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Altered homeostasis of regulatory T lymphocytes and differential regulation of STAT1 and STAT5 in CD4+ T lymphocytes in childhood-onset SLE

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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 analysed T helper lymphocyte (Th) subsets, STAT1/STAT5 signalling 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 cSLE patients 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 naïve-resting Treg (rTreg) subset compared to HC were found. The percentages of CD25$^-$cells, possibly reflecting IL-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, was 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 homestasis, increased levels of STAT1 protein, and homeostatic STAT5 signalling appear to be intrinsic characteristics of the disease, present in cSLE and aSLE alike.

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Introduction

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 (DNT) is higher in SLE patients than in healthy controls (HC) and can induce auto-reactive B lymphocytes to produce anti-dsDNA antibodies and higher numbers of inflammatory cytokines[4]. Changes are also noticeable in individual subpopulations of $CD4^{+}$helper T lymphocytes (Th). It is suspected that disruption of the homeostasis between the effector Th (Teffs) and the regulatory Th (Tregs) can lead to various inflammatory and autoimmune diseases, including SLE[5].

According to the major transcription factors and cytokines needed for their differentiation, as well as the characteristic cytokines secreted by the activated cells, Teffs can be divided into Th1, Th2, Th17, and others[6]. Despite the considerable functional specialization of subtypes, there is some plasticity between different Teffs, which sometimes allows multifunctional subpopulations to arise. An example of the latter are Th1/Th17, which secrete interferon-gamma (IFN-γ), characteristic for Th1, and highly inflammatory IL-17, characteristic for Th17[7].

Tregs control the immune response by direct inhibition of Teffs, but also by reducing the antigen presentation potential of dendritic cells and altering their release of cytokines. The depletion of Tregs can thus contribute to the onset and maintenance of autoimmune diseases in two distinct ways. The removal of cells with a suppressor function can lead to unrestrained polyclonal activation of Teffs; 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 Tregs is usually reduced in SLE; moreover, an elevated number of FOXP3-

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expressing cells, which are functionally not suppressive, has been found[9,10]. Many recent studies have focused also on IL-17, expansion of Th17, and the Tregs:Th17 dynamic in the pathogenesis of SLE[11–13].

Proper functioning of Th depends on their successful signal transduction. Cytokine receptors receive signals of different extracellular molecules and trigger signal transduction through the JAK-STAT pathway[14]. Canonically it begins with three consecutive tyrosine phosphorylations, last being phosphorylation of STAT proteins. The phosphorylation rate of STATs can, therefore, offer relevant insight into the activation status of the cell. Aberrant cytokine signalling 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 IFNs. 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 lupus 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 gamma-chain cytokines (such as IL-2), and induces expression of FOXP3, a transcription factor crucial for the maturation of Tregs[23]. STAT5-dependant 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 Tregs and is thus likely to be involved in the

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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 long-term 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 and other organ systems[28].

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

MATERIALS AND METHODS

Study subjects

Seventeen cSLE patients with a median age of 18.0 years at enrolment 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 SLEDAI-2K scoring system[29]. As a control group, we included twenty healthy adolescents (HC) with a median age of 16.0 years and no history of allergies, acute infections, autoimmune disorders or medications that

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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 their 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 one aliquot and stored for later cytokine analysis. The remaining aliquots were prepared following three different protocols. Standard whole-blood staining methodology with premixed multicolour 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 (PhospflowLyse/Fix Buffer and Perm bufferIII). Staining of surface and intracellular antigens was in this case done simultaneously (Tube 5, Table 2). Tregs were detected after primary staining of surface antigens in whole blood, with subsequent fixation and permeabilisation 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, Aachen, Germany), anti-CXCR3-APC (BioLegend Inc., San Diego, USA) and anti-CD161-FITC (eBioscience Inc., San Diego, USA), were acquired from BDBiosciences, San Jose, USA. Cells were analysed with the FACSCantoII™ Flow Cytometer, equipped with blue and

red lasers, running FACSDiva software (both BDBioscience, San Jose, USA). Digital data were analysed using FlowJo software (Tree Star Inc., Ashland, USA).

Flow cytometric analysis of lymphocyte subpopulations

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Percentages of Th, Tc, DNT and HLA-DR$^+$T lymphocytes were analysed after gating on CD3$^+$CD45$^+$lymphocytes. Absolute cell counts were calculated using a dual-platform approach with panleukogating after measuring leukocytes on the BeckmanCoulterAcT8 Hematology Analyser (BDBiosciences, San Jose, USA). Analysis of all lymphocyte subpopulations 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 (BDBiosciences, San Jose, USA) to set the forward, side scatter and PMT 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 analysed in a single experiment. Cytokine concentrations were analysed 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, TNF-α, IFN-γ, MCP1) in a single sample at a single time point (Cytokine&Growth Factors Array I) with the Evidence Investigator immunoanalyser (both Randox, Crumlin, UK), according to the manufacturer's instructions.

Statistical analysis

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The Mann-Whitney test was used to test differences between two groups and the Kruskal-Wallis between three groups. For within-group comparisons the Wilcoxon matched-pairs signed rank test was used. Correlations between experimental results were examined using Spearman's 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., La Jolla, USA).

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] Significant lymphopenia was found in our patients with cSLE ($p<0.0001$) compared to HC as well. The number of analysed subsets including T lymphocytes, DNT, Th and Tc was significantly lower in cSLE (first three 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 DNT 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

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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).

As 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 (aTregs) cells, CD45RA^{+}FOXP3^{low} naïve-resting Tregs (rTregs), and the CD45RA^{-}FOXP3^{low} activated effector Tcon (abbreviated as non-Treg, even though they represent only a small and distinct subset of the non-Treg/Tcon fraction) subset among FOXP3-expressing cells.

In line with lymphopenia, numbers of aTregs and rTregs 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 Tregs (aTreg+rTreg) in the cSLE ($p<0.01$). However, in contrast to the absolute numbers, while percentages of aTregs were significantly decreased among FOXP3^{+}Th from cSLE and no significant difference was found in the rTregs, the percentage of non-Tregs was significantly increased in cSLE ($p<0.001$) compared to HC (Figure 2B).

As the recent 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-Tregs from both cSLE and HC, it was significantly increased only in aTregs 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 and 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

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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$^-$conventional T lymphocytes (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).

As 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 (Δ MFI and %) was also examined in cSLE[36]. Although no statistical differences in basal pSTAT5 between 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, as incubation of whole blood samples from SLE with neutralizing anti-IL7, but not anti-IL-2 antibodies, resulted in significant reduction of pSTAT5 ΔMFI values (Figure 3D).

Concentrations of plasma cytokines does not differ significantly between the groups

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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 MCP1 in plasma samples found no statistically significant differences between groups. (Suppl. Table). There was also no statistically significant correlation between SLEDAI-2K of patients at the time of the study and any of the analysed laboratory markers.

DISCUSSION

cSLE usually has a more aggressive clinical course than 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 signalling in aSLE[36,37]. In this study, we determined subpopulations of Tregs and Teffs, cytokine profile and basal STAT1/STAT5 expression and phosphorylation in a single centre 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 DNT 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 DNT, 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 aTregs were reported after alemtuzumab therapy[33].

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The Treg population should ideally be defined in a way to reliably exclude effector cells and $FOXP3^+CD127^{low}$cells are advocated to accurately identify real Tregs.[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. Down-regulation of CD127 is therefore not entirely specific for Tregs either (Suppl. Fig.). We performed identification and analysis of Tregs by using antibodies against CD4, CD25, FOXP3, and CD45RA antigens (Table 2) and used activation status of the $FOXP3^+$ cells – expression of CD45RA – as distinguishing marker for Tregs[34]. Increased percentages of $FOXP3^+$Th were found in cSLE patients (Figure 2E), but were not reflected in increased percentages of both Treg subsets: aTregs, which actively perform a suppressive function, and rTregs, which upon activation differentiate in aTregs. 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 disease-activity-related decrease in the expression of CD25 on Tregs[35]. In this case, distinction between cells, which are in reality just "exhausted" Tregs due to IL-2 deprivation, and FOXP3-expressing "false" Tregs, which actively excrete IFN-γ and IL-17, may be difficult. Indeed, in cSLE, $CD25^-$cells were enriched in non-Tregs 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 (Suppl. Table), however due to 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

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maintained in Tregs 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 cSLE patients (Figure 2B). In addition, the percentage of $CD25^-$cells, possibly also reflecting *in vivo* IL-2 deficiency, was significantly increased in the aTregs from cSLE (Figure 2C). As 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 G).

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[11–13,44]. 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

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cells and DNT in cSLE (Table 3), the plasma concentrations of cytokines secreted by these cells did not differ from the HC (Suppl. Table). 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–dependant dynamics of Th subpopulations in healthy subjects suggests that the numbers of Teffs are likely to increase in adulthood due 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,D) 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, aTregs 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 (Suppl. Table), 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 aTregs in cSLE. This could assist with a break of peripheral

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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.

In conclusion, 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 both in the childhood- and adult-onset disease.

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Figure 1: Differences in numbers and percentages of basic subpopulations of T lymphocytes between patients and healthy controls.

Numbers of basic lymphocyte subpopulations in cSLE and HC (A). Percentages of same basic lymphocyte subpopulations in cSLE and HC (B). 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

Figure 2: Th subsets difference among groups.

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 (A). Percentages of rTreg, non-Treg and aTreg within FoxP3$^+$Th lymphocytes in cSLE and HC. Each symbol represents a single sample. The graphs show the median values with interquartile range (B). 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 interquartile range (C). 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 interquartile range (D). Percentages of FOXP3$^+$cells among Th in cSLE and HC. Each symbol represents a single sample. The graphs show the median values with interquartile range (E). Purified CD4$^+$T cells from healthy donors (n=7) were cultured for 3 days in the presence or absence of IL-7 (0.1 ng/mL). Bar graph (mean with SD) depicts the percentage of FOXP3$^+$cells among Th for treated (IL-7) and untreated (−) samples (F). Percentages of CD25$^+$cells among FOXP3$^-$Tcon in cSLE and HC. Each symbol represents a single sample. The graphs show the median values with interquartile range (G). *: p<0.05; **: p<0.01; ***: p<0.001; cSLE: Childhood-onset Systemic Lupus Erythematosus; HC: Healthy Controls

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Figure 3: Expression and phosphorylation of STAT1 and STAT5

Basal expression STAT1 (ΔMFI) in cSLE and HC. Each symbol represents a single sample. The graphs show the median values with interquartile range (A). Basal phosphorylation of STAT5 in cSLE and HC (ΔMFI and percentage). Each symbol represents a single sample. The graphs show the median values with interquartile range. Representative histograms of pSTAT5 levels in $CD4^{+}$Th and $CD3^{-}$lymphocytes from cSLE patient and HC are shown on the right (B). 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 (C). Bar graphs (mean with SD) show the difference in pSTAT5 levels (ΔMFI) between $CD4^{+}$Th and $CD3^{-}$lymphocytes in samples from SLE patients, incubated with anti-IL-7 and anti-IL-2 antibodies for 30 minutes as compared to basal − untreated samples (n=7). Representative histograms of pSTAT5 levels in both subsets of lymphocytes from patient with SLE are shown on the right (D). STAT protein basal expression and phosphorylation were analysed in $CD3^{+}CD4^{+}$Th lymphocytes *: p<0.05; ****: p<0.0001; cSLE: Childhood-onset Systemic Lupus Erythematosus; HC: Healthy Controls; STAT: Signal Transducer and Activator of Transcription; pSTAT: Phosphorylated Signal Transducer and Activator of Transcription; MFI: Median Fluorescent Intensity

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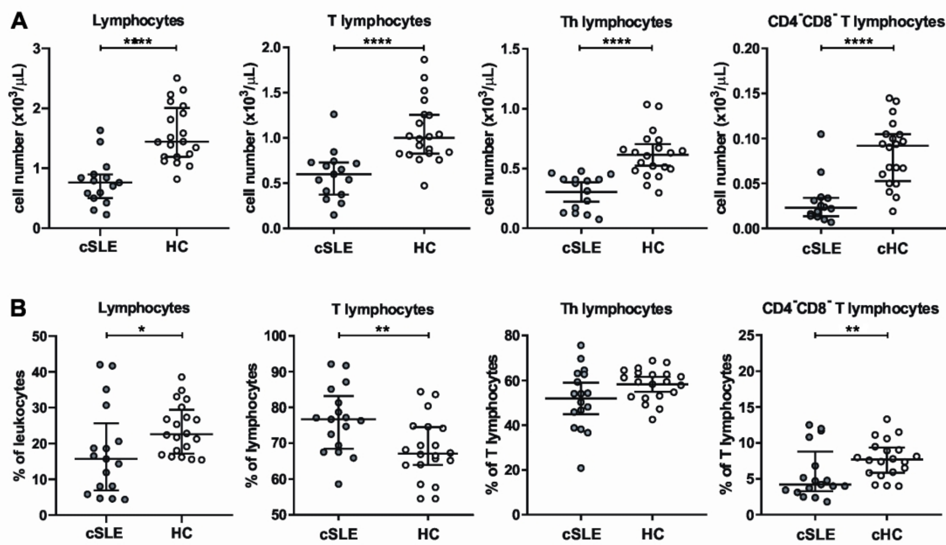

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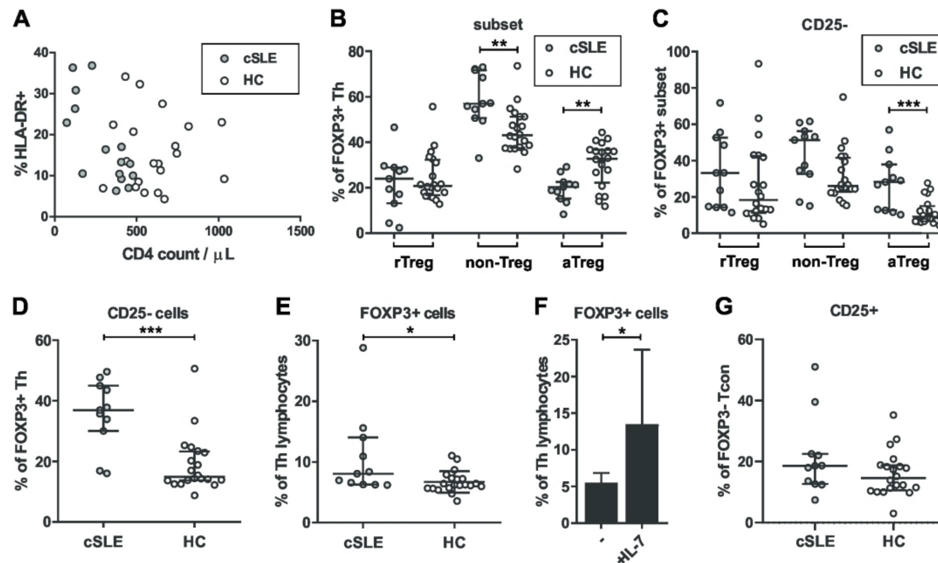

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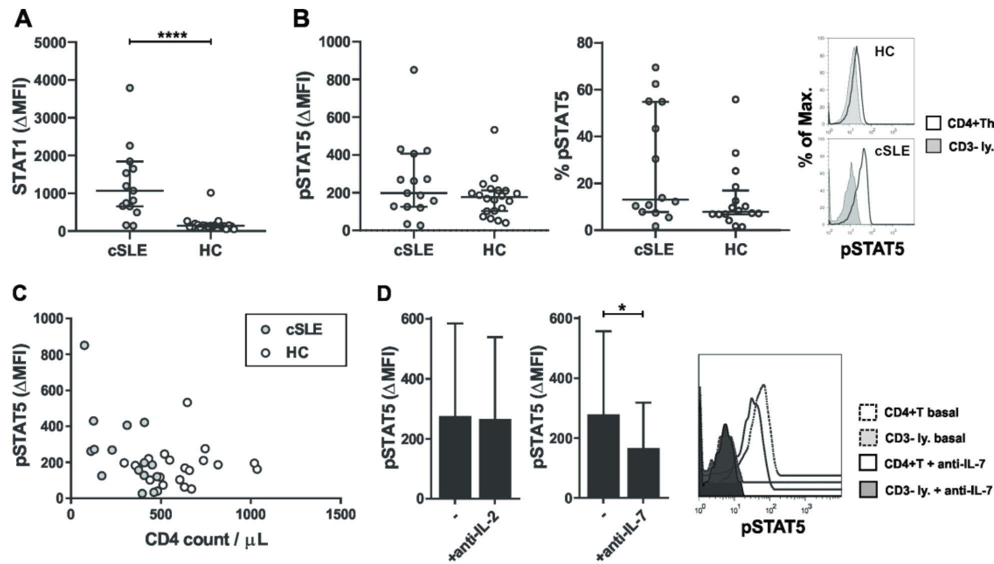

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Table 1: Study cohorts age, therapy, clinical and laboratory data

HC (n=20)	
Median age	Median (min-max)
at study entrance	16.0 years (15.5-20.9)
cSLE (n=17)	
Median age	Median (min-max)
at diagnosis	15.5 years (6.8-17.2)
at study entrance	18.0 years (8.8-21.2)
Therapy	No. of patients (percentage)
Antimalarials	12 (71%)
Corticosteroids	10 (59%)
Immunosuppressives	7 (41%)
Hypertension drugs	4 (24%)
Nonsteroidal anti-inflammatory drugs	2 (12%)
Anticoagulants	2 (12%)
Antipsychotics	1 (6%)
Anti-epileptic drugs	1 (6%)
Biologicals	/
Without therapy	2 (12%)
Laboratory and clinical signs	No. of patients (percentage)
Low complement	8 (47%)
reduced activation of classic pathway	6 (35%)
low concentration of C3	5 (29%)
low concentration of C4	3 (18%)
Anti-DNA 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%)
Median disease activity	Median (min-max) No. of patients (percentage)
SLEDAI-2K	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.

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

Lymphocyte subpopulation	Antybody combination
T lymphocytes	$CD3^{+}CD45^{+}$
Th lymphocytes	$CD3^{+}CD4^{+}CD45^{+}$
Tc lymphocytes	$CD3^{+}CD8^{+}CD45^{+}$
$CD4^{-}CD8^{-}$ T lymphocytes	$CD3^{+}CD4^{-}CD8^{-}CD45^{+}$

Tube 1: anti-CD3-FITC/anti-CD8-PE/anti-CD45-PerCP/anti-CD4-APC

Lymphocyte subpopulation	Antybody combination
$HLA\text{-}DR^{+}$ T lymphocytes	$CD3^{+}CD45^{+}HLA\text{-}DR^{+}$

Tube 2: anti-CD45-FITC/anti-CD3-PE/anti-HLA-DR-APC

Lymphocyte subpopulation	Antybody combination
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 3: anti-CD161-FITC/anti-CCR6-PE/anti-CD4-PerCP/anti-CXCR3-APC/anti-CCR4-Pe-Cy™7

Lymphocyte subpopulation	Antybody combination
aTreg lymphocytes	$CD4^{+}CD45RA^{-}FOXP3^{hi}$
rTreg lymphocytes	$CD4^{+}CD45RA^{+}FOXP3^{lo}$
Treg lymphocytes	$CD4^{+}CD45RA^{-}FOXP3^{hi}$, $CD4^{+}CD45RA^{+}FOXP3^{lo}$
non-Treg lymphocytes	$CD4^{+}CD45RA^{-}FOXP3^{lo}$
$FOXP3^{+}$ Th lymphocytes	$CD4^{+}CD45RA^{-}FOXP3^{hi}$, $CD4^{+}CD45RA^{+}FOXP3^{lo}$, $CD4^{+}CD45RA^{-}FOXP3^{lo}$
Tcon lymphocytes	$CD4^{+}FOXP3^{-}$

Tube 4: anti-CD25-PE/anti-CD45RA-APC/anti-CD4-PE-Cy™7 + anti-FOXP3-Alexa Fluor® 488

Lymphocyte subpopulation	Antybody combination
STAT1	$CD3^{+}CD4^{+}STAT1^{+}$
pSTAT5A	$CD3^{+}CD4^{+}STAT5A(pTyr694)^{+}$

Tube 5: anti-CD3-FITC/anti-STAT1-PE/anti-pSTAT5A(pTyr694)-Alexa Fluor 647/anti-CD4-PE-Cy™7

STAT: Signal Transducer and Activator of Transcription; pSTAT: Phosphorylated Signal Transducer and Activator of Transcription

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Table 3: Studied cell subsets of all three groups analyzed by flow cytometry

	SLE[A]			HC[B]		
Cell numbers [cells/µL]	n	median	1st - 3rd quartile	n	median	1st - 3rd quartile
Leukocytes	17	5900	4800-8800	20	6400	5575-7450
Lymphocytes	15	762.2	542.2-870.7	20	1443.3 [AB****]	1190.6-1948.5
T lymphocytes	15	599.9	393.4-723.3	20	1001 [AB****]	830.2-1252.4
Th lymphocytes	15	376.4	150.3-408.5	20	618.1 [AB****]	492.1-687.3
Tc lymphocytes	16	235.3	162.7-325.7	20	299.2 [AB*]	269.4-409.5
$CD4^-CD8^-$ T lymphocytes	14	22.9	14.4-33.0	20	91.9 [AB****]	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	24.6173.40	19	80.19 [AB*]	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 [AB**]	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 [AB***]	8.61-16.93
rTreg lymphocytes	11	6.51	2.03-10.48	20	8.49 [AB*]	6.21-11.36
Treg lymphocytes	11	11.13	6.69-17.01	20	23.35 [AB**]	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 [AB***]	465.3-650.1
Percentages						
(% of Leukocytes)						
Lymphocytes	17	15.700	8.000-18.800	20	22.597 [AB**]	17.735-28.008
(% of Lymphocytes)						
T lymphocytes	17	76.700	69.400-81.300	20	67.100 [AB**]	63.975-74.300
(% of 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 [AB*]	27.150-37.425
Th/Tc lymphocytes	17	1.301	0.923-1.818	20	1.844 [AB*]	1.429-2.232
$CD4^-CD8^-$ T lymphocytes	17	4.200	3.120-6.800	20	7.685 [AB**]	5.905-9.140
HLA-DR$^+$ T lymphocytes	17	16.400	10.400-22.900	20	13.250	8.188-22.100
(% of 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 [AB***]	2.265-3.385
FOXP3$^+$Th lymphocytes	11	8.060	6.440-12.490	20	6.250 [AB*]	5.724-7.255
Tcon lymphocytes	11	90.040	84.745-92.140	20	94.430 [AB****]	94.118-94.668
Treg/Tcon lymphocytes	11	0.040	0.026-0.059	20	0.039	0.031-0.047
(% of FOXP3$^+$ Th lymphocytes)						
$CD25^-$ lymphocytes	11	36.900	31.950-44.250	20	14.900 [AB***]	13.775-23.025
aTreg lymphocytes	11	20.350	16.692-22.094	20	32.796 [AB**]	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	43.091 [AB**]	37.758-51.218
(% of each FOXP3$^+$ Th subset)						
$CD25^-$ cells in aTreg subset	11	28.200	12.850-34.250	20	9.155 [AB***]	6.620-13.275

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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
(% of Tcon lymphocytes)						
CD25⁺ cells	11	18.600	13.150-22.250	20	14.550	11.075-18.525
Analysis of STATs						
STAT1 (ΔMFI)	15	1068.0	657.5-1744.5	19	145.0 [AB]****	111.0-160.5
pSTAT1 (ΔMFI)	9	220.4	44.0-390.0	6	5.0 [AB]***	2.5-8.4
STAT5 (ΔMFI)	9	112.0	39.0-209.0	12	13.0 [AB]***	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; STAT: Signal Transducer and Activator of Transcription; pSTAT: Phosphorylated Signal Transducer and Activator of Transcription; MFI: Median Fluorescent Intensity

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