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Original article

Lymphocyte subset abnormalities in early severe scleroderma favor a Th2 phenotype and are not altered by prior immunosuppressive therapy

Ankoor Shah[1], Jan Storek[2], Rob Woolson[3], Ashley Pinckney[3], Lynnette Keyes-Elstein[3], Paul K. Wallace[4], Gregory D. Sempowski[1], Peter McSweeney[5], Maureen D. Mayes[6], Leslie Crofford[7], M. E. Csuka[8], Kristine Phillips[7], Dinesh Khanna[9], Robert Simms[10], Karen Ballen[11], Sharon LeClercq[2], William St. Clair[1], Andrew B. Nixon[1], Richard Nash[5], Mark Wener[12,13], Richard Brasington[14], Richard Silver[15], Linda M. Griffith[16], Daniel E. Furst [13,17,18], Ellen Goldmuntz[16] and Keith M. Sullivan[1]

Abstract

Objectives. The Scleroderma: Cyclophosphamide or Transplantation (SCOT) trial compared hematopoietic stem cell transplant to CYC treatment in patients with early SSc with progressive skin and lung or kidney involvement. Here we describe lymphocyte phenotype abnormalities at study entry and the relation to prior DMARD therapy.

Methods. Lymphocyte subsets ($n = 26$) measured by flow cytometry were compared in 123 heathy controls and 71 SCOT participants, including those given ($n = 57$) or not given ($n = 14$) DMARDs within 12 months of randomization.

Results. Compared with healthy controls, individuals with SSc showed significant reductions in central memory CD8 T cells, activated total and CD4 T cells, γ/δ T cells, memory B cells, myeloid and plasmacytoid dendritic cells and $FOXP3^+CD25^+$ Treg cells and increases in naïve CD4 T cells, effector memory CD4 T cells and effector CD8 T cells. A greater bias towards a IL-4^+ Th2/T cytotoxic 2 (Tc2) phenotype based on the Th2:Th1 CD4 ratio and Tc2:Tc1 CD8 T cells was also found. Notably, no difference in any lymphocyte subset was observed between those given or not given prior DMARDs.

Conclusions. In patients with early, severe SSc, significant lymphocyte subset abnormalities were observed. Prior treatment with immunosuppressive therapy did not impact the immunophenotype, suggesting that lymphocyte disturbances in scleroderma appeared to be due to the disease itself.

Trial registration. ClinicalTrials.gov (https://clinicaltrials.gov), NCT00114530.

Key words: scleroderma, lymphocyte subsets, clinical trial, stem cell transplantation, CYC

BASIC SCIENCE

[1]Department of Medicine, Duke University, Durham, NC, USA, [2]Departments of Medicine and Oncology, University of Calgary, Calgary, AB, Canada, [3]Rho Federal System, Durham, NC, [4]Department of Oncology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, [5]Department of Hematology/Oncology, Colorado Blood Cancer Institute, Denver, CO, [6]Department of Medicine, University of Texas, Houston, TX, [7]Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, [8]Department of Medicine, Medical College of Wisconsin, Milwaukee, WI, [9]Department of Medicine, University of Michigan, Ann Arbor, MI, [10]Department of Medicine, Dartmouth-Hitchcock Medical Center, Lebanon, NH, [11]Department of Medicine, University of Virginia, Charlottesville, VA, [12]Department of Oncology, Fred Hutchinson Cancer Research Center, [13]Department of Medicine, University of Washington, Seattle, WA, [14]Department of Medicine, Washington University, St. Louis, MO, [15]Department of Medicine, Medical University of South Carolina, Charleston, SC, [16]National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, [17]Department of Medicine, University of California, Los Angeles, CA, USA and [18]University of Florence, Florence, Italy

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Correspondence to: Ankoor Shah, Department of Medicine, Duke University, DUMC Box 3874, Durham, NC 27710, USA.
E-mail: ankoor.shah@duke.edu

Rheumatology key messages

- Individuals with early, aggressive systemic sclerosis have a profoundly altered lymphocyte profile compared with healthy controls.
- T lymphocytes skewed towards an IL-4^+ Th2 or Tc2 phenotype.
- Prior immunosuppressive therapy does not appear to alter the lymphocyte profile.

Introduction

SSc is a systemic autoimmune disease marked by vascular endothelial damage and diffuse tissue fibrosis leading to skin thickening, pulmonary fibrosis, pulmonary hypertension and renal disease [1]. The autoimmune basis of disease is supported by the presence of serum autoantibodies, histological findings of lymphocytic infiltrates in the involved tissues before or soon after fibrosis has developed [2], disease improvement in some individuals treated with DMARDs such as CYC or MMF [3] and, in a preclinical model, dermal lymphocyte infiltration and fibrosis in healthy mice after lymphocyte transfer using tight-skin mouse models of human SSc [2, 4]. However, understanding of the immunopathogenesis of SSc remains incomplete. Recently the Scleroderma: Cyclophosphamide or Transplantation (SCOT) randomized clinical trial demonstrated long-term benefit in participants with early, progressive SSc with internal organ involvement receiving hematopoietic stem cell transplant compared with pulse i.v. CYC (as measured by a global ranked composite score). To examine the immunologic dysregulation in this population, we enumerated peripheral blood lymphocyte subsets before randomization and compared results with those of healthy controls. To test the hypothesis that prior treatment would influence baseline lymphocyte profiles, we compared SCOT participants who were exposed to immunosuppressive therapy in the prior 12 months with those who were treatment naïve.

Methods

Subjects and controls

The study population included 123 healthy controls of similar age and gender and 71 participants randomized on the multicenter SCOT trial. Peripheral blood was collected before participants underwent study procedures [5]. Entry criteria for the trial included diffuse cutaneous SSc of <5 years duration and significant pulmonary involvement or a history of scleroderma renal crisis. The history of immunosuppressant use was extracted from the prior medication listing of all randomized participants, including use of any of the following agents in the 12 months prior to randomization: prednisone (>10 mg/day), CYC, MTX, AZA, MMF, imatinib, D-penicillamine, anti-thymocyte globulin, tacrolimus, ciclosporin, TNF-α inhibitors, gold and/or LEF. The immunosuppressive-naïve group was comprised of participants whose only immunosuppression in the prior 12 months was prednisone at a dose of ≤ 10 mg/day.

Details of the SCOT trial, including inclusion and exclusion criteria, measurements of pulmonary function and skin involvement using the modified Rodnan skin score (mRSS), have been published previously [5]. All participants had scleroderma internal organ involvement and all but two had pulmonary involvement [baseline mean diffusing capacity of the lungs for carbon dioxide (DLCO) of 53% predicted]. The study was approved by the Duke University institutional review board and the institutional review boards of all participating centers (Supplementary Data S1, available at *Rheumatology* online). Written informed consent was obtained from all participants prior to study entry and again prior to treatment randomization.

Lymphocyte subset enumeration

Heparinized blood was collected from participants at participating SCOT centers and from healthy controls (collected separately from the published SCOT study) and shipped overnight to the Roswell Park Comprehensive Cancer Center (Buffalo, NY, USA). Similar collection, shipment and analytic methods were performed for participants enrolled in the SCOT trial and healthy controls. Leukocyte counts and differentials were performed using AcT10 hematology analyzer (Beckman Coulter, Miami, FL, USA). Leukocytes were labelled with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry using standard techniques [6–8]. Cytofluorometric analysis was performed using an FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) [9]. A minimum of 100 000 events were collected using DiVa software (BD Biosciences). Twenty-six distinct lymphocyte subset measures were defined by surface and intracellular immunophenotypes as shown in Table 1. Absolute numbers of lymphocyte subsets (per microliter of blood) were calculated as the product of the absolute lymphocyte count and the fraction of fluorescent tagged cells. For the enumeration of induced Th1, Th2, T cytotoxic 1 (Tc1) and Tc2 cells, blood was washed with and resuspended in complete medium (RPMI 1640 with 50% fetal calf serum) and then aliquoted into two tubes—an unstimulated control tube and a stimulated tube. Brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 10 μg/ml was added to both tubes and the cells in the stimulated tube were activated for 4 h with

Table 1 Definitions of lymphocyte subsets

Subset	Definition
T cells	$CD3^+$
CD4 T cells	$CD3^+CD4^+CD8^-$
CD8 T cells	$CD3^+CD4^-CD8^+$
Naïve CD4 T cells	$CD4^+CD27^+CD62L^+CD45RA^+CD45RO^-$
Central memory CD4 T cells	$CD4^+CD27^+CD62L^+CD45RA^-CD45RO^+$
Effector memory CD4 T cells	$CD4^+CD27^-CD62L^-CD45RA^-CD45RO^+$
Naïve CD8 T cells	$CD8^+CD27^+CD62L^+CD11a^{low}CD45RO^-$
Central memory CD8 T cells	$CD8^+CD27^+CD62L^+CD11a^{high}CD45RO^+$
Effector memory CD8 T cells	$CD8^+CD27^-CD62L^-CD11a^{high}CD45RO^+$
Effector CD8 T cells	$CD8^+CD27^-CD62L^-CD11a^{high}CD45RO^-$
Activated T cells	$CD3^+CD25^{low}CD69^+$
Activated CD4 T cells	$CD3^+CD4^+CD25^{low}CD69^+$
Recent thymic CD4 emigrants	$CD3^+CD4^+CD45RA^+CD45RO^-CD31^+$
γ/δ T cells	$CD3^+\delta,\gamma^+$
Induced Th1 CD4 T cells	$CD3^+CD4^+CD8^-$IFN-γ^+IL-4^- with PMA/ionomycin stimulation
Induced Th2 CD4 T cells	$CD3^+CD4^+CD8^-$IFN-γ^-IL-4^+ with PMA/ionomycin stimulation
Induced Tc1 CD8 T cells	$CD3^+CD4^-CD8^+$IFN-γ^+IL-4^- with PMA/ionomycin stimulation
Induced Tc2 CD8 T cells	$CD3^+CD4^-CD8^+$IFN-γ^-IL-4^+ with PMA/ionomycin stimulation
B cells	$CD19^+$
Naïve B cells	$CD19^+CD27^-$
Non-isotype-switched memory B cells	$CD19^+CD27^+IgM^+IgD^+$
Isotype-switched memory B cells	$CD19^+CD27^+IgM^-IgD^-$
Myeloid dendritic cells/precursors	$CD11c^+HLADR^+$lineage-[a]
Plasmacytoid dendritic cells/precursors	$CD11c^-CD123^+HLADR^+$lineage-[a]
Treg cells	$CD3^+CD4^+CD25^+FoxP3^+$
Treg cells/$CD127^-$	$CD3^+CD4^+CD25^+FoxP3^+CD127^-$

[a]Not expressing lineage-specific markers (CD3, CD14, CD16, CD19 or CD56).

phorbol myristate acetate (PMA; Sigma-Aldrich) and ionomycin (Sigma-Aldrich) at a final concentration of 25 ng/ml and 1 μg/ml, respectively. Th1 and Th2 cells were defined as $CD4^+$ cells if stained positive for IFN-γ and IL-4, respectively. Similarly, Tc1 and Tc2 cells were defined as $CD8^+$ cells that stained positive for IFN-γ and IL-4, respectively.

Statistics

Differences between subgroups were evaluated using the Mann–Whitney–Wilcoxon rank sum and Pearson's χ^2 statistics for quantitative and categorical variables, respectively. Given the multiple comparisons across multiple cell types, the false discovery rate was controlled via the method of Benjamini and Hochberg [10].

Results

Participants

Clinical characteristics of the SCOT participants at randomization are presented in Table 2. Of the 75 individuals in the SCOT trial, 71 had baseline lymphocyte immunophenotype data available for this analysis. Those participants who were DMARD exposed in the 12 months before randomization ($n = 57$) had received glucocorticoids ($n = 39$), CYC ($n = 23$), MMF ($n = 18$) or other DMARDs ($n = 18$). Of the 39 who had previously received glucocorticoids, 17 received doses >10 mg/day of prednisone, 2 of whom received only glucocorticoids and no other DMARD. Eight received prednisone at a dose of ≤10 mg/day. Several received more than one prior therapy. There were 14 DMARD-naïve patients. The median mRSS was notably lower in the naïve group (23 naïve, 30 treated; unadjusted *P*-value = 0.027). The median duration of disease was similar in the two DMARD-exposed groups: 1.9 years for naïve and 1.8 years for treated participants.

Lymphocyte subsets

Table 3 presents the median lymphocyte subset counts for healthy controls ($n = 123$) and SCOT participants ($n = 71$) grouped by those who were previously DMARD exposed ($n = 57$) or naïve ($n = 14$). Compared with healthy controls, SCOT participants had reduced numbers of CD8 T cells, activated total and CD4 T cells and γ/δ T cells. In addition, reductions in switched and non-switched memory B cells, central memory CD4 and CD8 T cells, myeloid and plasmacytoid dendritic cells and $FOXP3^+CD25^+$ Treg cells were observed in SCOT participants. Conversely, increased numbers of naïve and effector memory CD4 T cells, effector CD8 T cells and induced Th2 CD4 and Tc2 CD8 T cells were observed in SCOT participants. The Th2:Th1 CD4 and Tc2:Tc1 CD8 T cell ratios were increased in SCOT participants compared with healthy controls.

TABLE 2 Clinical characteristics of the SCOT participants at randomization

Characteristics	Immunosuppressive treatment naïve	Immunosuppressive treatment exposed	Total
Participants, *n*	14	57	71
Age, years, median (minimum–maximum)	44 (30–56)	47 (22–65)	47 (22–65)
Sex (male/female), *n*	3/11	23/34	26/45
Race (Caucasian/Black/Asian/Other), *n*	12/1/0/1	46/5/3/3	58/6/3/4
Disease duration between first non-Raynaud's symptom and randomization, years, median (minimum–maximum)	1.9 (0.9–4.1)	1.8 (0.5–5.4)	1.8 (0.5–5.4)
mRSS, median (minimum–maximum)	23(8–37)	30 (12–49)[b]	29 (8–49)
FVC, % predicted, median (minimum–maximum)	74 (53–96)	74 (36–107)	74 (36–107)
DLCO, % predicted[a], median (minimum–maximum)	51.4 (41.0–67.8)	54.2 (40.2–70.0)	53.1 (40.2–70.0)
Hemoglobin, g/dL, median (minimum–maximum)	12.9 (8.7–14.1)	12.1 (8.6–15.1)	12.3 (8.6–15.1)
ESR in 1 h, median (minimum–maximum)	19.0 (5.0–60.0)	24.0 (3.0–108.0)	22.0 (3.0–108.0)
Creatinine clearance, mL/min/1.73 m^2, median (minimum–maximum)	113.5 (33.6–197.0)	122.0 (25.0–305.6)	117.8 (25.0–305.6)

[a]Corrected for altitude and hemoglobin. [b]The unadjusted *P*-value for the Mann–Whitney statistic to compare groups = 0.027. No other *P*-values are <0.05.

Overall, among the SCOT participants, no statistically significant differences were observed between those who were immunosuppressive exposed as compared with naïve participants. An exploratory subgroup analysis was performed comparing controls to SCOT participants on the basis of their prior DMARD exposure. Results for the subgroup exposed to the immunosuppressive agents, as compared with controls, are completely consistent with findings observed for all SCOT participants. For the immunosuppressive-naïve subgroup, as compared with controls, the direction and magnitude of effects are analogous to those for the treatment-exposed subgroup for most lymphocyte subsets; the larger *P*-values and inability to detect differences between the naïve subgroup and healthy controls for some cell subsets may be attributable to the small number of naïve subjects. For two subsets, total CD8 T cells and γ/δ T cells, however, observed numbers for the immunosuppressive-naïve subgroup were similar to those for healthy controls compared with the immunosuppressive-exposed subgroup.

Association between lymphocyte subsets and disease severity

For select lymphocyte subset measures we investigated possible correlations with baseline characteristics and severity of SSc at study entry. Among the group of all SCOT participants, as well as the group of 57 DMARD-exposed participants, there were no noteworthy (defined as a Pearson correlation of <-0.5 or >0.5) correlations between any lymphocyte subset and age, mRSS, forced vital capacity (FVC) or DLCO (Table 4).

Discussion

In this study of early severe SSc with internal organ involvement, significant differences in peripheral blood lymphocyte subsets were observed between SCOT trial participants and healthy controls. This attests to the profound immune dysregulation in early severe scleroderma. Our results support other reports of patients with early diffuse SSc, including a recent investigation of the patients enrolled in the Abatacept Systemic SclErosis Trial (ASSET), which enrolled study participants with early SSc and no internal organ involvement. Enrolment criteria excluded individuals with prior DMARD therapy, although prednisone $\leq$10 mg/day was allowed. The investigators also found low $CD8^+$ T cells among study participants compared with controls [11]. In that study, no significant differences were seen between SSc and controls for Treg cells nor was a Th2 bias observed.

To evaluate the possibility that subset differences were due to prior DMARD treatments, we compared immunosuppressive-exposed and naïve participants and found that subset abnormalities were similar in the two groups, suggesting that abnormalities were primarily due to scleroderma itself rather than immune

Table 3 Lymphocyte subset counts in SCOT participants and healthy controls (cells per microliter)

Lymphocyte subset	Median (10th–90th percentiles)			
	Healthy controls ($n = 123$)	SCOT participants		
		All subjects ($n = 71$)	Immunosuppressive naïve ($n = 14$)	Immunosuppressive exposed ($n = 57$)
T cells	1447 (799–2247)	1226 (513–2386)	1181 (719–2504)	1226 (452–2386)
CD4 T cells	935 (503–1544	836 (335–1806)	731 (444–1924)	843 (334–1806)
CD8 T cells	395 (199–735)	261 (100–667)***	347 (232–556)	247 (99–721)***
Naïve CD4 T cells	88 (26–273)	187 (38–390)***	231 (38–375)*	173 (24–467)***
Central memory CD4 T cells	376 (199–683)	244 (82–518)***	227 (66–717)	252 (82–518)***
Effector memory CD4 Cells	16 (6–42)	21 (8–91)**	31 (4–94)	20 (9–91)*
Naïve CD8 T cells	35 (7–119)	36 (5–119)	81 (6–127)	27 (4–119)
Central memory CD8 T cells	108 (49–246)	15 (2–65)***	13 (4–49)***	15 (2–70)***
Effector CD8 T cells	5 (1–19)	26 (6–90)***	29 (9–106)***	24 (6–90)***
Activated T cells	99 (40–256)	27 (7–287)***	57 (13–402)	25 (6–259)***
Activated CD4 T cells	53 (20–138)	9 (2–148)***	16 (4–296)	9 (2–139)***
γ/δ T cells	39 (13–97)	22 (5–66)***	41 (4–94)	20 (5–63)***
Induced Th1 CD4 T cells	205 (64–543)	230 (28–667)	269 (24–1071)	217 (28–568)
Induced Th2 CD4 T cells	13 (2–49)	36 (4–95)***	31 (0–131)	36 (6–95)***
Th2:Th1 CD4 cells ratio	0.051 (0.018–0.150)	0.146 (0.033–0.724)***	0.146 (0.000–0.209)	0.153 (0.035–0.746)***
Induced Tc1 CD8 T cells	249 (79–585)	209 (47–633)	268 (22–633)	182 (47–702)
Induced Tc2 CD8 T cells	2 (1–13)	4 (1–21)*	4 (0–10)	4 (1–25)*
Tc2:Tc1 CD8 cells ratio	0.010 (0.002–0.056)	0.021 (0.004–0.213)**	0.015 (0.000–0.108)*	0.021 (0.004–0.254)***
B cells	197 (87–449)	161 (42–430)	208 (84–320)	159 (39–458)
Naïve B cells	151 (62–351)	148 (37–375)	183 (54–291)	141 (33–425)
Non-switched memory B cells	13 (5–29)	6 (1–18)***	7 (1–18)*	6 (1–22)***
Switched memory B cells	25 (9–57)	6 (2–17)***	10 (4–17)***	5 (2–22)***
Myeloid dendritic cells/ precursors	43 (24–88)	18 (8–49)***	17 (9–70)*	19 (8–42)***
Plasmacytoid dendritic cells/precursors	5 (2–9)	3 (0–7)***	2 (0–6)*	3 (0–9)**
$CD3^+CD4^+CD25^+$Fox $P3^+$ (Treg)	37 (7–134)	3 (0–17)***	4 (0–13)***	2 (0–17)***
$CD3^+CD4^+CD25^+FoxP3^{+-}$ $CD127^-$ (Treg)	34 (6–125)	2 (0–15)***	4 (0–10)***	2 (0–15)***

IS: immunosuppressive. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, compared with healthy controls. No significant differences were observed between immunosuppressive-exposed and naïve patients.

modulatory treatment. Even prior treatment with CYC resulted in a baseline lymphocyte profile that was not significantly different from those participants treated with other DMARDs.

Reduced thymopoiesis was not confirmed in our study. We found normal to increased naïve CD4 and CD8 T cells and normal recent thymic emigrants compared with healthy controls. Blood dendritic cells were found to be decreased as compared with healthy controls, similar to other autoimmune diseases. Our results showing increased numbers of induced Th2 CD4 T cells strengthen the theory that Th2 (rather than Th1) cells play a role in the pathogenesis of SSc. Our study also found higher Th2:Th1 cell ratios in SCOT participants as compared with healthy controls. A Th2 bias (defined as increased levels of Th2 cytokines such as IL-4, IL-10 and IL-13) in serum, skin and bronchoalveolar lavage (BAL) fluid (or exhaled breath condensate) or an increased number of Th2 cells in blood or BAL fluid has been described [12]. In other studies, the degree of Th2 bias correlated with the degree of skin fibrosis [13] or lung dysfunction [14, 15]. An increased frequency of Th2-biased CD4 T cells, identified by the production of IL-4, a profibrotic cytokine [16], has also been noted in individuals with SSc. It has also been demonstrated that B cell depletion with the anti-CD20 agent rituximab was able to significantly reduce peripheral and skin Th2 cells in a heterogeneous population of subjects with SSc (although the correlation with clinical improvement was not studied) [17]. A higher frequency of IL-4-producing CD8 T cells (designated as Tc2 cells in our study) has been found in the BAL fluid of patients with

TABLE 4 Pearson correlations between select lymphocyte subsets and clinical features

Lymphocyte subset	All SCOT participants ($n = 71$)	
	mRSS	FVC
T cells	−0.05	−0.11
CD4 T cells	−0.03	−0.05
CD8 T cells	−0.09	−0.18
Naïve CD4 T cells	0.05	0.05
Central memory CD4 T cells	−0.21	0.02
Naïve CD8 T cells	−0.15	0.25
Central memory CD8 T cells	−0.28	−0.12
Recent thymic CD4 emigrants	0.18	0.06
Induced Th1 CD4 T cells	−0.27	−0.18
B cells	−0.07	−0.13
Naïve B cells	−0.05	−0.12
Non-switched memory B cells	−0.13	−0.14
Switched memory B cells	−0.10	−0.16
Treg cells	−0.24	0.07
Treg cells/CD127−	−0.26	0.08

Pearson correlation <-0.5 or >0.5 is considered a noteworthy correlation.

SSc and was associated with a greater decline in lung function [14]. While we did not examine populations of Th17 cells in our study population, increased numbers of Th17 cells have been observed in the skin of patients with SSc. It has been proposed that these cells are in fact regulatory cells and act in opposition to the profibrotic Th2 cells [18].

Similar to our findings for central memory CD8 T cells, decreased peripheral counts of total CD8 T cells in patients with SSc have been reported [19–22]. The increased levels of the effector CD8 T cell subset may suggest that the low CD8 T cell count is not a result of decreased thymic production, but rather a result of decreased proliferation, redistribution from blood to tissues or an increased death rate of memory CD8 T cells. Given that T cells of patients with scleroderma are more resistant to apoptosis than T cells from healthy controls [23], an increased death rate may be a less plausible explanation.

Our study has several limitations. We did not measure target tissue levels of lymphocyte subsets from lung or skin, which may differ from results in peripheral blood. While the duration of SSc disease was similar in DMARD-naïve and exposed participants (1.9 *vs* 1.8 years, respectively), the severity of skin disease (mRSS) was lower in naïve participants. The reason for these patients not receiving immunosuppression before randomization is unknown.

The analysis of blood lymphocyte subsets detailed here is the largest and most comprehensive report in early severe SSc with organ involvement and the first to report individuals with and without prior DMARD therapy. Our study population was well defined and homogeneous, with a large healthy control population. We compared our immunophenotype data to clinically meaningful parameters of skin score and pulmonary function. Notably, no difference in any lymphocyte subset was observed between those given or not given prior DMARDs. These data suggest that in this population of severe SSc with internal organ involvement, the disease phenotype appears to have a greater influence on immunophenotype abnormalities than prior immunosuppression. Longitudinal analysis will provide a greater opportunity for comparisons with other immunologic and genomic assays.

Conclusions and future directions

Our study evaluated a well-defined homogeneous population of SCOT trial participants with early, aggressive SSc and internal organ involvement not confounded by variable disease durations and observed significant differences between participants at baseline when compared with healthy controls. Differences were observed among a variety of T and B cell populations, including Treg cells, and were found irrespective of prior exposure to immunosuppressive agents. Most notably, a greater bias towards an IL-4-producing phenotype (for both CD4 and CD8 T cells) as compared with controls was found, supporting the potential role of cytokines such as IL-4, IL-10 and IL-13 in disease pathogenesis. No differences in phenotypes were observed between those individuals who did and did not receive prior DMARD therapy.

Longitudinal analysis will provide a greater opportunity for comparisons with other immunologic and genomic assays. Subsequent serial analyses of lymphocyte subsets with clinical endpoints correlating [5, 24] to month 56 after randomization may provide insights into the predictive outcome value of baseline measures and portray the tempo and pattern of lymphocyte recovery in

relation to the effects of the two SCOT treatments. Such correlations of outcomes with baseline immunophenotype abnormalities and prior DMARD therapy may offer additional insights into the mechanisms of immune recovery and control of severe scleroderma over time.

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Data availability statement

The SCOT trial data are accessible at www.import.org for study SDY1039 (doi: 10.21430/M3SM4LTLH).

Supplementary data

Supplementary data are available at *Rheumatology* online.

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