA, MP, TCZ
7	F	48	dcSSc	Early	Yes	PF	Anti-Scl70	DPA, MP
8	F	37	lcSSc	Early	Yes	No	Anti-Ro52	CHQ
9	F	65	dcSSc	Late	No	No	Anti-CENP-A, Anti-CENP-B	MP, CHQ
10	F	36	lcSSc	Intermediate	Yes	No	Speckled	PMP, PCYP, DPA
11	F	47	dcSSc	Early	Yes	SRC	Anti-Scl70	PMP, PCYP
12	F	32	lcSSc	Early	Yes	PF	Speckled	MP, TCZ
13	F	62	dcSSc	Early	Yes	No	Speckled	PMP, PCYP
14	F	27	lcSSc	Late	Yes	No	Anti-PM/Scl-100	MTX
15	F	73	lcSSc	Intermediate	No	PF	Speckled	MP, MTX
16	F	32	dcSSc	Late	Yes	PF	Anti-Scl70, Anti-PM/Scl-75	PMP, PCYP
17	F	60	dcSSc	Late	Yes	No	Speckled	PMP, PCYP
18	F	34	dcSSc	Early	Yes	No	Anti-Scl70	MP, MTX
19	F	56	lcSSc	Late	No	E	Anti-CENP-B	MP
20	F	53	lcSSc	Early	Yes	No	Anti-PM/Scl-75	MP, AZA
21	F	30	lcSSc	Late	Yes	No	Speckled	MP, DPA
22	F	61	dcSSc	Late	No	E, PF, PH	Anti-Scl70	MP
23	F	39	lcSSc	Early	No	No	Anti-CENP-B	MTX
24	F	56	lcSSc	Intermediate	No	E	Speckled	MP, MTX

¹In cases where no SSc specific autoantibody was detected, the staining pattern of patient's serum on indirect immunofluorescence is shown. F: Female; M: Male; E: Esophageal dysmotility; PF: Pulmonary fibrosis; PH: Pulmonary hypertension; SRC: Scleroderma renal crisis; MP: Methylprednisolone; PMP: Pulse MP; MTX: Methotrexate; CYP: Cyclophosphamide; PCYP: Pulse CYP; DPA: D-penicillamine; CHQ: Chloroquine; TCZ: Tocilizumab; AZA: Azathioprine.

lcSSc group, the following subdivision was performed: Early lcSSc (< 5 years' duration), intermediate (5-10 years) and late (10+ years) stages. The disease activity was assessed according to the Preliminary Revised EUROSTAR Activity Index^[39]. Sixteen age and gender-matched healthy individuals served as controls. Patients' clinical data as well as treatment regimens are shown in Table 1.

Activation capacity of T-lymphocytes in response to PHA-M stimulation of in patients with SSc

Heparinized whole venous blood, 2 mL was collected (LH 68 IU BD-Plymouth, United Kingdom, 5 mL) from each subject and was separated equally into two tubes - control tube and a PHA-M stimulated sample. To the stimulated test tube 20 µg/mL PHA-M (Roche Diagnostics GmbH, Germany) was added and the two samples were incubated for 4 h at 37 °C, 5% CO₂. The samples were gently shaken, at regular intervals, on a multispeed vortex (MSV-3500 BioSan LV). Afterwards, 100 µL blood from each tube was labeled with monoclonal anti-CD3 FITC (for determination of the T lymphocytes) and anti-CD69 PE, an early activation marker for T cells (BD Biosciences, United States) and incubated for 30 min, at room temperature (RT) in the dark. Followed a lysis of erythrocytes (BD FACS Lysing Solution, BD Biosciences, United States), then centrifuging at 1300 rpm for 10 min and double washing (CellWash, BD Biosciences, United States). Subsequently, cells were fixed with 200 µL CellFIX (BD Biosciences, United States) and were analyzed with BD FACSCalibur flowcytometer using the Cell Quest

software for data acquisition and analysis. Then 20000 lymphocytes were counted and analyzed for expression of CD69. The results obtained for each patient and healthy subject were analyzed for PHA-stimulated and unstimulated lymphocytes.

Flow-cytometric analysis of Th17 cells in SSc patients

Peripheral whole venous blood, 1 mL was collected (K2E BD-Plymouth, United Kingdom, 5 mL) from each subject. Monoclonal anti-CD3 FITC, anti-CD161-PE, anti-CD4-PerCP and anti-CD196-Alexa Flour 647 antibodies (BD Biosciences, United States) were added to the blood samples and incubated for 30 min, at RT in the dark. Followed a lysis of erythrocytes with a lysing solution (BD FACS Lysing Solution, BD Biosciences, United States) and after double washing in a CellWash solution (BD Biosciences, United States) the cells were fixed (CellFIX, BD Biosciences, United States). The specific fluorescent labeling was analyzed with BD FACSCalibur flowcytometer and 10000 lymphocytes were counted and analyzed using the Cell Quest software program of the same company.

Flow-cytometric analysis of Tregs in SSc patients

Peripheral venous blood, 1 mL was collected (K2E BD Vacutainer, BD-Plymouth, United Kingdom, 5 mL) from each individual. Monoclonal anti-CD25 FITC and anti-CD4-PE (BD Biosciences, United States) were added to the blood samples and incubated for 30 min, at RT in the dark. Followed a lysis of erythrocytes with a lysing solution (BD FACS Lysing Solution, BD Biosciences, United States) and after double washing in a CellWash

Table 2 T helper subsets in systemic sclerosis patients and healthy controls

T cell subpopulation (%)	SSc patients	Healthy controls	P value
CD4+Foxp3+	14.24 ± 1.39 (5.68-28.73)	11.04 ± 1.22 (3.55-20.84)	0.052
CD4+CD25-Foxp3+	10.22 ± 1.21 (2.09-23.09)	6.88 ± 0.91 (1.42-12.79)	
CD4+CD25+Foxp3+	4.02 ± 0.52 (0.71-10.77)	4.16 ± 0.53 (2.08-8.05)	0.016
CD4+CD25+	6.30 ± 0.68 (1.40-13.36)	9.36 ± 1.08 (2.84-19.60)	
Th17	18.13 ± 1.55 (9.18-32.64)	13.73 ± 1.21 (4.30-20.99)	0.031

Data are expressed as means ± SE. SSc: Systemic sclerosis.

solution (BD Biosciences, United States) a Human FoxP3 Buffer set (BD Biosciences, United States) was used for permeabilization of the cell membranes, as described by the manufacturer's instructions. Afterwards, a monoclonal antibody against intracellular expression of FoxP3 was used (anti-FoxP3 PE). After double washing, the cells were re-suspended in a wash buffer and analyzed immediately with BD FACSCalibur flowcytometer. At least 20000 CD4 positive lymphocytes were acquired using the Cell Quest software program.

Evaluation of serum soluble cytokines

Serum from each subject, 5 mL was collected using serum separator tubes (Vacutainer BD-Plymouth, United Kingdom, 5 mL). Circulating cytokine levels (serum IL-6, IL-10, IL-17A, TGF- β 1) were measured using Diaclone Human ELISA kits (Diaclone SAS, France) according to the manufacturer's instructions and every sample was tested in duplicates.

Statistical analysis

For the analysis of the data's distribution, the Kolmogorov-Smirnov test was used. In cases of normal distribution, we determined mean ± SE, minimum, and maximum values and used a two-sample *t*-test and ANOVA for further statistical evaluation of the experimental data. In cases of non-normal distribution, median, interquartile range (IQR), minimum, and maximum values, were calculated and the Mann-Whitney test was applied. The strength of linear relationship between two continuous variables was examined using Pearson's correlation coefficient. Differences were considered as significant at $P < 0.05$. All statistical analyses were performed using IBM SPSS Statistics (IBM® SPSS® Statistics, Version 19).

RESULTS

PHA-activation of peripheral blood lymphocytes

We found no significant differences in the frequency of early activated T cells (CD3+CD69+) in unstimulated peripheral blood samples (control test tube) between healthy control subjects and SSc patients. However

CD4+CD25+ lymphocytes, which are considered to be activated cells, were represented in decreased percentage in patients when compared to controls ($P = 0.016$, Table 2). Regarding the disease phenotype, dcSSc patients demonstrated lower frequency of CD4+CD25+ T cells against healthy subjects ($5.95\% \pm 0.89\%$ vs $9.36\% \pm 1.08\%$, respectively, $P = 0.025$).

In the PHA-stimulated samples, CD3+CD69+ cells were represented in decreased percentage in patients when compared to controls ($13.35\% \pm 2.90\%$ vs $37.03\% \pm 2.33\%$, respectively, $P < 0.001$) (Figure 1). As regards the lcSSc and dcSSc, there was no difference between the two phenotypes and in comparison with the healthy subjects.

Th17 cells

With regard to the Th17 cells, we found an up-regulated percentage in patients as opposed to controls ($P = 0.031$; Table 2). Accordingly, an increased percentage of Th17 cells was detected within the lcSSc subset vs controls ($20.46\% \pm 2.41\%$ vs $13.73\% \pm 1.21\%$, respectively, $P = 0.025$) (Figure 2). We detected no difference regarding the percentage of Th17 cells between the dcSSc and lcSSc phenotypes nor, when compared to controls.

Treg cells

There was no difference between patients and healthy individuals regarding CD4+Foxp3+ cells. We detected a certain trend toward increased percentage of these cells within the dcSSc subgroup as opposed to controls ($14.73\% \pm 1.71\%$ vs $11.04\% \pm 1.22\%$, respectively, $P = 0.083$). There was also no difference between patients and healthy individuals, regarding CD4+CD25+Foxp3+ T cells, nor within the distinct subtypes of SSc (Table 2). The percentage of CD4+CD25-Foxp3+ was marginally higher in patients ($P = 0.052$; Table 2) compared to controls. Although their percentage was increased in dcSSc vs controls ($10.94\% \pm 1.65\%$ vs $6.88\% \pm 0.91\%$, respectively, $P = 0.032$) (Figure 3). Still, we did not found differences between the dcSSc and lcSSc subsets.

Circulating cytokines

Regarding the peripheral cytokine profile, we detected increased levels of IL-6 [2.10 (1.05 - 4.60) pg/mL vs 0.00 pg/mL, $P < 0.001$], TGF- β 1 (19.94 ± 3.35 ng/mL vs 10.03 ± 2.25 ng/mL, $P = 0.02$), IL-10 (2.83 ± 0.44 pg/mL vs 0.68 ± 0.51 pg/mL, $P = 0.008$), and IL-17A [6.30 (2.50 - 15.60) pg/mL vs 0 (0.00 - 0.05) pg/mL, $P < 0.001$] in patients when compared to healthy controls (Table 3). Furthermore, we found increased circulating IL-10, TGF- β , IL-6 and IL-17A in the lcSSc subset vs control subjects, as it follows: IL-10 (3.32 ± 0.59 pg/mL vs 0.68 ± 0.51 pg/mL, $P = 0.003$), TGF- β 1 (22.82 ± 4.99 ng/mL vs 10.03 ± 2.25 ng/mL, $P = 0.031$), IL-6 [2.08 (1.51 - 4.69) pg/mL vs 0.00 pg/mL, $P < 0.001$], and IL-17A [14.50 (8.55 - 41.65) pg/mL vs 0.00 (0.00 - 0.05) pg/mL, $P < 0.001$]. Furthermore, circulating IL-17A was higher in lcSSc as opposed to

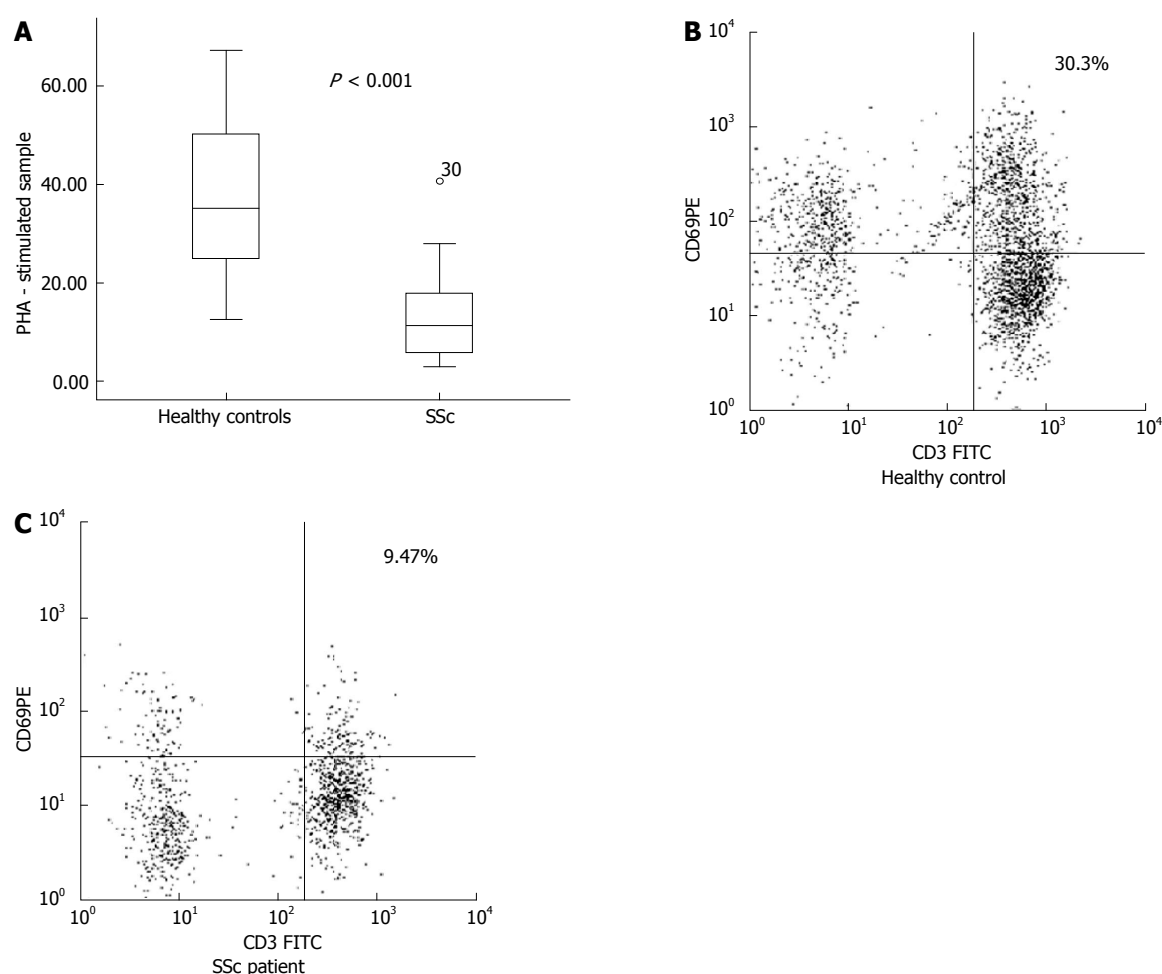


Figure 1 Decreased percentage of CD3+CD69+ cells upon PHA stimulation in the circulation of patients with systemic sclerosis as opposed to healthy controls. A: Percentage of CD3+CD69+ cells in PHA-stimulated samples from SSc patients ($n = 24$) and healthy controls ($n = 16$), as it follows: $13.35 \pm 2.90\%$ vs $37.03 \pm 2.33\%$, $P < 0.001$. The boxplots represent mean \pm SD; B and C: PHA stimulated sample of one representative subject from each group is shown. The percentage of CD69+ cells in the whole T cell pool (CD3+ cells) is depicted. SSc: Systemic sclerosis.

dcSSc subset (31.99 ± 13.29 pg/mL vs 7.14 ± 3.01 pg/mL, $P = 0.008$). Within the dcSSc subset, raised levels of IL-17A and IL-6 were detected vs healthy controls: IL-17A [2.60 (0.45 - 9.80) pg/mL vs 0.00 (0.00 - 0.05) pg/mL, $P < 0.001$], IL-6 [2.80 (1.03 - 7.23) pg/mL vs 0.00 pg/mL, $P < 0.001$].

The findings on circulating cytokines regarding the comparison of the two SSc phenotypes and vs healthy controls are depicted in Figure 4.

Relationship between activity, stage of SSc, presence of visceral organ involvement and the investigated immune parameters

Patients were divided in two groups depending on the disease activity. Sixteen patients had active disease, while eight patients were with stable/inactive SSc (Table 1). We identified no differences between the two groups, regarding Tregs, Th17 cells and levels of the serum soluble cytokines.

The distribution of the patients according to the stage of SSc was as follows: Early SSc, $n = 10$, intermediate SSc, $n = 5$, and late SSc $n = 9$ (Table 1). The stage of the disease did not influence the percentage of Tregs, nor

the frequency of Th 17 cells in patients' peripheral blood. As regards to the circulating cytokines, only TGF- β 1 serum levels were increased in early stage against late stage, independently from the SSc phenotype (30.03 ± 4.59 ng/mL vs 13.08 ± 4.50 ng/mL, $P = 0.017$).

Twelve patients enrolled in the study had visceral organ involvement, the distribution was as follows: Pulmonary arterial hypertension (PAH), $n = 1$; pulmonary fibrosis (PF), $n = 7$; esophageal dysmotility (E), $n = 5$; scleroderma renal crisis (SRC), $n = 1$ (Table 1). No differences were observed regarding the peripheral immune parameters in cases of presence of visceral organ involvement.

DISCUSSION

For the purposes of our study, we used PHA-M to activate resting T cells. PHA is a classical mitogen leading to selective nonspecific T-cell activation and proliferation^[40]. In the mid-1970s, it was found that T-cell proliferation induced by PHA requires the presence of monocytes. Ceuppens *et al.*^[41] confirmed this statement and identified that the addition of purified human IL-6,

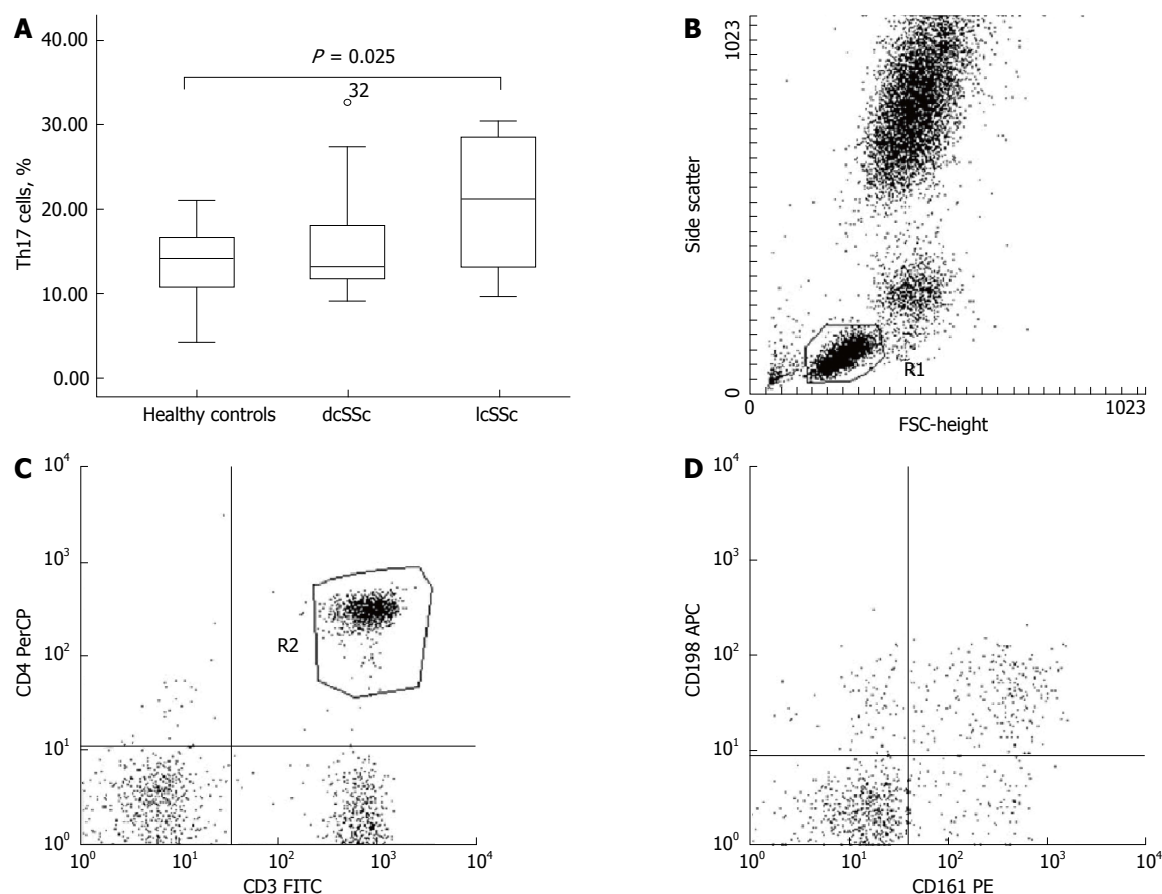


Figure 2 Increased percentage of Th17 cells within limited cutaneous systemic sclerosis subset vs healthy controls. A: Percentage of Th17 cells for lcSSc ($n = 11$), dcSSc ($n = 13$), and healthy controls ($n = 16$) is presented. Increased percentage of Th17 cells within lcSSc patients as opposed to controls, respectively, $20.46\% \pm 2.41\%$ vs $13.73\% \pm 1.21\%$, $P = 0.025$. Boxplots are expressed as means \pm SD; B-D: Panel B depicts the flow cytometric analysis of Th17 cells. A representative patient with lcSSc phenotype is shown. The lymphocytes were gated according to their physical characteristics (FSC and SSC) in R1; afterwards T helper cells (CD3+CD4+) were gated in R2. T helpers, which were detected double positive for CD161 and CD196 surface expression (R3, upper right quadrant) were defined as Th17 cells. lcSSc: Limited cutaneous systemic sclerosis.

along with monocytic supernatant, to PHA-stimulated cell cultures has led to effective T-cell activation and proliferation.

Aiming to approach our study to the conditions *in vivo*, we used heparinized venous blood samples. Moreover, we identified increased serum levels of IL-6 in our SSc patients, which as previously mentioned, is a factor involved in T-cell activation. In the PHA-stimulated samples, we detected a decreased percentage of CD3+CD69+ cells in patients when compared to healthy controls.

The circulating cytokine profile in our SSc patients might relate to the decreased ability of T cells to be activated. Our data has revealed increased levels of IL-10, TGF- β , and IL-6 in peripheral blood of SSc patients and all these cytokines are engaged directly or not in the process of suppression of T-cell activation.

IL-10 is a pleiotropic cytokine with important anti-inflammatory and immunoregulatory functions, which inhibits the activity of Th1 cells^[42,43]. Along with the tolerogenic dendritic cells and Treg subsets, other immunocompetent cells secreting IL-10 has been studied, including B cells, NK cells, neutrophils, and macrophages. The role of Th2 cells that produce IL-10

is also well-established^[44]. However, recent data have paradoxically demonstrated that Th1 and Th17 cells are also able to secrete IL-10. It is thought that these "double-natured" T cell subsets use the secretion of IL-10 to suppress their own proinflammatory activity, directly, or in concert with tolerogenic antigen-presenting cells^[43]. Some studies suggest that IL-10 (alone or in combination with IFN- γ) also has an inhibitory function regarding the fibrotic process in SSc^[45]. Based on the literature, IL-6 inhibits the differentiation of monocytes in dendritic cells alone or through induced autocrine secretion of IL-10^[46,47]. Likewise, both IL-6 and IL-10 restrain the antigen-presenting function of dendritic cells, which ultimately results in a formation of immature tolerogenic myeloid cells secreting IL-10 and their antigen-presenting capacity results in T lymphocytes' anergy^[48]. Along with IL-10, TGF- β also exerts an inhibitory action on T cells. TGF- β inhibits the IL-2 promoter/enhancer activity, which results in a block of IL-2 gene expression in T cells^[49].

TGF- β inhibitory effect on T cells may be mediated through up-regulation of cyclin-dependent kinase inhibitors p15, p21, and p27 expression^[50] and down-regulation of C-myc, cyclin D2, and cyclin E expression, too^[51]. The concept for the suppressive role of the

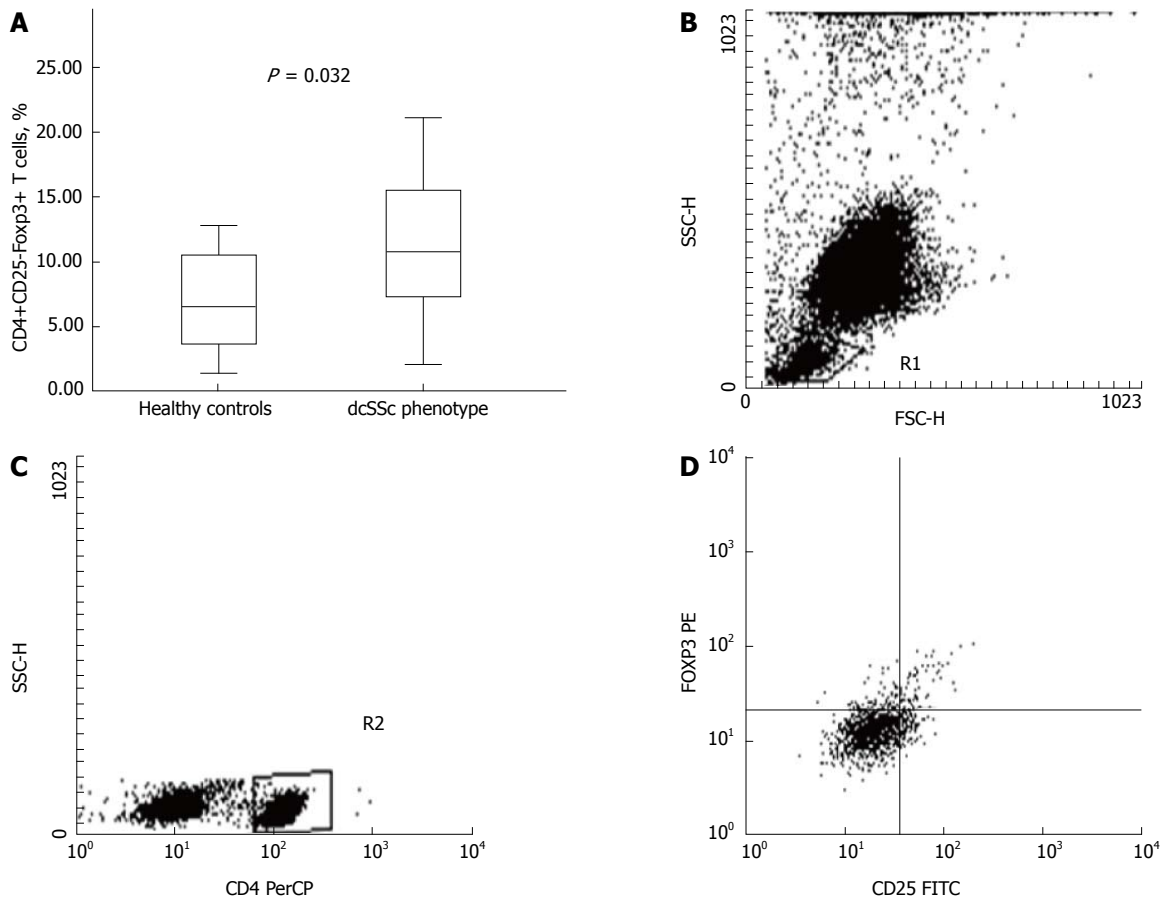


Figure 3 Increased percentage of CD4+CD25-Foxp3+ T cells within diffuse cutaneous systemic sclerosis phenotype vs healthy controls. A: Increased percentage of CD4+CD25-Foxp3+ T cells within dcSSc patients ($n = 13$) as opposed to controls ($n = 16$), respectively, $10.94\% \pm 1.65\%$ vs $6.88\% \pm 0.91\%$, $P = 0.032$. Boxplots are expressed as means \pm SD; B-D: Panel B depicts the flow cytometric analysis of CD4+CD25-Foxp3+ T cells. A representative patient with dcSSc phenotype is shown the lymphocytes were gated according to their physical characteristics (FSC and SSC) in R1; afterwards T helper cells were gated in R2. T helpers, which were found negative for CD25 surface expression and positive for Foxp3 intracellular expression (R3, upper left quadrant) were defined as CD4+CD25-Foxp3+ T cells. dcSSc: Diffuse cutaneous systemic sclerosis.

Table 3 Circulating cytokines in systemic sclerosis patients and healthy controls

Cytokine	SSc patients	Healthy controls	P value
IL-10, pg/mL	2.83 ± 0.44 (0.10-6.90)	0.68 ± 0.51 (0.00-5.20)	0.008
IL-17A, pg/mL	$6.30 [2.50-15.60]$ (0.20-124.90)	$0.00 [0.00-0.05]$ (0.00-1.36)	< 0.001
TGF- β 1, ng/mL	19.94 ± 3.35 (0-52.80)	10.03 ± 2.25 (1.16-21.80)	0.02
IL-6, pg/mL	$2.10 [1.05-4.60]$ (0.45-198.10)	0 (0.00-0.27)	< 0.001

Data represents means \pm SE (range) or medians [IQR] (range). SSc: Systemic sclerosis; IL: Interleukin; TGF: Tissue growth factor.

circulating cytokine milieu in SSc, regarding the T-cell activation, is in agreement with data reporting inhibited activation of Tregs from healthy donors or SSc by SSc plasma^[10].

On the other hand, the peripheral T cell anergy upon PHA-stimulation in our SSc patients may be due to the immunosuppressive therapy administered. Most of the patients enrolled in the study were under treatment

with glucocorticoids (GCs) (Table 1).

Normally, stimulation of T cells by cross-linking of both T-cell receptor TCR/CD3 and CD28 up-regulates both the nuclear factor of activated T cells (NFAT) and activating protein 1 (AP-1) transcription factors, resulting in increased transcription of the interleukin-2 (IL-2) gene and activation^[52]. One of the important genomic mechanisms of GC action includes the interaction of activated cytosolic GC receptor (cGCR) monomers with transcription factors. The GC/cGCR complex modulates the activity of AP-1, NFAT, and NF- κ B (nuclear factor- κ B)^[53]. The inhibition of their nuclear translocation and function leads to blockage of the expression of many proinflammatory cytokines, e.g., IL-2, IL-6, TNF- α ^[54]. This genomic mechanism of GC action may explain the decreased percentage of peripheral CD4+CD25+ cells in our SSc patients compared to healthy subjects, bearing in mind that CD25 along with a marker for T cell activation is an IL-2 receptor alpha chain as well. Moreover, we found decreased peripheral CD4+CD25+ cells in dcSSc patients, all of which had been under treatment with methylprednisolone.

Based on our results, we are not able to answer unconditionally to the question who exactly is responsible

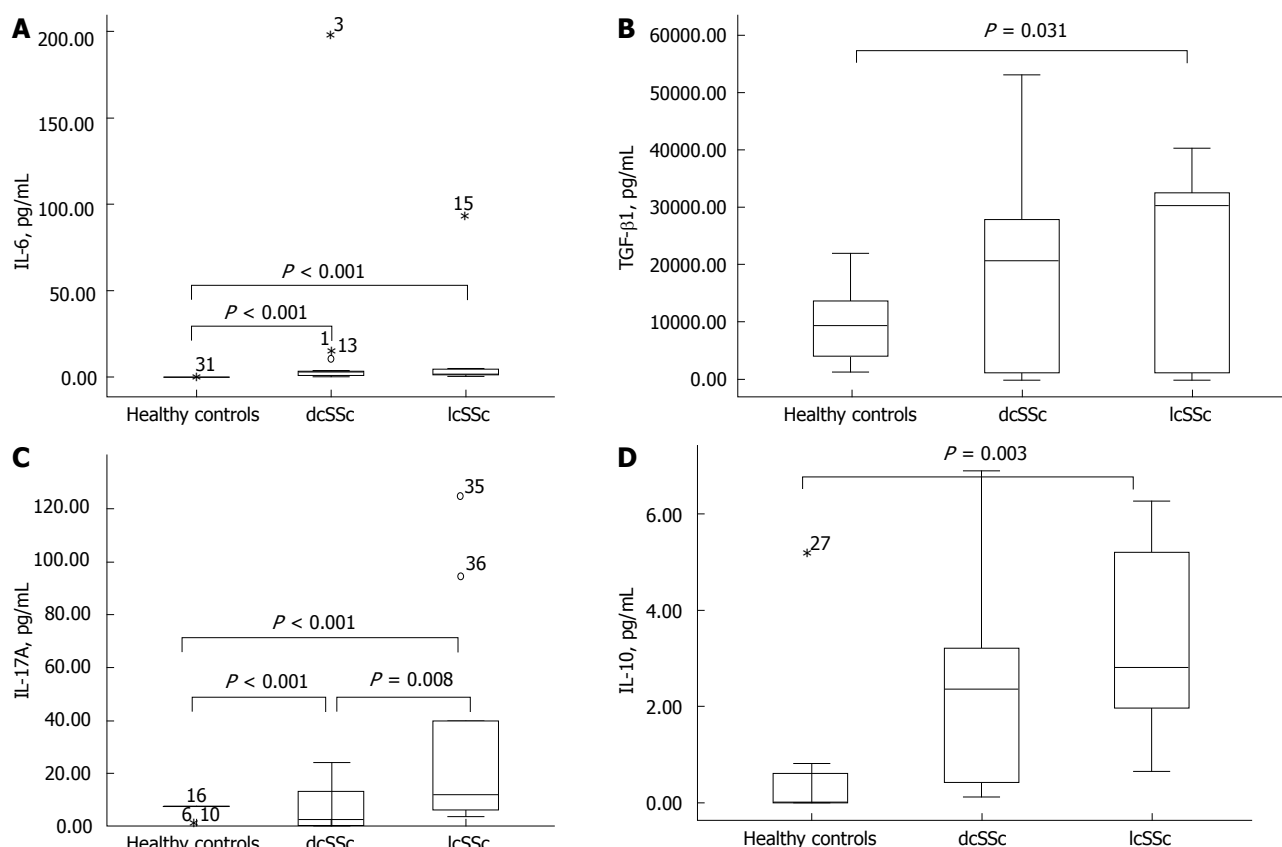


Figure 4 Elevated serum levels within the limited cutaneous systemic sclerosis ($n = 11$) and diffuse cutaneous systemic sclerosis ($n = 13$) phenotypes vs healthy controls ($n = 16$). Boxplots are expressed as means \pm SD. A: Increased serum levels of IL-6 in lcSSc phenotype vs controls, $P < 0.001$. Raised serum levels of IL-6 in dcSSc patients vs controls, $P < 0.001$; B: Raised serum levels of TGF- β 1 in lcSSc patients vs controls, $P = 0.031$; C: Elevated serum levels of IL-17 A in lcSSc phenotype vs dcSSc patients, $P = 0.008$, and vs controls, $P < 0.001$. The IL-17A were also increased in dcSSc patients vs controls, $P < 0.001$; D: Raised serum levels of IL-10 in lcSSc patients vs controls, $P = 0.003$. lcSSc: Limited cutaneous systemic sclerosis; dcSSc: Diffuse cutaneous systemic sclerosis; TGF- β 1: Tissue growth factor- β 1; IL: Interleukin.

for the decreased activation capacity of T cells in SSc patients - the therapeutic regimen, cytokines, both of them or perhaps, additional factors get involved.

One of the most considerable findings of our study is the increased percentage of Th17 cells and the elevated serum levels of their respective cytokine, IL-17.

Many papers have reported a higher frequency of Th17 cells in the peripheral blood of SSc patients as opposed to healthy controls^[13-15,30] which corresponds to our results.

Overproduction of IL-17 by T cells in the peripheral blood and in both skin and lung Kurasawa *et al.*^[18] have described overproduction of IL-17 by T cells in the peripheral blood and in both skin and lung lesions from SSc patients. These results suggest the central role that IL-17 overproduction plays in the pathogenesis of SSc, especially in the early stages of the disease, by enhancing the fibroblast proliferation and the production of IL-1 and the expression of adhesion molecules on endothelial cells^[18]. Our data have not revealed any difference in the level of serum IL-17, regarding the stage of SSc. However, we describe for the very first time elevated serum levels of IL-17 in patients with lcSSc when compared to the dcSSc phenotype.

Even though IL-17 enhances the fibroblast pro-

liferation, this cytokine does not induce collagen production in dermal fibroblasts, but rather decreases the ability of TGF- β to activate them. Furthermore, the number of IL-17 positive cells in SSc skin has been reported to correlate inversely with the extent of global skin thickness^[27]. Thus, in humans IL-17 may instead act as an antifibrotic inflammatory mediator. It is worth mentioning that prostanoids currently used to treat SSc vasculopathy, including prostaglandin I₂, increase *in vivo* the number of Th17 cells^[55]. Therefore they could be beneficial to the vascular compartment, particularly to endothelial cells, and might be crucial for the modulation of the inflammatory response.

Whether Th17 cells and IL-17 might have indirect pro-fibrotic effects *via* interaction with endothelial/epithelial cells or *via* the enhanced production of pro-angiogenic factors, such as IL-8, CCL-2, remains to be investigated. Similarly, the role of Th17 cells in autoantibody generation in SSc has not been investigated so far. However, in animal studies IL-17 has been shown to promote autoantibody generation in BXD2 mice by orchestrating the spontaneous formation of autoreactive germinal centers^[56].

Recent data has revealed that IL-6 plays an important role in the regulation of the balance between

IL-17-producing Th17 cells and Tregs^[30,35]. Our results demonstrate increased serum levels of IL-6 in both of lcSSc and dcSSc patients compared to controls with no difference between the two clinical subsets. IL-6 in concert with TGF- β induces the expression of ROR γ t in naïve T cells, transforming them in Th17 cells; in contrast, IL-6 inhibits TGF- β -induced Treg differentiation^[57].

Even though Th17 cells are crucial in the modulation of the inflammatory response, the Treg subset might also play a central role in the pathogenesis of SSc. Our results demonstrate nonsignificant increase in CD4+Foxp3+ Tregs in SSc patients when compared to controls and no difference between patients and healthy individuals regarding the percentage of CD4+CD25+Foxp3+ Tregs. There is controversial data in literature concerning the Treg numerical and functional alterations in SSc. Some of the papers have announced elevated circulating CD4+CD25+Foxp3+ Treg cells^[10,28] particularly in active and severe disease^[29]. Besides the up-regulation, Tregs from SSc patients demonstrate a defective suppressive capacity, which has been reported to correlate with a diminished CD69 expression and TGF- β levels^[10]. One study has not detected any differences between SSc patients and control groups^[15]. Finally, several studies have demonstrated a decreased frequency/impaired function of Tregs in SSc^[30-32].

However, our data reveals an increased percentage of CD4+CD25-Foxp3+ cells in our dcSSc patients in comparison with the healthy controls. Recent studies have reported up-regulated CD25 negative CD4+Foxp3+ cells in the peripheral blood of patients with systemic lupus erythematosus (SLE)^[58-60]. Both CD4+CD25-Foxp3+ T cells and CD4+CD25+ Foxp3+ Treg cells from SLE patients have demonstrated a similar pattern regarding the expression of CD62L, CD95, GITR, CD127, and CTLA-4, which are typical markers for the Treg phenotype^[61]. A considerable suppressive activity of CD4+CD25-Foxp3+ cells, comparable to the suppressive capacity exerted by the classical Tregs (CD4+CD25+Foxp3+ cells) has been reported^[62]. According to another hypothesis, CD4+CD25-Foxp3+ T cells subset could represent a peripheral reservoir of the CD4+CD25+ Foxp3+ Treg cell subset^[61]. In case of autoimmune reactivation, such as in SLE patients, CD25 negative Foxp3+ T cells could regain the expression of CD25, trying to reverse a homeostatic imbalance shift to more aggressive expansion of autoreactive T cells and B cells^[61]. However, another paper have considered CD4+CD25-Foxp3+ cells as functionally incompetent in SLE^[63].

The GC treatment of our dcSSc patients could also unravel the up-regulated peripheral CD4+CD25-Foxp3+ cells that we have found. The CD4+CD25-Foxp3+ cell subset has been reported increased in patients with rheumatoid arthritis treated with GCs and have correlated inversely with the disease parameters^[64]. GC-treated patient carriers of the high IL-10 genotype demonstrated higher levels of CD4+CD25-Foxp3+ cells, which finding

corresponds to our results.

In conclusion, our study demonstrates a decreased capacity for PHA-induced peripheral T-cells activation in patients with SSc. We also describe for the first time an up-regulated percentage of CD4+CD25-FoxP3+ cells in patients with dcSSc. Regarding the circulating cytokine profile in SSc, we originally identify increased serum levels of IL-17 in lcSSc as opposed to patients with dcSSc phenotype. The rest of our data, concerning the elevated circulating IL-6, IL-10, and TGF in SSc, confirms literature-based results.

COMMENTS

Background

Systemic sclerosis (SSc) is a generalized debilitating connective tissue disease affecting the skin and internal organs characterized by vasculopathy, fibrosis, and autoimmune alterations. The autoimmune dysregulation in SSc comprises lymphocyte activation that leads to the generation of autoantibodies, abnormal production of cytokines and chemokines, and impairment of the innate immunity. Over the last decade, the accumulating data has shown the central role of T lymphocytes in the pathogenesis of SSc. There is strong evidence in literature for altered T-cell activation and T helper cells abnormalities in SSc.

Research frontiers

There is accumulating data for numerical and functional alterations of Tregs and Th17 cells in patients with SSc. However, a functional heterogeneity exists between the T lymphocytes in the peripheral blood of patients with SSc and the corresponding T cell subsets in skin lesions or internal organs. The cytokine production by T cells affects the function of fibroblasts and endothelial cells, thereby influencing the vascular disease progression and the fibrosis development. Many efforts have been made to identify the cytokine patterns in SSc. Nevertheless, important issues remain unresolved, among them, identification of the trigger of the autoimmune response in SSc and the immunological differences between the dcSSc and lcSSc.

Innovations and breakthroughs

This is the first study demonstrating an up-regulated percentage of CD4+CD25-FoxP3+ cells in patients with dcSSc as compared to healthy subjects. Another of the original contributions of this research demonstrates a decreased capacity for PHA-induced peripheral T-cells activation in patients with SSc. Regarding the peripheral cytokine profile in SSc, this research group describes for the first time elevated serum levels of IL-17A in the lcSSc as opposed to the dcSSc subset of the disease.

Applications

It is likely that the altered percentage of Th17 and CD4+CD25-FoxP3+ cells may play a key role in the disease progression along with the peripheral cytokine profile in SSc patients.

Terminology

SSc is an abbreviation for systemic sclerosis as well as lcSSc and dcSSc are abbreviations for the limited cutaneous and the diffuse cutaneous subsets of the disease. Tregs represent the T regulatory lymphocytes (CD4+FoxP3+ cells), a T helper cell subset which is crucial for the establishment of immunological self-tolerance and for the prevention of autoimmunity.

Peer-review

The study represents an interesting continuum to the research series towards unveiling the immunological profile in SSc.

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