Disease Manifestation and Inflammatory Activity as Modulators of Th17/Treg Balance and RORC/FoxP3 Methylation in Systemic Sclerosis

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Keywords
Th17 · Tregs · Methylation · Systemic sclerosis · Suppression

Abstract

Introduction

Scleroderma is an inflammatory disease of the skin and internal organs resulting in fibrosis and organ dysfunction. There is much evidence that T cells are strongly involved in the pathogenesis of systemic and localized forms of scleroderma (SSc). A dysbalance between FoxP3+ regulatory CD4+ T cells (Tregs) and inflammatory T-helper (Th) 17 cells has been suggested. Methods: The study aimed (1) to investigate the phenotypical and functional characteristics of Th17 and Tregs in SSc patients depending on disease manifestation (limited vs. diffuse cutaneous SSc, dcSSc) and activity, and (2) the transcriptional level and methylation status of Th17- and Treg-specific transcription factors. Results: There was a concurrent accumulation of circulating peripheral IL-17-producing CCR6+ Th cells and FoxP3+ Tregs in patients with dcSSc. At the transcriptional level, Th17- and Treg-associated transcription factors were elevated in SSc. A strong association with high circulating Th17 and Tregs was seen with early, active, and severe disease presentation. However, a diminished suppressive function on autologous lymphocytes was found in SSc-derived Tregs. Significant relative hypermethylation was seen at the gene level for RORC1 and RORC2 in SSc, particularly in patients with high inflammatory activity. Conclusions: Besides the high transcriptional activity of T cells, attributed to Treg or Th17 phenotype, in active SSc disease, Tregs may be insufficient to produce high amounts of IL-10 or to control proliferative activity of effector T cells in SSc. Our results suggest a high plasticity of Tregs strongly associated with the Th17 phenotype. Future directions may focus on enhancing Treg functions and stabilization of the Treg phenotype.

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forms of scleroderma. A predominance of T helper (Th) 1- and interleukin-17 (IL-17)-producing Th17 cells was found in early inflammation and a shift towards Th2 cytokine signatures in late lesions [1–5]. However, the interpretation of the role of Th17 in systemic sclerosis (SSc) remains controversial. Increased expression of IL-17 mRNA by lymphocytes obtained from the skin and lung of SSc patients and elevated serum IL-17 levels suggest a role of IL-17 in the pathogenesis of SSc [3, 6]. While some studies reported Th17 responses predominate in active disease courses and diffuse manifestations of scleroderma [7], others noted an absence of IL-17 production but a distinct T-cell subpopulation characterized by synthesis of IL-22, i.e. Th22 cells [8].

An imbalance between forkhead-box-protein-3 (FOXP3)+ CD4+ regulatory T cells (Tregs) and inflammatory Th17 cells has been suggested. Fewer FoxP3+ Tregs and IL-10+ cells were found in the skin of scleroderma patients than controls as well as reduced serum IL-10 levels [9]. IL-10 is regarded as an important suppressive cytokine [10] produced by type I Tregs [11]. Others reported normal [12] or increased [13–15] frequencies of circulating FoxP3+ T cells. Frequencies of Th17 cells and FoxP3+ cells were increased in skin biopsies of early SSc manifestation with the number of Th17 cells closely related to disease activity [16]. The recent literature suggests that Th17 and Treg activity is a hallmark of SSc, as Th17-type cytokines can induce both inflammation and fibrosis [17, 18]. Tissue-selective trafficking of these inflammatory Th cells is mediated by chemokine receptor expression, such as CCR6 in Th17 cells [19].

The Th17-associated transcription factor RORC (RAR-related-orphan-receptor-C) and the Treg-associated transcription factor FOXP3 are both upregulated in naive T cells and compete for dominance early in T-cell differentiation. While a cytokine milieu dominated by IL-1β, IL-6, and IL-23 promotes Th17 differentiation, transforming-growth-factor (TGF)-β favors development of Tregs [20]. RORγ and RORγt are gene products of RORC1 and RORC2 genes, respectively, with RORγt being strongly involved in polarization of Th cells towards Th17. Epigenetic regulation of T-cell-specific transcription factors by methylation of regulatory CpG sites has been suggested to influence the pathogenesis of SSc [21–25].

This study aims (1) to investigate the phenotypical and functional characteristics of Th17 and Tregs in SSc patients depending on disease manifestation and activity, and (2) to correlate these findings with the levels and methylation status of Th17- and Treg-specific transcription factors.

Patients and Methods

Study Population

Blood samples were obtained from patients classified with limited cutaneous (lcSSc) and diffuse cutaneous SSc (dcSSc) [26, 27] and healthy controls (HC) at the Departments of Internal Medicine II and Dermatology, Venereology, and Allergology (University Hospital Würzburg) upon written informed consent. The study was performed according to the principles of the Declaration of Helsinki 2010 and approved by the local ethics committee (protocol No. 242/11).

Lymphocyte Separation

Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation (FicoLite-H, Linaris, Wertheim, Germany) from freshly drawn venous blood. After washing steps, cells were resuspended in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin.

Cell Surface and Intracellular Staining of T Cells

PBMCs were resuspended in FACS buffer containing phosphate-buffered saline (pH 7.2), 0.5% bovine serum albumin, and 0.01% NaN3 and stained with fluorochrome-labeled monoclonal antibodies against CD3, CD4, CD8, CD45RA, CD45RO, CD28, CD161 (all purchased from BioLegend, London, UK), chemokine receptor (CCR) 7, CCR6, CD25 (all purchased from BD, Franklin Lakes, NJ, USA), and CD127 (eBioscience, San Diego, CA, USA) for 30 min at 4°C. For intracellular staining of FoxP3 or RORγt (both purchased from BD), cells were fixed using fixation buffer (BioLegend) for 20 min at room temperature followed by a washing step with permeabilization buffer (BioLegend). Cells were then incubated with monoclonal antibody against FoxP3 or RORγt for 30 min at 4°C and resuspended in FACS buffer.

Intracellular cytokine production was determined following stimulation with phorbol 12-myristate 13-acetate (0.03 μg/mL), ionomycin (1 μg/mL), and brefeldin A (10 μg/mL) for 4 h at 37°C. Cells were fixed for 20 min at room temperature using fixation buffer (BD) followed by a washing step with permeabilization buffer and stained using monoclonal antibodies against IL-4, IL-10, IL-17, and IFNγ (all purchased from BioLegend). Surface and intracellular expression was assessed by flow cytometry (FACSCanto II; BD). Data analysis was performed using FACSDiva software V6 (BD).

Serum Cytokine Levels

Serum cytokine levels were measured by flow cytometry with the FlowCytomix multiple analyte detection system for human Th1/Th2/Th9/Th17/Th22 (eBioscience).

Suppression Assay

As significant differences regarding Tregs were found between dcSSc and HC, CD4+CD25+CD127− cells (Tregs) were isolated from PBMCs obtained from 6 dcSSc and 4 HC using the MACS regulatory T-cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. To track the proliferative capacity of autologous PBMCs in coculture with Tregs, PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE) (10 μM); 30,000 Tregs and 30,000 CFSE-labeled PBMCs were cocultured at a ratio of 1:1 in RPMI 1640 medium supplemented with 1% penicillin/streptomycin and 7% FCS.

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Table 1. Demographics of the study groups

<table>
<thead>
<tr>
<th>Patients, n</th>
<th>SSc</th>
<th>lcSSc</th>
<th>dcSSc</th>
<th>p valuea</th>
<th>HC</th>
<th>p valueb</th>
<th>p valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females/males, n</td>
<td>26</td>
<td>17</td>
<td>9</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>59 ± 10 (55; 42 – 77)</td>
<td>60 ± 12 (56; 42 – 77)</td>
<td>57 ± 6 (55; 48 – 71)</td>
<td>0.751</td>
<td>54 ± 6 (55; 37 – 61)</td>
<td>0.182</td>
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<tr>
<td>Leukocytes, ×1,000/μL</td>
<td>6.8 ± 1.9</td>
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<tr>
<td>Lymphocytes, % of leukocytes</td>
<td>19.1 ± 8.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CRP, mg/dL</td>
<td>0.5 ± 0.9 (0.16; 0.02 – 3.31)</td>
<td>0.3 ± 0.7 (0.14; 0.02 – 2.97)</td>
<td>0.7 ± 1.1 (0.26; 0.08 – 3.31)</td>
<td>0.312</td>
<td>n.a.</td>
<td>n.a.</td>
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<tr>
<td>ESR, mm/h</td>
<td>28 ± 21 (23; 5 – 79)</td>
<td>28 ± 20 (20; 7 – 65)</td>
<td>30 ± 24 (25; 5 – 79)</td>
<td>0.846</td>
<td>n.a.</td>
<td>n.a.</td>
<td></td>
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<tr>
<td>CRP, mg/dL</td>
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<td></td>
</tr>
<tr>
<td>Leukocytes, ×1,000/μL</td>
<td>6.8 ± 1.9</td>
<td>6.5 ± 1.6</td>
<td>7.2 ± 2.2</td>
<td>0.597</td>
<td>6.6 ± 2.9</td>
<td>0.718</td>
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<tr>
<td>Lymphocytes, % of leukocytes</td>
<td>19.1 ± 8.2 (21.6; 3.2 – 30.7)</td>
<td>22.1 ± 5.8 (22.6; 10.3 – 30.7)</td>
<td>14.1 ± 9.4 (11.3; 3.2 – 28.7)</td>
<td>0.041</td>
<td>28.7 ± 7.8 (27.5; 18.8 – 47.1)</td>
<td>0.001</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Values are given as means ± SD (medians; ranges). Ab, antibodies; n.a., not analyzed. a lcSSc vs. dcSSc. b SSc vs. HC. c dcSSc vs. HC. d Drugs: methotrexate (dose 10 – 15 mg/m2 per week; maximum of 25 mg/week); azathioprine (dose 1 – 3 mg/kg/day); glucocorticoids (prednisone equivalent dosage <0.5 mg/kg/day; maximum of 60 mg/day); cyclophosphamide (dosage: bolus of 800 mg/m2).

The following definitions of organ involvement were used [22]: renal: histologically proven renal thrombotic microangiopathy, histologically proven glomerulonephritis, documented scleroderma renal crisis; heart: pericardial effusion without alternative evident cause; biopsy-proven myocardial involvement, signs and symptoms of cardiomyopathy or conduction abnormalities with typical findings in MRI without alternative evident cause; pulmonary arterial hypertension: defined by right heart catheterization according to European Society of Cardiology/European Respiratory Society guidelines; lung: compatible intestinal lung disease in computed tomography; gastrointestinal system: documentation of required hyperalimentation, and pseudo-obstruction; esophageal dysmotility was not included as GI system involvement as it is known to be very common among SSc patients. Meeting at least one feature of these definitions was required to allow categorization as “organ involvement.”

Exclusion criteria were other autoimmune disorders, immunodeficiency, genetic syndromes, clinically symptomatic infections during the 6 weeks prior to inclusion and the administration of blood or plasma products (erythrocyte or thrombocyte concentrates, or intravenous immunoglobulins) in the past 3 months. Female and male patients did not significantly differ with respect to disease duration, age, medications, inflammatory parameters (ESR or CRP), leukocyte counts, relative lymphocytes or presence of auto-antibodies (Scl-70 or anticentromere). No correlation was noted between disease duration and patient age.

and stimulated with 0.5 μg/mL anti-CD3/anti-CD28 for 5 days. Different ratios of Tregs:PBMCs were tested in preliminary experiments to prove specificity. A ratio of 1:1 was confirmed to have the highest effect. After harvesting cells, proliferating and resting PBMCs were stained with fluorochrome-labeled anti-CD4 and analyzed by flow cytometry. The stimulation index was defined as proliferation of CD4+ T cells in coculture with Tregs divided by the proliferation of PBMCs or CD4+ T cells without Tregs. From flow-cytometric results, significant alterations were expected for Th17/Treg Balance in SSc

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PCR
Total RNA was extracted from PBMCs derived from 5 patients with dcSSc and 5 HC using the RNasy mini kit (Qiagen). Complementary DNA (cDNA) was generated from 1 μg RNA using oligo(dT)18 Primers (Thermo Scientific, Waltham, MA, USA) for reverse transcription with Maxima reverse transcriptase (Thermo Scientific). Real-time PCR was performed using the applied Biosystems® Real-Time PCR7500 (Applied Biosystems, Darmstadt, Germany) utilizing iTaq universal SYBR Green according to the manufacturer’s instructions (Bio-Rad, Ismaning, Germany) (for primer sequences see online suppl. Table 3). Amplification was conducted for 40 cycles. Relative expression of FOXP3, RORC, STAT3 (signal transducer and activator of transcription) 3, STAT5, CCR6, IL-10, and IL-17 were determined by normalizing expression of each gene to β2-microglobulin.

Bisulfite Pyrosequencing

Assays quantifying the methylation levels of CpGs in the target regions were designed with the PyroMark Assay Design software (Qiagen, Hilden, Germany). Primers and sequences analyzed are listed in online supplementary Tables 1 and 2. Data analysis was done with the Pyro Q-CpG software (Qiagen).
**Statistical Analysis**

After testing the distribution of variables with the Shapiro-Wilk test, the Mann-Whitney U test was applied for nonnormally distributed independent variables using SPSS (version 22; SPSS, Chicago, IL, USA). The Wilcoxon rank test was used for nonnormally distributed dependent variables. To avoid bias by multiple testing, a value of $p \leq 0.05$ was considered statistically significant using the less conservative Benjamini-Hochberg correction to reduce false-positive results. Correlations were analyzed by the Spearman rank correlation. In search for independent factors influencing Th17 or Treg functions, step-down multiple regression analysis was performed including age, gender, disease duration, presence of autoantibodies (Scl70/anti-centromere antibodies), organ involvement, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and different therapies (methotrexate, glucocorticoids, and azathioprine).

**Results**

**dcSSc Patients Present with Relative Lymphopenia**

Despite similar leukocyte counts, relative lymphopenia was seen in dcSSc when compared to lcSSc and HC (Table 1). In dcSSc, the reduction in lymphocyte proportions correlated with increased ESR ($R = -0.667; p = 0.05$) and CRP ($R = -0.0748; p = 0.02$). ESR and CRP were associated with organ involvement (correlation for ESR: $R = 0.725, p = 0.027$; CRP: $R = 0.678, p = 0.045$). dcSSc received more intense medical treatments than lcSSc (Table 1).
Increased Circulating Th17 and CCR6+ T Cells in dcSSc

CCR6 expression was used as a characteristic marker of IL-17-producing cells [3–8] (Fig. 1A). Significantly higher proportions of IL-17-producing CD4+ T cells (Fig. 2D, 3D) and CCR6+ (Fig. 3A) T cells were found in dcSSc.

In search for an independent factor influencing proportions of CD4+CCR6+ in SSc, multiple regression analysis ($R^2 = 0.324; p = 0.038$) was performed. Organ involvement ($p = 0.015$) was identified as an independent factor leading to a proportional increase in CD4+CCR6+ in all SSc patients. In the subgroup of dcSSc, female sex ($p = 0.013$), organ involvement ($p = 0.025$), and Scl-70 seropositivity ($p = 0.037$) were significant factors for elevated proportions of CD4+CCR6+. In a multiple regression model ($R^2 = 0.995; p = 0.015$) performed in dcSSc, higher IL-17 production by CD4+CCR6+ was significantly influenced by lower chronological age ($p = 0.004$), shorter disease duration ($p = 0.033$), organ involvement ($p = 0.012$), and increased inflammation parameters (ESR, $p = 0.021$; CRP, $p = 0.037$). There was a trend towards the influence of female sex ($p = 0.084$).

A trend towards double positivity for CD161 and CCR6 ($p = 0.077$) (Fig. 3B) and higher RORγτ expression was also seen in CD4+ dcSSc ($p = 0.061$) (Fig. 3C). In both

Fig. 2. Representative flow-cytometric staining for intracellular cytokines. Representative examples of flow-cytometric staining for CD4 (y-axis) and cytokines (x-axis) are shown for 1 HC (upper row), 1 lcSSc patient (middle row), and 1 dcSSc patient (lower row). Numbers in the right upper quadrants represent percentages of cytokine-positive CD4+ T cells. Intracellular IFNy was used as a key cytokine for Th1 cells (A), IL-4 for Th2 cells (B), IL-10 for Tregs (C), and IL-17 for Th17 cells (D).
SSc types and HC, CD161 and CCR6 expression strongly correlated with each other ($R = 0.835$, $p = 0.0001$, and $R = 0.454$, $p = 0.013$, respectively) and was associated with higher age ($R = 0.0321$, $p = 0.012$) and organ involvement ($R = 0.0493$, $p = 0.012$). In dcSSc, RORγτ expression strongly correlated with elevated ESR ($R = 0.712$, $p = 0.030$). A trend for a negative correlation with disease duration was recognized for RORγτ+ in CD4+CCR6+ ($R = -0.655$, $p = 0.055$).

Intracellular IFNγ and IL-4 productions, key cytokines of Th1 and Th2 cells, respectively, were not significantly different between SSc patients and HC (Fig. 2A, B, 3F, G). Also, typical Th1 and Th2 cytokines were not different between serum samples of SSc patients and HC.

**Abundance of Circulating FoxP3+ Tregs with Impaired Suppressive Function in SSc**

Significantly higher proportions of CD25++CD127−FoxP3+ Tregs were seen in lcSSc and dcSSc compared to HC (Fig. 1B, 4A). Multiple regression analysis ($R^2 = 0.981$, $p = 0.05$) revealed that elevated proportions of Tregs in dcSSc were significantly influenced by increased CRP ($p = 0.008$), female sex ($p = 0.031$), and shorter disease duration ($p = 0.040$). There was a trend towards the influence of elevated ESR ($p = 0.080$) and Scl-70+ ($p = 0.077$).
**Table 2. Serum cytokine concentrations**

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-1β</th>
<th>IL-12</th>
<th>TNFa</th>
<th>IFNγ</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-10</th>
<th>IL-17A</th>
<th>IL-22</th>
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<tbody>
<tr>
<td>SSc</td>
<td>16</td>
<td>10</td>
<td>5</td>
<td>11</td>
<td>7</td>
<td>15</td>
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<td>14</td>
<td>16</td>
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<td>n</td>
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<tr>
<td>p valuea</td>
<td>0.903</td>
<td>0.436</td>
<td>0.520</td>
<td>0.164</td>
<td>0.759</td>
<td>0.461</td>
<td>0.092</td>
<td>0.112</td>
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<td></td>
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<tr>
<td>p valueb</td>
<td>0.403</td>
<td>0.494</td>
<td>0.457</td>
<td>0.931</td>
<td>0.840</td>
<td>0.546</td>
<td>0.934</td>
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</table>

Values are given as means ± SD (medians; ranges) in pg/mL. The numbers of patients who had detectable concentrations of the specific cytokines are indicated for each group. IL-22 was positive in only 1 lcSSc patient. IL-6 and TGFβ were negative in all donors. IL-1β, IL-6, IL-17A, and IL-22 were considered to be Th17 cytokines, IL-2 and IL-10 as cytokines of induced Tregs, IL-12, TNFa, and IFNγ Th1 cytokines, and IL-4, IL-5, and IL-13 Th2 cytokines.

*a lcSSc vs. SSc.  b SSc vs. HC.  c dcSSc vs. HC.*

**Table 3. Percentages of IL-10+CD4+ T-cell subpopulations**

<table>
<thead>
<tr>
<th>Group</th>
<th>SSc (n = 26)</th>
<th>lcSSc (n = 17)</th>
<th>dcSSc (n = 9)</th>
<th>p valuea</th>
<th>HC (n = 29)</th>
<th>p valueb</th>
<th>p valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45RA+CD28CCR7+ (naive)</td>
<td>3.09 ± 5.89</td>
<td>1.97 ± 1.94</td>
<td>3.68 ± 10.60</td>
<td>0.346</td>
<td>1.01 ± 1.22</td>
<td>0.003</td>
<td>0.027</td>
</tr>
<tr>
<td>CD45RA+CD28CCR7+ (early memory)</td>
<td>3.07 ± 10.70</td>
<td>0.81 ± 0.77</td>
<td>4.27 ± 13.20</td>
<td>0.500</td>
<td>0.64 ± 0.59</td>
<td>0.194</td>
<td>0.668</td>
</tr>
<tr>
<td>CD45RA+CD28CCR7+ (late memory)</td>
<td>6.44 ± 13.77</td>
<td>1.07 ± 1.30</td>
<td>9.28 ± 16.45</td>
<td>0.089</td>
<td>1.34 ± 2.35</td>
<td>0.048</td>
<td>0.723</td>
</tr>
<tr>
<td>CD45RA+CD28CCR7+ (effector)</td>
<td>3.58 ± 13.34</td>
<td>1.18 ± 1.60</td>
<td>4.84 ± 16.49</td>
<td>0.148</td>
<td>0.66 ± 1.55</td>
<td>0.096</td>
<td>0.024</td>
</tr>
<tr>
<td>CD45RA+CD28CCR7+ (TEMRA)</td>
<td>4.15 ± 10.01</td>
<td>3.64 ± 4.10</td>
<td>4.42 ± 12.16</td>
<td>0.134</td>
<td>1.91 ± 3.61</td>
<td>0.245</td>
<td>0.027</td>
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</table>

Values are given as means ± SD (medians; ranges) in percentages of IL-10+CD4+ T-cell subpopulations. n, numbers of patients. * lcSSc vs. dcSSc.  b SSc vs. HC.  c dcSSc vs. HC.*

**Th17/Treg Balance in SSc**

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IL-10 was poorly produced by Tregs of lcSSc (Fig. 4B) but to a significant extent in CD4+ T cells (Fig. 2C, 3E) and CD4+ T-cell subpopulations, particularly in naive, late memory-effector and terminally differentiated effector-memory CD4+ T cells reexpressing CD45RA (TEMRA) of dcSSc (Table 3). In dcSSc, serum IL-10 tended to be higher than in lcSSc (Table 2). Scl-70+ patients were more likely to have lower IL-10+CD4+ T-cell proportions.

**Fig. 4.** Proportions of Tregs and suppressive function. Percentages of Tregs (CD25++FoxP3+CD127−) (A) and IL-10+ (B), or IL-17+ cells (C) in Tregs are shown in HC, lcSSc, and dcSSc. Bars represent means ± SD. *p* values indicate comparisons with HC (Mann-Whitney U test). D Results from suppression assays are shown. The stimulation index was defined as proliferation of CD4+ T cells in coculture with Tregs divided by the proliferation of CD4+ T cells without Tregs. E Representative examples of a suppression assay from 1 patient with dcSSc and 1 HC are shown. CFSE staining of lymphocytes and CD4+ T cells is shown on the x-axis. The cutoff for proliferating cells was adjusted to negative controls. Numbers in histograms represent the percentages of proliferated cells.
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Th17/Treg Balance in SSc

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No correlation was seen between Tregs and IL-17-producing CCR6+CD4+ in any group. Tregs demonstrated to be IL-17 producers to a low extent, too, but no significant difference could be found between SSC and HC (Fig. 4C). However, a positive association was found between IL-17+ Tregs and organ involvement (R = 0.403, p = 0.046). IL-17+ Tregs positively correlated with CCR6+CD161+CD4+ in dcSSc (R = 0.767, p = 0.016). However, removing IL-17+ Tregs from the FoxP3+ Tregs in flow-cytometric analysis did not change the result that higher proportions of Tregs were found in SSC compared to HC in all SSc patients, IL-10+ Tregs also correlated with CCR6+ (R = 0.453, p = 0.023) and CCR6+CD161+CD4+ T cells (R = 0.421, p = 0.036).

Despite the peripheral abundance of Tregs found in SSC, the functional activity of Tregs was unclear. Investigating the suppressive function of Tregs, lower suppression of autologous lymphocytes or CD4+ T cells was demonstrated by Tregs derived from SSC compared to Tregs derived from HC (Fig. 4D, E).
Table 4. Methylation status of Treg- and Th17-specific transcription factors

<table>
<thead>
<tr>
<th></th>
<th>dcSSc male (n = 3)</th>
<th>dcSSc female (n = 6)</th>
<th>HC male (n = 4)</th>
<th>HC female (n = 6)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXP3 sequence 1</td>
<td>65.38±4.47</td>
<td>68.69±4.41</td>
<td>63.59±6.54</td>
<td>63.91±2.74</td>
<td>0.917</td>
</tr>
<tr>
<td>FOXP3 sequence 2</td>
<td>72.88±1.74</td>
<td>78.32±5.92</td>
<td>71.09±4.02</td>
<td>76.29±3.10</td>
<td>0.465</td>
</tr>
<tr>
<td>FOXP3 enhancer sequence 1</td>
<td>6.68±4.24</td>
<td>43.46±3.15</td>
<td>5.40±2.66</td>
<td>41.80±5.05</td>
<td>0.347</td>
</tr>
<tr>
<td>FOXP3 enhancer sequence 2</td>
<td>4.98±3.16</td>
<td>38.91±2.78</td>
<td>4.90±3.64</td>
<td>34.82±10.46</td>
<td>0.466</td>
</tr>
<tr>
<td>STAT5A sequence 1</td>
<td>8.01±3.22</td>
<td>(6.77; 4.87 – 14.39)</td>
<td>9.02±2.20</td>
<td>(8.64; 6.21 – 11.51)</td>
<td>0.140</td>
</tr>
<tr>
<td>RORC1 sequence 1</td>
<td>37.04±6.64</td>
<td>(35.41; 25.16 – 47.60)</td>
<td>28.24±4.59</td>
<td>(28.52; 19.98 – 33.22)</td>
<td>0.003</td>
</tr>
<tr>
<td>RORC1 sequence 2</td>
<td>24.38±4.41</td>
<td>(24.40; 15.55 – 29.83)</td>
<td>18.14±3.14</td>
<td>(17.52; 12.55 – 21.95)</td>
<td>0.004</td>
</tr>
<tr>
<td>RORC1 sequence 3</td>
<td>39.04±4.85</td>
<td>(37.54; 33.75 – 49.68)</td>
<td>31.87±3.45</td>
<td>(33.27; 24.58 – 35.84)</td>
<td>0.001</td>
</tr>
<tr>
<td>RORC2 sequence 1</td>
<td>40.12±6.73</td>
<td>(39.55; 29.22 – 49.59)</td>
<td>32.17±4.50</td>
<td>(31.98; 26.05 – 40.38)</td>
<td>0.010</td>
</tr>
<tr>
<td>RORC2 sequence 2</td>
<td>25.71±4.33</td>
<td>(27.10; 19.05 – 31.79)</td>
<td>20.67±3.69</td>
<td>(20.78; 17.53 – 20.78)</td>
<td>0.016</td>
</tr>
<tr>
<td>STAT3 sequence 1</td>
<td>4.64±3.10</td>
<td>(3.45; 2.17 – 12.49)</td>
<td>4.42±3.05</td>
<td>(2.84; 2.17 – 11.21)</td>
<td>0.806</td>
</tr>
</tbody>
</table>

Values are given as means ± SD (medians; ranges) in mean percentages of methylation.

Alterations at the Transcriptional Level of Th17 and Tregs of dcSSc

CCR6 (Fig. 5A) and IL-17 expression (Fig. 5B) was elevated in dcSSc. IL-10 expression was similar to HC (Fig. 5B). The Th17-specific transcription factor RORC and the downstream regulator of Th17 differentiation STAT3, as well as the Treg-specific transcription factor FOXP3 and the downstream regulator for Treg differentiation STAT5, were not found to be differently expressed in peripheral lymphocytes of dcSSc compared to HC (Fig. 5C). However, FOXP3+ expression correlated positively with IL-10 expression (R = 0.900, p = 0.037), RORC expression correlated negatively with IL-10 expression (R = -0.900, p = 0.037). CCR6 expression was positively associated with STAT3 expression (R = 0.900, p = 0.037).

Hypermethylation of RORC in dcSSc Patients

The methylation status of Th17 and Treg-specific transcription factors was analyzed to determine transcriptional activity. Significant hypermethylation was found for all RORC1 and RORC2 CpG positions in dcSSc (Table 4; online suppl. Table 4) which correlated with each other (R = 0.846, p = 0.001). Mean methylation at RORC1 was negatively associated with PCR products of RORC (R = -0.900, p = 0.037).

As epigenetic regulation may be influenced by inflammatory parameters, methylation levels were statistically correlated to proportions of different CD4+ subpopulations, to inflammatory disease activity stratified by elevated ESR ≥10 mm/h (Fig. 5D), and to prevalence of specific antibodies. Hypermethylation of RORC1 and RORC2 correlated with CD161+C CR6+CD4+ and ESR, with strongest effects in sequence 2 of RORC2 (CD161+ CCR6+CD4+: R = 0.806, p = 0.005; ESR: R = 0.970, p = 0.0001). Higher methylation levels at RORC1 and RORC2 were also seen in Scl-70+ compared to Scl-70- patients (p = 0.020).

Multiple regression analysis (R² = 0.989, p = 0.026) revealed that higher inflammatory parameters (ESR: p = 0.016; CRP: p = 0.028), older age (p = 0.064), and Scl-70 seropositivity (p = 0.084) had the strongest influence on RORC2 sequence 2 methylation levels.

Due to the X-chromosomal location of FOXP3, highly significant methylation of FOXP3 promoter and enhancer regions were seen in female compared to male dcSSc and HC (female vs. male: p = 0.008). Relative hypermethylation at the FOXP3 promoter locus was seen only in female dcSSc but not female HC (Table 4; online suppl. Table 4). Lower methylation at sequence 2 within the FOXP3 promoter was associated with higher inflammatory parameters (ESR: R = -0.862, p = 0.006; CRP: R = -0.653, p = 0.057), which also correlated with sequence 1 and 2 of the enhancer regions (R = -0.728, p = 0.026, and R = -0.594, p = 0.092, respectively). In dcSSc, methylation levels between sequence 1 and 2 of FOXP3 promoter regions (R = 0.0585, p = 0.046) and enhancer regions (R = 0.0746, p = 0.005) correlated with each other. In dcSSc, higher mean methylation levels at the sequence 2 FOXP3 promoter region correlated with lower methylation levels at sequence 1 of RORC1 regions (R = -0.643, p = 0.024).
Mean methylation at FOXP3 enhancer region sequence 2 was negatively associated with PCR products of the FOXP3 ($R = -0.900, p = 0.037$).

Although there was no significant difference in methylation levels at STAT3 between dcSSc and HC, mean methylation at the STAT3 locus was associated with increased disease duration ($R = 0.675, p = 0.032$). In dcSSc, STAT3 methylation levels were also positively correlated with STAT5A ($R = 0.671, p = 0.017$) and RORC1 sequence 1 methylation ($R = 0.706, p = 0.010$), and negatively with FOXP3 promoter sequence 1 methylation ($R = -0.900, p = 0.037$). Correlation to the other RORC1 and RORC2 sequences was less significant ($R = 0.529, p = 0.077$). Hypomethylation of STAT5A at position 1 was seen in dcSSc compared to HC (online suppl. Table 4).

**Discussion**

Our findings demonstrated a concomitant abundance of peripheral IL-17-producing CCR6+ Th cells and FoxP3+ Tregs in patients with dcSSc. Associations of Th17- and Treg-associated characteristics were seen with early, active, and severe disease. However, a diminished suppressive function on autologous lymphocytes was found in SSc-derived Tregs. At the transcriptional level, Th17-associated factors were elevated in dcSSc. Significant relative hypermethylation at the gene level for RORC1 and RORC2 in dcSSc was strongly associated with elevated ESR.

Our results confirmed earlier findings that Th17 responses predominate in active disease courses with elevated inflammatory blood parameters and diffuse manifestations of SSc [3–5, 7, 28]. Moreover, our findings demonstrated higher Th17-associated markers with lower chronological age or shorter disease duration, indicating an important role of Th17 in the early stages of the disease. Percentages of CD161+CD4+ were increased in SSc [29–32] and correlated with percentages of IL-17A-producing cells [33] with high expression of CCR6 [34]. Our study demonstrated a trend to higher proportions of CD161 and CCR6 double-positive CD4+ in dcSSc. Scl-70-seropositivity was associated with CCR6+CD4+, which was also shown by others [35].

Some inconsistencies exist regarding serum IL-17 levels in SSc [3–5, 36]. In contrast to others [3–5], elevated serum levels of IL-17 or other Th17-associated inflammatory cytokines were not found in our study. Several studies have reported a reduced IL-10 production from PBMCs [37, 38], others have found augmented IL-10 concentrations [39, 40]. However, our data reflect the intracellular production of IL-17 or IL-10 in CD4+ Th cell subpopulations and are therefore more likely to explain intrinsic dysfunctions of Th cells than serum cytokine concentrations may do.

Concomitantly with Th17, Tregs were increased in the peripheral blood of dcSSc correlating with disease activity in agreement with others [8]. Increased frequencies of Th17 and FoxP3+ cells were also reported in skin biopsies of early SSc manifestations with the number of Th17 cells closely related to disease activity [16] and severity [41].

Regarding the functional abilities of Tregs, Foxp3+ CD25+ Tregs were found to act suppressive on proliferating cells, as shown by others [42, 43]. Similar to our results, a diminished suppressive function of Tregs [28] was seen by an overall increased number of Tregs in SSc [13]. Alterations in the Th17 compartment correlated with Treg alterations in each patient [28]. In our study, no correlation between IL-17-producing CCR6+CD4+ and Tregs could be found. Several groups have reported the conversion of Tregs to Th17 cells in both the mouse and humans [44–46], and that Th17 cells derived from Tregs share common features with Th17 cells generated from naive precursors including expression of CCR6 [32, 47]. Thus, a concurrent abundance of Tregs and Th17 in SSc was attributed to the possibility of Tregs to change into Th17 cells. Indeed, increased IL-17-producing cells with high expression of CD25 were observed in SSc [5]. Although a small proportion of Tregs appeared to be IL-17 producers in our study, no differences could be shown for HC and SSc. However, a positive correlation was found between proportions of IL-17+ Tregs and organ involvement, as well as a positive correlation with CCR6+ T cells.

A high plasticity was attributed to the Treg phenotype. Isolation of pure Tregs is a challenge as there are high percentages of CD127low cells that did not express FoxP3 and, conversely, there was a high percentage of CD127+ cells that expressed FoxP3 [48]. Staining for FoxP3 and CD127 may not always represent the same Treg population and may result in a highly varying number of Tregs in SSc [48]. In our study, removing IL-17+ Tregs from the FoxP3+ Treg pool by retrospective flow-cytometric analysis did not change the finding of increased FoxP3+ Tregs in SSc patients. Studies on the plasticity of CD4+FoxP3+ T cells found that upon stimulation by inflammatory cytokines CD4+FoxP3+ cells may also downregulate FoxP3 expression and produce inflammatory cytokines such as IL-17 and IFNγ [49, 50]. Increased Tregs in SSc [13] were explained by a possible influence of glucocorticoids which may increase CD4+CD25+ cells [51, 52]. In our study, no significant influence of relatively low-dose systemic glu
corticoids or of any medications on Tregs or Th17 cells was demonstrated.

At the transcriptional level, IL-17 and CCR6 were elevated in peripheral lymphocytes of SSc. The discrepancy between nonsignificantly elevated IL-17 serum levels and the ability of T cells to produce high levels of IL-17 may be explained by a predominant local cytokine production in scleroderma lesions, as seen by others [3, 53]. IL-17 levels were negatively associated with disease duration [53], with elevated IL-17 levels in early SSc disease [3, 53]. Again, concomitant with RORC expression, a significant upregulation of FOXP3 was seen in SSc in our study.

Downregulation of FOXP3 gene expression and hypermethylation of the FOXP3 promoter regions were described in SSc patients [25]. In our findings, elevated ESR was associated with hypomethylation at FOXP3 promoter and enhancer CpG regions and hypermethylation at RORC1 and RORC2 CpGs. These findings are compatible with the idea of a concurrent abundance of Foxp3+ and Th17 cells. Our findings are in contrast to previously published results showing downregulation of FOXP3 gene expression and hypermethylation of the FOXP3 promoter regions in SSc patients [25]. However, our findings indicate a clear immunological pattern of active T cells displaying a regulatory T-cell phenotype going ahead with IL-17-producing CCR6+ T cells in active and early SSc disease. Hypomethylation at the FOXP3 promoter allows stable FOXP3 expression [54]. In functional assays of SSc-derived Tregs, suppressive function on CD25– T cells was low in our study. Thus, besides the high transcriptional activity of T cells, which is attributed to the Treg or Th17 phenotype, Tregs may be insufficient to produce high amounts of IL-10 or to control proliferative activity of effector T cells in SSc.

In our study, hypermethylation of RORC regions presented concomitant with higher inflammatory activity in dcSSc patients. Besides the hypermethylation of RORC and correlation with reduced PCR products of RORC, Th17 subsets identified by CCR6 cell expression and IL-17 production were not impaired. The likely explanation for this is that RORC, albeit being a lineage-specific transcription factor for Th17 cells, may not be the sole factor for Th17 differentiation. Moreover, Th17 polarization is influenced by nuclear receptor RORα, STAT3 gene expression, and various interactions between inhibitory factors, such as TBX21 (characteristic for Th1 lineage), FOXP3 (Tregs), or GATA3 (Th2 lineage) [55, 56]. Data regarding the epigenetic regulation of Th17 cell plasticity in mice revealed that permissive and repressive marks exist at the IFNγ and IL-17A promoter regions, and may itself influence IL-17 production. The histone methylation patterns at promoters of lineage-specific transcription factors TBX21 and RORC in Th17 cells and Tregs have been shown to display evenly poised bivalent marks which correlate to undergo transdifferentiation in response to the local cytokine milieu [55]. This may explain the difference in RORC methylation levels between dcSSc patients with high systemic inflammatory activity (ESR ≥ 10 mm/h) and patients with ESR < 10 mm/h. A second explanation for the trend towards higher RORγτ expression in CD4+CCR6+ T cells and intracellular IL-17 production despite hypermethylation at RORC regions in dcSSc is that in our assay, DNA from total PBMCs due to small sample volumes of dcSSc patients and HC was taken to analyze methylation levels at RORC1, RORC2, FOXP3, STAT3, and STAT5A regions, but not from isolated T-cell subsets. Recently, it has been shown that RORC2 is differentially methylated in classic CD4+CD161–CCR6–IFNγ+ Th1 cells, and transiently in Th17/Th1 (CD4+CD161+CCR6+IL-17A+IFNγ+) cells and nonclassic CD4+CD161+CCR6+IFNγ+ Th1 cells [57]. Thus, hypermethylation at RORC regions may result rather from other Th cell subpopulations, such as Th1 or Th2, than from the relatively small peripheral Th17 subpopulation. However, in our study, hypomethylation at CpGs within FOXP3 promoter regions goes ahead with inflammatory activity in dcSSc patients. FOXP3 promoter was not found to have repressive effects on Th1 or Th17 cells, which was interpreted that FOXP3 can be more widely and transiently expressed in Tregs as well as in activated T cells [55].

Novel therapeutic strategies targeting the IL-17 axis or IL-6 as an important proinflammatory cytokine inducing Th17 cells [49] have been suggested for the treatment of SSc patients. Our results also suggest a high plasticity of Tregs that is strongly associated with the Th17 phenotype. Future research directions may focus on stabilization of the Treg phenotype and on enhancing Treg functions, as our results revealed a proportional increase in T cells showing features of Tregs at the transcriptional and phenotypical level.

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Disclosure Statement

There are no conflicts of interest for any author.
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