Altered Homeostasis of Regulatory T Lymphocytes and Differential Regulation of STAT1/STAT5 in CD4+ T Lymphocytes in Childhood-onset Systemic Lupus Erythematosus

Marija Holcar, Aleš Goropevšek, and Tadej Avčin

ABSTRACT. ***Objective.*** Childhood-onset systemic lupus erythematosus (cSLE) is usually a more severe and aggressive disease than adult-onset SLE (aSLE), but cellular and subcellular reasons for these differences are not well understood. The present study analyzed Th subsets, STAT1/STAT5 signaling response, and cytokine profiles of cSLE.

Methods. FOXP3+ regulatory (Treg) and effector Th subsets, expression and phosphorylation of STAT1/STAT5 in Th, and cytokine profiles were measured in the peripheral blood of patients with cSLE and healthy controls (HC), using flow cytometry and immunoassay on a biochip.

Results. Significant correlation between expression of the activation marker HLA-DR and decreased Th counts, an increase in the percentage of FOXP3+ Th, and a decrease in the activated Treg (aTreg) subset among them were found in cSLE. In contrast to our previous findings in aSLE, no significant differences in percentages and a significant decrease in the numbers of the naive-resting Treg (rTreg) subset compared to HC were found. The percentages of CD25– cells, possibly reflecting interleukin 2 depletion, were significantly increased in cSLE aTreg, but not in the rTreg subset. Consistent with the results of our previous studies in aSLE, increased expression of STAT1, along with significant correlation between decreased Th counts and their increased basal phosphorylation of STAT5, were also found in cSLE.

Conclusion. Our results suggest that the key difference in Treg homeostasis between cSLE and aSLE is in the rTreg subset. However, perturbed aTreg homeostasis, increased levels of STAT1 protein, and homeostatic STAT5 signaling appear to be intrinsic characteristics of the disease, present in cSLE and aSLE alike. (J Rheumatol First Release December 15 2019; doi:10.3899/jrheum.181418)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS
CHILDHOOD ONSET
FLOW CYTOMETRY
TREG
EFFECTOR TH
TH17

Systemic lupus erythematosus (SLE) is a chronic multisystem autoimmune inflammatory disease with diverse clinical and laboratory signs[1]. The exact etiopathogenesis of SLE is not yet fully known[2]. Pathogenic T lymphocytes from SLE can demonstrate many signs of altered function, which in the end stimulates the autoimmune inflammatory state[3]. The population of CD4–CD8– double negative T lymphocytes is higher in patients with SLE than in healthy controls (HC) and can induce autoreactive B lymphocytes to produce anti-dsDNA antibodies and higher numbers of inflammatory cytokines[4]. Changes are also noticeable in individual subpopulations of helper T lymphocytes (Th). It is suspected that disruption of the homeostasis between the effector Th (Teff) and the regulatory Th (Treg) cells can lead to various inflammatory and autoimmune diseases, including SLE[5].

According to the major transcription factors, cytokines needed for their differentiation, as well as the characteristic cytokines secreted by the activated cells, Teff can be divided into Th1, Th2, Th17, and others[6]. Despite the considerable functional specialization of subtypes, there is some plasticity between different Teff, which sometimes allows multifunctional subpopulations to arise. Examples of the latter are Th1Th17, which secrete interferon-γ (IFN-γ), characteristic

---

From the Department of Allergology, Rheumatology and Clinical Immunology, University Children's Hospital, University Medical Centre Ljubljana, Ljubljana; Department of Laboratory Diagnostics, University Medical Centre Maribor, Maribor; Department of Pediatrics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia.

This work was partially supported by the Slovenian Research Agency grant (grant number L7-8274) and the University Medical Centre Ljubljana research grant (grant number 20180093).

M. Holcar, PhD, Department of Allergology, Rheumatology and Clinical Immunology, University Children's Hospital, University Medical Centre Ljubljana; A. Goropevšek, MD, PhD, Department of Laboratory Diagnostics, University Medical Centre Maribor; T. Avčin, MD, PhD, Department of Allergology, Rheumatology and Clinical Immunology, University Children's Hospital, University Medical Centre Ljubljana, and Department of Pediatrics, Faculty of Medicine, University of Ljubljana.

Address correspondence to Dr. T. Avčin, Head, Department of Allergology, Rheumatology and Clinical Immunology, University Children's Hospital, University Medical Centre Ljubljana, Bohoričeva 20, SI-1525 Ljubljana, Slovenia. E-mail: tadej.avcin@kclj.si

Accepted for publication June 20, 2019.

for Th1, as well as highly inflammatory interleukin (IL)-17, characteristic for Th17[7].

Treg control the immune response by direct inhibition of Teff, but also by reducing the antigen presentation potential of dendritic cells and altering their release of cytokines. The depletion of Treg can thus contribute to the onset and maintenance of autoimmune diseases in 2 distinct ways. The removal of cells with a suppressor function can lead to unrestrained polyclonal activation of Teff; additionally, systemic expansion and maturation of dendritic cells can increase the likelihood of the presentation of self-antigens[8]. The total number and activation of Treg is usually reduced in SLE; moreover, an elevated number of FOXP3-expressing cells, which are functionally not suppressive, has been found[9,10]. Many previous studies have focused also on IL-17, expansion of Th17, and the Treg:Th17 dynamic in the pathogenesis of SLE[11,12,13].

Proper functioning of Th depends on their successful signal transduction. Cytokine receptors receive signals of different extracellular molecules and trigger signal transduction through the Janus kinase and STAT pathway[14]. Canonically it begins with 3 consecutive tyrosine phosphorylations, last being phosphorylation of STAT proteins. The phosphorylation rate of STAT can therefore offer relevant insight into the activation status of the cell. Aberrant cytokine signaling is associated with many immune-mediated disorders, in particular, inflammatory conditions and autoimmune diseases such as SLE[15,16].

The primary roles of STAT1 are the transmission of IFN signals and the activation of antiviral inflammatory response by excretion of new IFN. It regulates the cytokine production of Th1 and controls the proliferation and apoptosis of other immune cells[17]. In SLE, as well as in the SLE mice model, basal expression of STAT1 was raised[18,19]. Phosphorylation of STAT1 was also slightly increased and in positive correlation with the disease duration in mice T lymphocytes, but not in humans with adult-onset SLE (aSLE)[20]. Higher expression of STAT1 mRNA was reported in lupus nephritis and was correlated with disease activity and IFN-dependent gene expression[21,22]. The rise in basal expression and phosphorylation of STAT1 can thus be a result of — and a help in maintaining — the inflammatory environment of affected cells.

STAT5 transmits signals of common γ-chain cytokines (such as IL-2), and induces expression of FOXP3, a transcription factor crucial for the maturation of Treg[23]. STAT5-dependent genes are tissue-specific, but generally STAT5 controls survival, proliferation, differentiation, and regulation of the cell cycle. It plays a central role in maintaining peripheral tolerance through activation of Treg and is thus likely to be involved in the pathogenesis of autoimmune diseases[24]. Accordingly, the levels of phosphorylated STAT5 (pSTAT5) were elevated in aSLE and were in correlation with their disease activity[19]. STAT3 and STAT5 can bind to many identical binding sites on the gene for IL-17. STAT5 can competitively block STAT3-dependent transcription of IL-17 and inhibit Th17 polarization, leading to a decrease of inflammation[25,26].

Childhood-onset SLE (cSLE) is usually a more severe form of the disease compared to aSLE. It can involve more organs and require more aggressive treatments, increasing the possibility of longterm drug toxicity and disease damage. Systemic manifestations of the disease and development of anti-dsDNA antibodies are also more common in cSLE[27]. The causes of disease onset at different ages and the clinical differences between cSLE and aSLE are not yet well understood, but factors contributing to the more severe childhood form of the illness are likely to be predominantly genetic predisposition, a higher incidence of acute infections, and immaturity of the immune system and other organ systems[28].

In our study, subpopulations of Th, their expression and phosphorylation of STAT1 and STAT5, and plasma cytokine concentrations were analyzed in cSLE. Comparison of our results with findings on aSLE enhances understanding of the similarities and differences between the 2 types of the disease.

MATERIALS AND METHODS

Study subjects. Seventeen cSLE patients with a median age of 18.0 years at enrollment were included in the study. All patients were followed at the Children's Hospital, University Medical Centre Ljubljana, Slovenia. Disease activity was assessed using the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) scoring system[29]. As a control group, we included 20 healthy adolescents (HC) with a median age of 16.0 years and no history of allergies, acute infections, autoimmune disorders, or medications that could affect the immune system. Demographic, clinical, and laboratory data are presented in Table 1. The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (approval number: 27/11/11); each participant or a legal guardian signed an informed consent form.

Antibodies and sample staining for analysis of lymphocyte subpopulations. Whole venous EDTA blood was aliquoted. Plasma was collected from 1 aliquot and stored for later cytokine analysis. The remaining aliquots were prepared following 3 different protocols. Standard whole-blood staining methodology with premixed multicolor panels (Tube 1–3; Table 2), according to the manufacturer's instructions, was used when staining solely surface antigens. STAT expression/phosphorylation was studied following the BD Phosflow protocol (Phosflow Lyse/Fix Buffer and Perm Buffer III). Staining of surface and intracellular antigens was in this case done simultaneously (Tube 5; Table 2). Treg were detected after primary staining of surface antigens in whole blood, with subsequent fixation and permeabilization using BD HumanFOXP3 Buffer set and staining of FOXP3 (Tube 4; Table 2).

All reagents, except for anti–CD3-PerCP, pSTAT5A-AlexaFluor647 (antibodies-online GmbH), anti-CXCR3-APC (BioLegend Inc.), and anti-CD161-FITC (eBioscience Inc.), were acquired from BD Biosciences. Cells were analyzed with the FACSCantoII Flow Cytometer, equipped with blue and red lasers, running FACSDiva software (both BD Biosciences). Digital data were analyzed using FlowJo software (Tree Star Inc.).

Flow cytometric analysis of lymphocyte subpopulations. Percentages of Th, cytotoxic lymphocyte T (Tc), CD4–DC8– T lymphocytes, HLA-DR+ T lymphocytes were analyzed after gating on CD3+CD45+ lymphocytes. Absolute cell counts were calculated using a dual-platform approach with panleukogating after measuring leukocytes on the Beckman Coulter AcT8 Hematology Analyzer (BD Biosciences). Analysis of all lymphocyte subpop-

Table 1. Study cohorts' age, therapy, clinical, and laboratory data.

Variables	Values
Age, yrs, median (min–max)	
HC, at study entrance, n = 20	16.0 (15.5–20.9)
cSLE, n = 17	
At diagnosis	15.5 (6.8–17.2)
At study entrance	18.0 (8.8–21.2)
Therapy, n (%)	
Antimalarials	12 (71)
Corticosteroids	10 (59)
Immunosuppressives	7 (41)
Hypertension drugs	4 (24)
Nonsteroidal antiinflammatory drugs	2 (12)
Anticoagulants	2 (12)
Antipsychotics	1 (6)
Anti-epileptic drugs	1 (6)
Biologics	–
Without therapy	2 (12)
Laboratory and clinical signs, n (%)	
Low complement	8 (47)
Reduced activation of classic pathway	6 (35)
Low concentration of C3	5 (29)
Low concentration of C4	3 (18)
Anti-dsDNA antibodies	4 (24)
Proteinuria	3 (18)
Pyuria	2 (12)
Arthritis	2 (12)
Seizures	1 (6)
Psychosis	1 (6)
Headache	1 (6)
Hematuria	1 (6)
New rash	1 (6)
Pleural effusion	1 (6)
Fever	1 (6)
Leukopenia	1 (6)
Disease activity, n (%)	
SLEDAI-2K, median (min–max)	5.1 (0–16)
SLEDAI-2K: 0	5 (29)
SLEDAI-2K: 1–5	7 (41)
SLEDAI-2K: ≥ 6	5 (29)

cSLE: childhood-onset systemic lupus erythematosus; HC: healthy controls; SLEDAI-2K: Systemic Lupus Erythematosus Disease Activity Index 2000.

ulations and measurement of STAT expression/phosphorylation were conducted after gating on the CD3+CD4+ cell population. The exact antibodies combination used to identify each subset can be found in Table 2. Gating strategies were the same as previously described[30]. Median fluorescent intensity (MFI) was used to measure expression and phosphorylation of STAT proteins.

Samples were obtained and studied individually; standard calibration beads (BD Biosciences) to set the forward, side scatter, and photomultiplier voltage were used for consistency before each experiment. MFI was normalized by subtraction of MFI values of CD3–CD4– cells in each sample (ΔMFI).

Plasma cytokine array on a biochip. All plasma samples were stored at –80°C and analyzed in a single experiment. Cytokine concentrations were analyzed using a kit with predesigned multiplex cytokine immunoassay on a biochip with pre-applied spatially discrete test regions, allowing for simultaneous determination of 10 cytokines [IL-1α, IL-1ß, IL-2, IL-4, IL-6, IL-8, IL-10, tumor necrosis factor–α (TNF-α), IFN-γ, monocyte chemoattractant protein 1 (MCP-1)] in a single sample at a single timepoint (Cytokine & Growth Factors Array I) with the Evidence Investigator immunoanalyser (both Randox), according to the manufacturer's instructions.

Statistical analysis. The Mann-Whitney U test was used to test differences between 2 groups and the Kruskal-Wallis among 3 groups. For within-group comparisons, the Wilcoxon matched-pairs signed-rank test was used. Correlations between experimental results were examined using Spearman rank test. A value of $p < 0.05$ was considered significant in all statistical tests. Statistical data analysis was performed using the GraphPad Prism software (GraphPad Software Inc.).

RESULTS

Severe lymphopenia leads to lower numbers of lymphocyte subsets in cSLE, but percentages of Th lymphocytes do not differ significantly. Lymphocyte number and their function are strongly implicated in the pathogenesis of SLE[31,32]. As well, significant lymphopenia was found in our patients with cSLE ($p < 0.0001$) compared to HC. The number of analyzed subsets including T lymphocytes, CD4–DC8– T lymphocytes, Th, and Tc was significantly lower in cSLE (first 3 subsets: $p < 0.0001$, Tc: $p < 0.05$; Figure 1A, Table 3). Lymphocytes were decreased also relatively in cSLE (% of leukocytes, $p < 0.01$), as were T lymphocytes (% of lymphocytes, $p < 0.01$; Figure 1B). While percentages of Th did not differ between groups, we found decrease within CD4–DC8– T lymphocytes and Tc in cSLE ($p < 0.01$ and $p < 0.05$; Figure 1B, Table 3).

Treg and Teff subsets show signs of perturbed homeostasis leading to decline in Treg function. Cells undergoing homeostatic lymphopenia-induced proliferation were shown to develop an activated phenotype[33]. To determine whether this effect influenced T lymphocyte reconstitution in cSLE Th depletion, we examined the expression of the marker of activation HLA-DR. Significant negative correlation was found between Th counts and the percentage of HLA-DR+ T lymphocytes from cSLE, but not HC (Figure 2A).

Because Treg reconstitution in cSLE could also be characterized by a shift in the activated/effector status of these cells, we performed Treg quantification as proposed by Miyara, *et al*[34], differentiating between CD45RA–FOXP3^{high} activated-effector Treg (aTreg) cells, CD45RA+FOXP3^{low} naive-resting Treg (rTreg), and the CD45RA–FOXP3^{low} activated effector T lymphocytes [abbreviated as non-Treg, even though they represent only a small and distinct subset of the conventional T lymphocyte (Tcon) fraction] subset among FOXP3-expressing cells.

In line with lymphopenia, numbers of aTreg and rTreg were significantly lower in cSLE than HC ($p < 0.001$ and $p < 0.05$, respectively; Table 3), which was reflected in the significant decrease of the total number of Treg (aTreg + rTreg) in cSLE ($p < 0.01$). However, in contrast to the absolute numbers, while percentages of aTreg were significantly decreased among FOXP3+ Th from cSLE and no significant difference was found in the rTreg, the percentage of non-Treg was significantly increased in cSLE ($p < 0.001$) compared to HC (Figure 2B).

Table 2. Combinations of antibodies used for identifying and analysis of different lymphocyte subpopulations.

Lymphocyte Subpopulations	Antibody Combination
Tube 1: anti-CD3-FITC/anti-CD8-PE/anti-CD45-PerCP/anti-CD4-APC	
T lymphocytes	CD3+CD45+
Th lymphocytes	CD3+CD4+CD45+
Tc lymphocytes	CD3+CD8+CD45+
CD4–CD8– T lymphocytes	CD3+CD4–CD8–CD45+
Tube 2: anti-CD45-FITC/anti-CD3-PE/anti-HLA-DR-APC	
HLA-DR+ T lymphocytes	CD3+CD45+HLA-DR+
Tube 3: anti-CD161-FITC/anti-CCR6-PE/anti-CD4-PerCP/anti-CXCR3-APC/anti-CCR4-Pe-Cy7	
Th1-like lymphocytes	CD4+CXCR3+CCR4–CCR6–
Th2-like lymphocytes	CD4+CXCR3–CCR4+CCR6–
Th1Th17-like lymphocytes	CD4+CXCR3+CCR4–CCR6+
Th17-like lymphocytes	CD4+CXCR3–CCR4+CCR6+
Tube 4: anti-CD25-PE/anti-CD45RA-APC/anti-CD4-PE-Cy7 + anti-FOXP3-Alexa Fluor 488	
aTreg lymphocytes	CD4+CD45RA–FOXP3^{high}
rTreg lymphocytes	CD4+CD45RA+FOXP3^{low}
Treg lymphocytes	CD4+CD45RA–FOXP3^{high}, CD4+CD45RA+FOXP3^{low}
non-Treg lymphocytes	CD4+CD45RA–FOXP3^{low}
FOXP3+ Th lymphocytes	CD4+CD45RA–FOXP3^{high}, CD4+CD45RA+FOXP3^{low}, CD4+CD45RA–FOXP3^{low}
Tcon lymphocytes	CD4+FOXP3–
Tube 5: anti-CD3-FITC/anti-STAT1-PE/anti-pSTAT5A(pTyr694)-Alexa Fluor 647/anti-CD4-PE-Cy7	
STAT1	CD3+CD4+STAT1+
pSTAT5A	CD3+CD4+STAT5A(pTyr694)+

Tc: cytotoxic T lymphocytes; Treg: regulatory Th; rTreg: resting Treg; aTreg: activated Treg; Tcon: conventional T lymphocytes; non-Treg: CD45RA–FOXP3^{low} activated effector T lymphocytes; pSTAT: phosphorylated STAT.

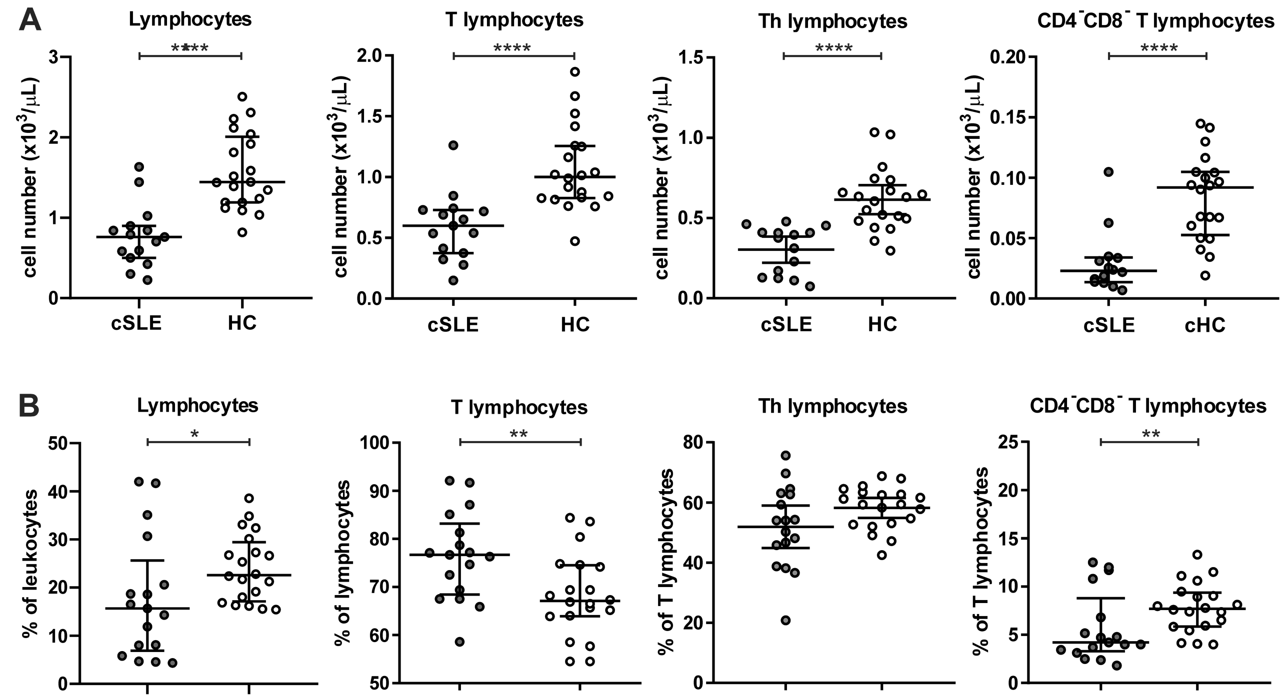

Figure 1. Differences in numbers and percentages of basic subpopulations of T lymphocytes between patients and HC. A. Numbers of basic lymphocyte subpopulations in cSLE and HC. B. Percentages of same basic lymphocyte subpopulations in cSLE and HC. Each symbol represents a single sample. The graphs show the median values with interquartile range. * $p < 0.05$. ** $p < 0.01$. **** $p < 0.0001$. cSLE: childhood-onset systemic lupus erythematosus; HC: healthy controls.

Table 3. Studied cell subsets analyzed by flow cytometry.

Cell Subsets		SLE			HC	
	N	Median	1st–3rd Quartile	N	Median	1st–3rd Quartile
Cells/μl						
Leukocytes	17	5900	4800-8800	20	6400	5575–7450
Lymphocytes	15	762.2	542.2–870.7	20	1443.3****	1190.6–1948.5
T lymphocytes	15	599.9	393.4–723.3	20	1001****	830.2–1252.4
Th lymphocytes	15	376.4	150.3–408.5	20	618.1****	492.1–687.3
Tc lymphocytes	16	235.3	162.7–325.7	20	299.2*	269.4–409.5
CD4–CD8– T lymphocytes	14	22.9	14.4–33.0	20	91.9****	57.7–104.4
HLA-DR+ T lymphocytes	16	80.4	57.6–128.6	20	135.7	70.1–252.2
Th1-like lymphocytes	7	49.35	24.6–173.40	19	80.19 *	65.92–102.10
Th2-like lymphocytes	7	16.75	9.26–28.90	20	18.19	14.49–26.07
Th1Th17-like lymphocytes	7	17.23	12.00–30.90	20	55.53**	39.42–69.02
Th17-like lymphocytes	7	15.59	11.23–18.08	20	21.99	13.46–32.99
aTreg lymphocytes	11	4.64	3.37–6.87	20	10.44***	8.61–16.93
rTreg lymphocytes	11	6.51	2.03–10.48	20	8.49*	6.21–11.36
Treg lymphocytes	11	11.13	6.69–17.01	20	23.35**	16.20–25.93
non-Treg lymphocytes	11	15.91	8.11–40.20	20	15.69	12.30–22.87
FOXP3+ Th lymphocytes	11	26.46	16.98–52.95	20	30.57	26.25–42.03
Tcon lymphocytes	11	349.90	137.9–383.8	20	583.2***	465.3–650.1
Percentages						
Leukocytes						
Lymphocytes	17	15.700	8.000–18.800	20	22.597**	17.735–28.008
Lymphocytes						
T lymphocytes	17	76.700	69.400–81.300	20	67.100**	63.975–74.300
T lymphocytes						
Th lymphocytes	17	54.000	45.800–62.700	20	59.350	53.000–62.875
Tc lymphocytes	17	40.000	34.700–50.600	20	33.200*	27.150–37.425
Th/Tc lymphocytes	17	1.301	0.923–1.818	20	1.844*	1.429–2.232
CD4–CD8– T lymphocytes	17	4.200	3.120–6.800	20	7.685**	5.905–9.140
HLA-DR+ T lymphocytes	17	16.400	10.400–22.900	20	13.250	8.188–22.100
Th lymphocytes						
Th1-like lymphocytes	7	21.300	10.095–23.850	20	14.900	12.925–17.375
Th2-like lymphocytes	7	5.290	2.300–26.000	20	3.235	2.745–4.253
Th1/Th2-like lymphocytes	7	1.546	0.841–6.619	20	5.251	3.200–5.975
Th1Th17-like lymphocytes	7	6.920	4.520–8.905	20	8.540	7.108–11.600
Th17-like lymphocytes	7	5.060	3.885–6.060	20	3.960	2.538–5.100
aTreg lymphocytes	11	2.180	1.280–2.650	20	1.860	1.695–2.483
rTreg lymphocytes	11	1.710	0.930–2.930	20	1.240	1.025–2.140
Treg lymphocytes	11	4.210	2.850–5.560	20	3.685	2.981–4.350
non-Treg lymphocytes	11	4.760	4.370–6.720	20	2.835***	2.265–3.385
FOXP3+ Th lymphocytes	11	8.060	6.440–12.490	20	6.250*	5.724–7.255
Tcon lymphocytes	11	90.040	84.745–92.140	20	94.430****	94.118–94.668
Treg/Tcon lymphocytes	11	0.040	0.026–0.059	20	0.039	0.031–0.047
FOXP3+ Th lymphocytes						
CD25– lymphocytes	11	36.900	31.950–44.250	20	14.900***	13.775–23.025
aTreg lymphocytes	11	20.350	16.692–22.094	20	32.796**	25.258–36.362
rTreg lymphocytes	11	23.932	15.123–28.478	20	20.839	17.529–31.477
non-Treg lymphocytes	11	56.938	52.566–70.045	20	43.09**	37.758–51.218
Each FOXP3+ Th subset						
CD25– cells in aTreg subset	11	28.200	12.850–34.250	20	9.155***	6.620–13.275
CD25– cells in rTreg subset	11	33.200	14.500–50.600	20	18.300	11.975–42.150
CD25– cells in non-Treg subset	11	51.300	33.600–54.750	20	26.050	23.900–40.125
Tcon lymphocytes						
CD25+ cells	11	18.600	13.150–22.250	20	14.550	11.075–18.525
Analysis of STAT						
STAT1 (ΔMFI)	15	1068.0	657.5–1744.5	19	145.0****	111.0–160.5
pSTAT1 (ΔMFI)	9	220.4	44.0–390.0	6	5.0***	2.5–8.4
STAT5 (ΔMFI)	9	112.0	39.0–209.0	12	13.0***	6.0–19.0
pSTAT5 (%)	14	13.03	7.735–54.86	16	7.854	6.775–16.99
pSTAT5 (ΔMFI)	15	198.0	126.5–339.0	20	176.0	103.5–210.8

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$. cSLE: childhood-onset systemic lupus erythematosus; HC: healthy controls; Tc: cytotoxic T lymphocytes; Treg: regulatory Th; rTreg: resting Treg; aTreg: activated Treg; Tcon: conventional T lymphocytes; non-Treg: CD45RA-FOXP3^{low} activated effector T lymphocytes; pSTAT: phosphorylated STAT; MFI: median fluorescent intensity.

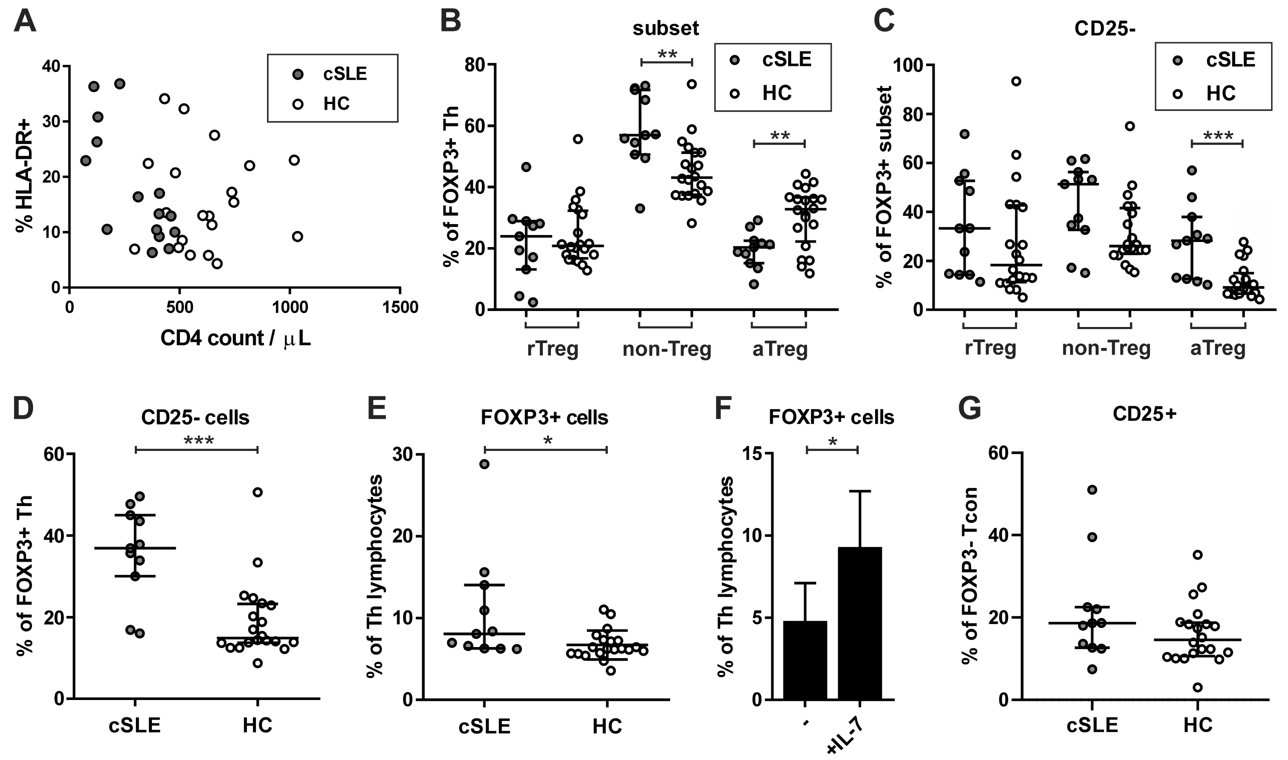

Figure 2. Th subsets difference among groups. A. Correlation between HLA-DR+ T lymphocytes and Th numbers in cSLE ($r_s = -0.643$, $p = 0.0116$) and HC ($r_s = -0.003$, $p = 0.9900$). Each symbol represents a single sample. B. Percentages of rTreg, non-Treg, and aTreg within FoxP3+ Th lymphocytes in cSLE and HC. Each symbol represents a single sample. The graph shows the median values with IQR. C. Percentages of CD25– cells among rTreg, non-Treg, and aTreg subsets in cSLE and HC. Each symbol represents a single sample. The graphs show the median values with IQR. D. Percentages of CD25– cells among all FOXP3+ Th in cSLE and HC. Each symbol represents a single sample. The graphs show the median values with IQR. E. Percentages of FOXP3+ cells among Th in cSLE and HC. Each symbol represents a single sample. The graphs show the median values with IQR. Purified CD4+T cells from healthy donors (n = 7) were cultured for 3 d in the presence or absence of IL-7 (0.1 ng/ml; Supplementary Data 1, available from the authors on request). F. Bar graph (mean with SD) depicts the percentage of FOXP3+ cells among Th for treated (IL-7) and untreated (-) samples. G. Percentages of CD25+ cells among FOXP3– Tcon in cSLE and HC. Each symbol represents a single sample. The graphs show the median values with IQR. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. cSLE: childhood-onset systemic lupus erythematosus; HC: healthy controls; Treg: regulatory Th; rTreg: resting Treg; non-Treg: CD45RA–FOXP3^{low} activated effector T lymphocytes; aTreg: activated Treg; IL: interleukin; IQR: interquartile range; Tcon: activated effector conventional T lymphocytes.

Because data suggest that an acquired insufficiency of IL-2 in SLE accounts for the reduced expression of CD25 in FOXP3+ Treg and the inverse increase in the proportions of the CD25– subset[35], we also analyzed CD25– cells in the rTreg, non-Treg, and aTreg subsets from cSLE and HC. While the highest percentage of CD25– cells was found in the non-Treg from both cSLE and HC, it was significantly increased only in aTreg from cSLE compared to HC (Figure 2C).

Percentages of CD25– cells among all FOXP3+ Th, as well as the percentage of FOXP3+ cells among Th, increased significantly in cSLE compared to HC (Figures 2D–E).

The percentage of FOXP3+ cells among Th was also significantly increased after *in vitro* treatment of purified Th from healthy donors with IL-7 (Figure 2F). Therefore, if diminished *in vivo* availability of IL-2 accounts for the increase in the CD25– subset from SLE, the other homeostatic STAT5 signaling cytokine IL-7 could be responsible for the increase in the frequency of FOXP3+ cells among Th.

In contrast to FOXP3+ Th, the percentage of CD25+ cells among FOXP3– Tcon was higher in cSLE, but the difference was not statistically significant ($p = 0.10$; Figure 2G).

Analysis of cell numbers showed a significant decrease in the number of Tcon, Th1, and Th1Th17-like cells in cSLE ($p < 0.001$, $p < 0.05$, and $p < 0.01$, respectively). Percentages of the latter effector Th populations, Th2, and Th17-like cells were, however, not significantly different compared to HC (Table 3).

Increased expression of STAT1 and homeostatic IL-7–dependent STAT5 activation are present in cSLE. We found significantly increased STAT1 expression in Th from cSLE (Figure 3A).

Because significant increases in Th, but not CD3– lymphocyte levels of basal pSTAT5, were observed previously by our research group in aSLE, the increase in Th pSTAT5 (ΔMFI and %) was also examined in cSLE[36]. Although no statistical differences in basal pSTAT5 between

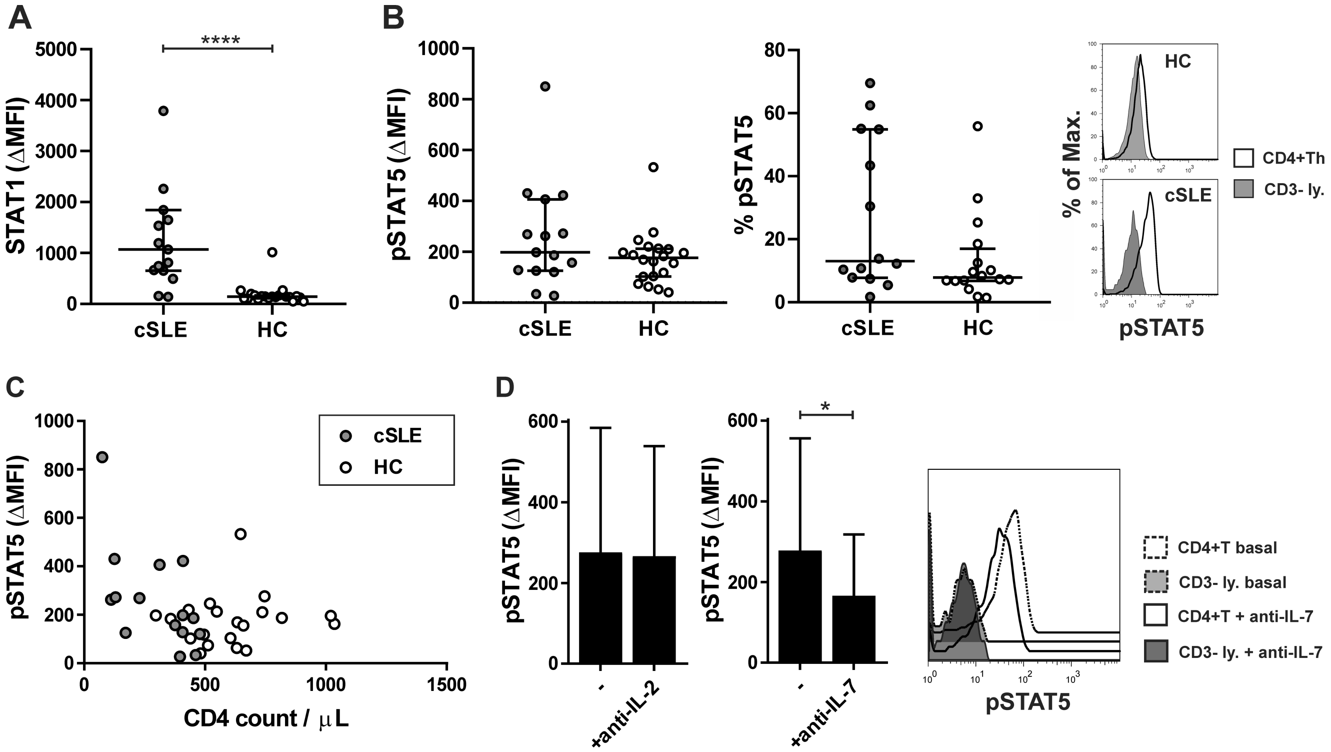

Figure 3. Expression and phosphorylation of STAT1 and STAT5. A. Basal expression STAT1 (ΔMFI) in cSLE and HC. Each symbol represents a single sample. The graphs show the median values with IQR. B. Basal phosphorylation of STAT5 in cSLE and HC (ΔMFI and percentage). Each symbol represents a single sample. The graphs show the median values with IQR. Representative histograms of pSTAT5 levels in CD4+ Th and CD3– lymphocytes from cSLE patient and HC are shown on the right. C. Correlation between basal pSTAT5 and Th numbers in cSLE ($r_s = -0.579$, $p = 0.026$) and HC ($r_s = 0.129$, $p = 0.587$). Each symbol represents a single sample. D. Bar graphs (mean with SD) show the difference in pSTAT5 levels (ΔMFI) between CD4+ Th and CD3– lymphocytes in samples from patients with SLE, incubated with anti–IL-7 and anti-IL-2 antibodies for 30 min as compared to basal/untreated samples (n = 7; Supplementary Data 1, available from the authors on request). Representative histograms of pSTAT5 levels in both subsets of lymphocytes from patient with SLE are shown on the right. STAT protein basal expression and phosphorylation were analyzed in CD3+CD4+ Th lymphocytes. * $p < 0.05$. **** $p < 0.0001$. cSLE: childhood-onset systemic lupus erythematosus; HC: healthy controls; pSTAT: phosphorylated STAT; MFI: median fluorescent intensity; IL: interleukin; IQR: interquartile range.

groups were found (Figure 3B), higher pSTAT5 ΔMFI was significantly correlated to lower CD4 counts in cSLE, but not HC (Figure 3C).

Finally, the increase in pSTAT5 levels in Th was dependent on homeostatic cytokine IL-7, because incubation of whole-blood samples from SLE with neutralizing anti–IL-7, but not anti–IL-2 antibodies, resulted in significant reduction of pSTAT5 ΔMFI values (Figure 3D).

Concentrations of plasma cytokines do not differ significantly between the groups. A comparison of the number of cytokines IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ, and MCP-1 in plasma samples found no statistically significant differences between groups (Supplementary Table 1, available from the authors on request). There was also no statistically significant correlation between SLEDAI-2K of patients at the time of the study and any of the analyzed laboratory markers.

DISCUSSION

A more aggressive clinical course usually occurs with cSLE compared to aSLE, but pathogenetic reasons for this difference are not entirely understood. Our previous studies have identified significant disruption of immune cell homeostasis and changes of cytokine signaling in aSLE[36,37]. In this study, we determined subpopulations of Treg and Teff, cytokine profile, and basal STAT1/STAT5 expression and phosphorylation in a single-center cohort of cSLE.

Lymphopenia is frequent in all SLE, but more likely to appear in cSLE, with T lymphocytes, especially Th, more affected than B lymphocytes[28,32]. Our results showed the same trend of pronounced lymphopenia in cSLE, including decreased numbers of T lymphocytes, Th, and even the CD4–DC8– T lymphocytes subpopulation (Figure 1A), which was shown to be expanded in aSLE and to produce IL-17 and IFN-γ *in vivo*[4]. In addition, percentages of CD4–DC8– T lymphocytes, but not Th among T lymphocytes, were significantly decreased in cSLE compared to HC (Figure 1B).

Lymphopenia with associated compensatory homeostatic proliferation in response to IL-7 can release autoreactive Th from inhibitory networks[38]. T lymphocyte recovery was driven by homeostatic proliferation also in patients with multiple sclerosis treated with the lymphocyte-depleting monoclonal antibody alemtuzumab, and their T lymphocytes showed evidence of chronic activation[33]. Consistent with that, we found significant correlation between decreased Th numbers in cSLE and expression of the activation marker HLA-DR (Figure 2A). However, in contrast to our findings on cSLE and aSLE (previous study[36,37]), increased aTreg were reported after alemtuzumab therapy[33].

The Treg population should ideally be defined in a way that reliably excludes effector cells, and FOXP3+CD127^{low} cells are advocated to accurately identify real Treg[39]. However, stimulation of purified Th with IL-7 significantly decreased the difference in CD127 (IL-7R) expression between FOXP3+ and FOXP3– subsets, while it increased percentage of FOXP3+ cells among Th. Downregulation of CD127 is therefore not entirely specific for Treg either (Supplementary Figures 1–2, available from the authors on request). We performed identification and analysis of Treg by using antibodies against CD4, CD25, FOXP3, and CD45RA antigens (Table 2) and used activation status of the FOXP3+ cells — expression of CD45RA — as the distinguishing marker for Treg[34]. Increased percentages of FOXP3+ Th were found in patients with cSLE (Figure 2E), but were not reflected in increased percentages of both Treg subsets: aTreg, which actively perform a suppressive function, and rTreg, which upon activation differentiate in aTreg. However, the non-Treg subset was significantly increased among Th from cSLE (Figure 2B). The same subset, which we found to be increased after stimulation with IL-7 *in vitro* before, was increased also in our aSLE[36,40].

Systemic reduction of IL-2 levels in the early stages of the disease promoted Tcon hyperactivity and accelerated disease progression in a mouse model, highlighting the importance of the Treg-IL-2 axis[41]. As shown by von Spee-Mayer, *et al*, in the aSLE population, deficiency of IL-2 can lead to a disease activity–related decrease in the expression of CD25 on Treg[35]. In this case, distinction between cells may be difficult; they are in reality just “exhausted” Treg owing to IL-2 deprivation, and FOXP3-expressing “false” Treg, which actively excrete IFN-γ and IL-17. Indeed, in cSLE, CD25– cells were enriched in non-Treg and were significantly increased among FOXP3+ Th from cSLE compared to HC (Figure 2C). IL-2 levels in plasma from cSLE patients were, in our study, not decreased compared to HC (Supplementary Table 1, available from the authors on request). However, because of the short half-life of IL-2, measurement of plasma IL-2 is probably not an adequate method to detect shortage of IL-2 *in vivo*[35]. It has recently been suggested that in lymphoid tissues FOXP3 expression is maintained in Treg by STAT5-signaling cytokines, such as IL-2 and IL-7. These signals are lost during recirculation in the bloodstream, resulting in decay of FOXP3 in many Treg cells[42]. Therefore, increased levels of IL-7, which were described previously in our aSLE study and were associated with Th depletion[36], may be responsible for the increase in FOXP3+ cells (Figure 2F) and the non-Treg subset among Th. However, IL-7–dependent basal pSTAT5 levels were in our aSLE study[36] not significantly increased in the most suppressive aTreg subset[34], which was relatively decreased in the pool of FOXP3+ Th from our patients with cSLE (Figure 2B). In addition, the percentage of CD25– cells, possibly also reflecting *in vivo* IL-2 deficiency, was significantly increased in the aTreg from cSLE (Figure 2C). As for FOXP3, CD25 expression is also regulated by the STAT5[43]. An increased Tcon/aTreg pSTAT5 ratio, found previously in aSLE[36], could explain a higher percentage of CD25– cells among aTreg and CD25+ cells among Tcon also in cSLE (Figure 2C and Figure 2G).

Our previous study revealed a significant increase of CD25–FOXP3+ Th in healthy children, compared to healthy adolescents and adults, suggesting that occurrence of this subpopulation could be influenced by the immaturity of the immune system[30]. The same could be true for the rTreg subset, which showed (in contrast to our previous findings on aSLE[36,37]) decreased numbers and did not significantly increase among FOXP3+ Th in cSLE compared to HC (Figure 2C).

To our knowledge, numbers and percentages of peripheral blood circulating Th17 were previously not directly investigated in cSLE, but a comparison of numbers of plasma cytokines, associated with Th1, Th2, and Th17, showed strong evidence of crucial involvement of IL-17 in aSLE pathogenesis[11,12,13,44]. Unlike in aSLE, we found no significant differences in percentages of Th17-like cells in cSLE compared to HC (Table 3). However, our analysis of plasma cytokine concentrations indirectly indicated onset of functional changes in these cells. Despite the significant decrease in numbers of Th1 and Th1Th17-like cells and CD4–DC8– T lymphocytes in cSLE (Table 3), the plasma concentrations of cytokines secreted by these cells did not differ from those in the HC (Supplementary Table 1, available from the authors on request). The cSLE cells were, therefore, probably hyperactive in their response to the stimulus. Lower numbers of Teff subsets could also be a consequence of their recruitment to the site of inflammation, out of the periphery. The low SLEDAI-2K score (Table 1) of cSLE patients without extensive actively inflamed sites at the time of the study and normal plasma cytokine levels, however, make this theory less probable. Our previous study of the age-dependent dynamics of Th subpopulations in healthy subjects suggests that the numbers of Teff are likely to increase in adulthood, owing to the aging process itself[30].

On the other hand, STAT1 and STAT5 have both been shown to be capable of suppressing Th17 responses[26,45]. Consistent with results of our study on aSLE[37], an increased expression of STAT1 (Figure 3A) and homeostatic IL-7–dependent STAT5 activation in Th (Figure 3C–3D) were also found in cSLE despite the low disease activity scores of the patients at the time of the study (Table 1), which could be the reason for the absence of expected Th17 expansion. Th depletion was in cSLE associated with higher pSTAT5 levels (Figure 3C), which were shown to confer a worse prognosis in aSLE[36]. The increased levels of STAT1 expression do not necessarily reflect rapid changes in the clinical activity of the disease, but probably demonstrate an "interferon signature," and may be associated with increased sensitivity to the new inflammatory signals[20,46,47].

Our previous study on aSLE showed that among Th, aTreg are the most sensitive to IFN-α stimulation. They exhibited the highest IFN-α–induced pSTAT1 response combined with decreased proliferation assessed by Ki-67 expression[37]. IFN-γ, which signals mainly through STAT1, was not increased in plasma from cSLE (Supplementary Table 1, available from the authors on request), but the concentration of IFN-α was not measured in our study. Elevated levels of IFN-α, characteristic for SLE, could inhibit the proliferation of aTreg in cSLE. This could assist with a break of peripheral tolerance and lead to further increase in STAT1 expression and maintenance of an inflammatory condition. Further study of cSLE should perform detailed analysis of STAT1/STAT5 expression and phosphorylation in different subpopulations of FOXP3– Tcon and FOXP3+ Th.

According to our results, decreased numbers of the rTreg subset and lack of significant expansion of Teff subsets compared to HC could, therefore, be interpreted as the key difference in Treg/Teff homeostasis between cSLE and aSLE, while perturbed aTreg homeostasis, higher expression of STAT1 and homeostatic IL-7–dependent basal STAT5 activation, associated with Th depletion, appear to be present in both the childhood- and adult-onset disease.

REFERENCES

1. Carter EE, Barr SG, Clarke AE. The global burden of SLE: prevalence, health disparities and socioeconomic impact. Nat Rev Rheumatol 2016;12:605–20.
2. Mok CC, Lau CS. Pathogenesis of systemic lupus erythematosus. J Clin Pathol 2003;56:481–90.
3. Tenbrock K, Juang YT, Kyttaris VC, Tsokos GC. Altered signal transduction in SLE T cells. Rheumatology 2007;46:1525–30.
4. Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, et al. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. J Immunol 2008;181:8761–6.
5. Purnama C, Camous X, Larbi A. An overview of T cell subsets and their potential use as markers of immunological ageing. Int Trends Immun 2013;1:21–32.
6. Crome SQ, Clive B, Wang AY, Kang CY, Chow V, Yu J, et al. Inflammatory effects of ex vivo human Th17 cells are suppressed by regulatory T cells. J Immunol 2010;185:3199–208.
7. Gonzalez Y, Herrera MT, Juárez E, Salazar-Lezama MA, Bobadilla K, Torres M. CD161 expression defines a Th1/Th17 polyfunctional subset of resident memory T lymphocytes in bronchoalveolar cells. PLoS One 2015;10:e0123591.
8. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol 2010;11:7–13.
9. Bonelli M, Savitskaya A, Steiner CW, Rath E, Smolen JS, Scheinecker C. Phenotypic and functional analysis of CD4+CD25-Foxp3+ T cells in patients with systemic lupus erythematosus. J Immunol 2009;182:1689–95.
10. Lieberman LA, Tsokos GC. The IL-2 defect in systemic lupus erythematosus disease has an expansive effect on host immunity. J Biomed Biotechnol 2010;2010:740619.
11. Xing Q, Wang B, Su H, Cui J, Li J. Elevated Th17 cells are accompanied by FoxP3+ Treg cells decrease in patients with lupus nephritis. Rheumatol Int 2012;32:949–58.
12. Martin JC, Baeten DL, Josien R. Emerging role of IL-17 and Th17 cells in systemic lupus erythematosus. Clin Immunol 2014; 154:1–12.
13. Talaat RM, Mohamed SF, Bassyouni IH, Raouf AA. Cytokine Th1/Th2/Th17/Treg cytokine imbalance in systemic lupus erythematosus (SLE) patients: correlation with disease activity. Cytokine 2015;72:146–53.
14. Liongue C, Ward AC. Evolution of the JAK-STAT pathway. JAKSTAT 2013;2:e22756.
15. Villarino AV, Kanno Y, Ferdinand JR, O'Shea JJ. Mechanisms of Jak/STAT signaling in immunity and disease. J Immunol 2015;194:21–7.
16. Böhmer FD, Friedrich K. Protein tyrosine phosphatases as wardens of STAT signaling. JAKSTAT 2014;3:e28087.
17. Miklossy G, Hilliard TS, Turkson J. Therapeutic modulators of STAT signalling for human diseases. Nat Rev Drug Discov 2013;12:611–29.
18. Dong J, Wang QX, Zhou CY, Ma XF, Zhang YC. Activation of the STAT1 signalling pathway in lupus nephritis in MRL/lpr mice. Lupus 2007;16:101–9.
19. Huang X, Guo Y, Bao C, Shen N. Multidimensional single cell based STAT phosphorylation profiling identifies a novel biosignature for evaluation of systemic lupus erythematosus activity. PLoS One 2011;6:e21671.
20. Hale MB, Krutzik PO, Samra SS, Crane JM, Nolan GP. Stage dependent aberrant regulation of cytokine-STAT signaling in murine systemic lupus erythematosus. PLoS One 2009;4:e6756.
21. Karonitsch T, Feierl E, Steiner CW, Dalwigk K, Korb A, Binder N, et al. Activation of the interferon-gamma signaling pathway in systemic lupus erythematosus peripheral blood mononuclear cells. Arthritis Rheum 2009;60:1463–71.
22. Lu MC, Lai NS, Chen HC, Yu HC, Huang KY, Tung CH, et al. Decreased microRNA(miR)-145 and increased miR-224 expression in T cells from patients with systemic lupus erythematosus involved in lupus immunopathogenesis. Clin Exp Immunol 2013;171:91–9.
23. Zheng SG, Wang J, Wang P, Gray JD, Horwitz DA. IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. J Immunol 2007;178:2018–27.
24. Passerini L, Allan SE, Battaglia M, Di Nunzio S, Alstad AN, Levings MK, et al. STAT5-signaling cytokines regulate the expression of FOXP3 in CD4+CD25+ regulatory T cells and CD4+CD25- effector T cells. Int Immunol 2008;20:421–31.
25. Yang XP, Ghoreschi K, Steward-Tharp SM, Rodriguez-Canales J, Zhu J, Grainger JR, et al. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. Nat Immunol 2011;12:247–54.
26. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity 2007;26:371–81.
27. Amador-Patarroyo MJ, Rodriguez-Rodriguez A, Montoya-Ortiz G. How does age at onset influence the outcome of autoimmune diseases? Autoimmune Dis 2012; 2012:251730.
28. Aggarwal A, Srivastava P. Childhood onset systemic lupus erythematosus: how is it different from adult SLE? Int J Rheum Dis 2015;18:182-91.
29. Gladman DD, Ibañez D, Urowitz MB. Systemic lupus

erythematosus disease activity index 2000. J Rheumatol 2002;29:288–91.

30. Holcar M, Goropevšek A, Ihan A, Avčin T. Age-related differences in percentages of regulatory and effector T lymphocytes and their subsets in healthy individuals and characteristic STAT1/STAT5 signalling response in helper T lymphocytes. J Immunol Res 2015;2015:352934.
31. Ohl K, Tenbrock K. Inflammatory cytokines in systemic lupus erythematosus. J Biomed Biotechnol 2011;2011:432595.
32. Faddah S, Elwakd M, Aboelenein A, Hussein M. Lymphopenia and systemic lupus erythematosus, a preliminary study: Correlation with clinical manifestations, disease activity and damage indices. Egypt Rheumatol 2014;36:125–30.
33. Jones JL, Thompson SA, Loh P, Davies JL, Tuohy OC, Curry AJ, et al. Human autoimmunity after lymphocyte depletion is caused by homeostatic T-cell proliferation. Proc Natl Acad Sci 2013;110:20200–5.
34. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity 2009;30:899–911.
35. von Spee-Mayer C, Siegert E, Abdirama D, Rose A, Klaus A, Alexander T, et al. Low-dose interleukin-2 selectively corrects regulatory T cell defects in patients with systemic lupus erythematosus. Ann Rheum Dis 2016;75:1407–15.
36. Goropevšek A, Gorenjak M, Gradišnik S, Dai K, Holc I, Hojs R, et al. STAT5 phosphorylation in CD4 T cells from patients with SLE is related to changes in their subsets and follow-up disease severity. J Leukoc Biol 2017;101:1405-18.
37. Goropevšek A, Gorenjak M, Gradišnik S, Dai K, Holc I, Hojs R, et al. Increased levels of STAT1 protein in blood CD4 T cells from systemic lupus erythematosus patients are associated with perturbed homeostasis of activated CD45RA–FOXP3^{hi} regulatory subset and follow-up disease severity. J Interf Cytokine Res 2017;37:254–68.
38. Monti P, Scirpoli M, Maffi P, Ghidoli N, De Taddeo F, Bertuzzi F, et al. Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory T cells. J Clin Invest 2008;118:1806–14.
39. Hartigan-O'Connor DJ, Poon C, Sinclair E, McCune JM. Human CD4+ regulatory T cells express lower levels of the IL-7 receptor alpha chain (CD127), allowing consistent identification and sorting of live cells. J Immunol Methods 2007;319:41–52.
40. Goropevšek A, Holcar M, Pahor A, Avčin T. STAT signaling as a marker of SLE disease severity and implications for clinical therapy. Autoimmun Rev 2019;18:144–54.
41. Humrich JY, Morbach H, Undeutsch R, Enghard P, Rosenberger S, Weigert O, et al. Homeostatic imbalance of regulatory and effector T cells due to IL-2 deprivation amplifies murine lupus. Proc Natl Acad Sci U S A 2010;107:204–9.
42. Tabares P, Berr S, Langenhorst D, Sawitzki B, ten Berge I, Tony HP, et al. Short-term cytokine stimulation reveals regulatory T cells with down-regulated Foxp3 expression in human peripheral blood. Eur J Immunol 2018;48:366–79.
43. Mahmud SA, Manlove LS, Farrar MA. Interleukin-2 and STAT5 in regulatory T cell development and function. JAKSTAT 2013;2:e23154.
44. Ballantine LE, Ong J, Midgley A, Watson L, Flanagan BF, Beresford MW. The pro-inflammatory potential of T cells in juvenile-onset systemic lupus erythematosus. Pediatr Rheumatol Online J 2014;12:4.
45. Villarino AV, Gallo E, Abbas AK. STAT1-activating cytokines limit Th17 responses through both T-bet-dependent and -independent mechanisms. J Immunol 2010;185:6461–71.
46. Hu X, Ivashkiv LB. Cross-regulation of signaling and immune responses by IFN-γ and STAT1. Immunity 2009;31:539–50.
47. Landolt-Marticorena C, Bonventi G, Lubovich A, Ferguson C, Unnithan T, Su J, et al. Lack of association between the interferon-α signature and longitudinal changes in disease activity in systemic lupus erythematosus. Ann Rheum Dis 2009;68:1440–6.