

Received: 2014.05.19  
Accepted: 2014.06.13  
Published: 2014.06.30

**Authors' Contribution:**

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

## Peripheral blood Th17/Treg imbalance in patients with low-active systemic lupus erythematosus\*

### Zaburzenia równowagi między komórkami Th17 i Treg we krwi obwodowej chorych na toczeń rumieniowaty układowy z niską aktywnością choroby

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#### Summary

**Introduction:**

The balance between proinflammatory Th17 cells and regulatory T cells plays an important role in the pathogenesis of autoimmune diseases, including systemic lupus erythematosus (SLE). In particular, an increased ratio of Th17/Treg cells has been shown to correlate with active SLE and specific organ involvement. The aim of our study was to assess Th17 and Treg cell populations in peripheral blood (PB) of patients with clinically quiescent SLE, and to evaluate their correlation with organ involvement.

**Material/Methods:**

We performed flow cytometric analysis of studied T CD4<sup>+</sup> cell subpopulations in PB from 21 patients with SLE and 13 healthy controls. Disease activity was measured with the SELENA-SLEDAI index; organ involvement was divided into renal, neurological and hematological.

**Results:**

A statistically significant difference ( $p < 0.01$ ) between the mean percentages of CD4<sup>+</sup>CD25<sup>hi</sup>FoxP3<sup>+</sup> Treg cells in SLE patients (18.57%) and healthy controls (32.08%) was observed. Similarly, proportions of functional CTLA-4<sup>+</sup> Treg cells were markedly lower in SLE patients than in healthy controls – 19.3% vs. 23.82% ( $p = 0.03$ ). In contrast, SLE patients exhibited a significantly increased frequency of circulating Th17 cells with the phenotype CD4<sup>+</sup>IL-17<sup>+</sup> compared to controls – 1.36% vs 0.19% ( $p < 0.01$ ). Also the ratio of Th17 cells to Th1 cells was markedly higher in SLE patients than in the control group ( $p < 0.01$ ). We did not find any correlation of PB Th cell distribution with organ involvement in SLE patients examined.

**Conclusions:**

Our report showed for the first time that systemic Th17/Treg imbalance occurred also in patients with low disease activity and in remission. We suggest that immunological alterations may precede clinical and laboratory symptoms of the disease activity.

**Keywords:**

Systemic lupus erythematosus • Treg cells • Th17 cells • disease activity

\* The study was supported by Wrocław Medical University during the years 2010-2012 (grant no. ST 460).

<b>Full-text PDF:</b>	<a href="http://www.phmd.pl/fulltxt.php?CID=1111127">http://www.phmd.pl/fulltxt.php?CID=1111127</a>
<b>Word count:</b>	3179
<b>Tables:</b>	2
<b>Figures:</b>	1
<b>References:</b>	19

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## INTRODUCTION

Systemic lupus erythematosus is an autoimmune disease characterized by autoantibody production and deposition of immune complexes in numerous organs, causing tissue injury. The pathogenesis of SLE is not completely understood, with various types of immune cells being involved. SLE is characterized by the expansion of autoreactive B cells and autoantibody production, which is promoted by IL-10 and B-lymphocyte stimulator (BLyS); CD4<sup>+</sup> T cells were shown to provide help to autoreactive B cells. Recently, new light on the pathogenesis of SLE was shed by the discovery of new T-cell subsets, such as proinflammatory Th17 cells and regulatory T cells (Tregs).

Th17 cells are a distinct subset of helper T cells (Th cells) with proinflammatory properties. Th cell differentiation depends on the cytokine milieu as well as the expression of certain transcription factors. Initial differentiation of Th17 cells from naïve CD4<sup>+</sup> T cells depends on IL-1 $\beta$ , IL-6 and TGF $\beta$ , while their expansion is promoted by IL-23 and IL-21 [14,16].

Th17 cells are a source of multiple cytokines, such as IL-17 (IL-17A), IL-17F, IL-21 and IL-22. IL-17 can drive inflammation and autoimmunity, by inducing the production of chemokines and cytokines such as G-CSF, GM-CSF, and IL-8. It is also able to promote the proliferation, maturation and recruitment of neutrophils and recruit other inflammatory cells, such as macrophages and lymphocytes. IL-17 can induce proinflammatory cytokines, such as TNF- $\alpha$  and IL-6, and can cause tissue damage by up-regulating matrix metalloproteinases. It has been recently demonstrated that human Th17 cells express on their surface chemokine receptors CCR4, CCR6, and CD61 [14].

Elevated levels of circulating Th17 cells and IL-17 have been described in various autoimmune diseases, such as rheumatoid arthritis (RA), multiple sclerosis (MS), Sjögren syndrome and psoriasis [1,2,14]. There are also several reports on the increased number of Th17 cells and IL-17 levels in peripheral blood of SLE patients, which in addition correlated with disease activity and renal involvement [1,9,13,14]. The role of IL-17 secreted

by Th17 cells in SLE is not fully elucidated. Lupus patients had increased expression of IL-17 as well as IL-17-producing double negative T cells in the kidneys and increased expression of the IL-17 gene was found in urine sediment, which suggest a pathogenic role of IL-17 in lupus nephritis [18,19]. It has been demonstrated that IL-17 upregulates humoral immunity, as alone or together with BLyS (B lymphocyte stimulating factor) it increased the survival and differentiation of autoreactive B cells [3]. Its role in the pathogenesis of SLE was also supported by an experiment performed in an autoimmune model of BXD mice with IL-17 blockade, which resulted in a diminished number of germinal centre B cells and decreased production of autoantibodies [5].

Treg cells are involved in self-tolerance and their impaired function is associated with the development of autoimmunity. Different subpopulations of Treg cells have been described so far, including natural Tregs (nTregs) developing in the thymus in the early phases of life, known to be IL-2-dependent. Inducible Tregs (iTregs) are converted from conventional CD4<sup>+</sup>CD25<sup>-</sup> T cells in the presence of TGF $\beta$  in the periphery. Other types of regulatory T cells have been found, such as regulatory Tr1 lymphocytes secreting IL-10 and TGF $\beta$ , and Th3 cells synthesizing membrane bound TGF $\beta$ , which result from the differentiation of naïve T cells in secondary lymphoid organs during the entire life [10,16].

Treg cells fail to proliferate or secrete cytokines in response to polyclonal or antigen-specific stimulation and show an anergic phenotype. They have suppressor properties and can inhibit the activation of conventional CD4<sup>+</sup>CD25<sup>-</sup> cells or CD8<sup>+</sup> cells. By blocking the activity of self-reactive T lymphocytes Tregs prevent the onset of the aberrant self-response. Other important features of Tregs are the induction of tolerance for antigens associated with tumor, and down regulation of the excessive response to infection and allergy.

The mechanisms of the suppressor action of Tregs are not fully elucidated. Inducible Tregs can act through the synthesis of immunosuppressive cytokines such as IL-10 and TGF $\beta$ . Treg suppression seems to be cell contact dependent and appears to be mediated by granzyme B. In addition, the transfer of down-regulating signals may

be caused via various molecules expressed on the Treg cell surface, such as CTLA4 and membrane bound TGF $\beta$  [9,10]. Regulatory T cells co-expressing CTLA-4 (CTLA-4<sup>+</sup> Tregs) are defined as a functional Treg subpopulation, since CTLA-4 expression is required for Treg cells to sufficiently exert the suppressor activity [4].

The aim of the study was to assess the percentages of Th17 and Treg cells in peripheral blood (PB) from patients with clinically quiescent systemic lupus erythematosus and to search for their correlation with specific organ involvement.

## PATIENTS AND METHODS

### Patients

The study group consisted of 21 patients with systemic lupus erythematosus fulfilling the revised ACR criteria for SLE who were admitted to the Department of Rheumatology and Internal Diseases in Wrocław Medical University during the years 2011-2012. The patient group consisted of 20 female and 1 male patients, of average age 44.5 (min 25, max 77 years) and with mean disease duration of 8.5 years. Disease activity was measured by the SLEDAI index; accordingly, active disease was defined as SLEDAI  $\geq$  12.0. The mean value of the SLEDAI index in patients enrolled in the study was 6.92.

The clinical data obtained included patients' age, sex, disease duration, and disease activity.

The patients were further divided according to the major organ involvement: 7 patients had renal involvement, 11 had central nervous system involvement and 15 had hematological involvement, with some patients having more than one organ involved. The control group consisted of 13 healthy volunteers matched for age and sex. The study was approved by Wrocław Medical University Ethics Committee and carried out in accordance with the Helsinki protocol. All the patients were enrolled after giving their informed consent.

Patients' characteristics are shown in Table 1.

**Table 1.** General characteristics of SLE patients

	Values
Age (mean; std. dev.)	44.54; 13.71
Sex – female (no.)	20 (95%)
Disease duration (mean; std. dev.)	8.44; 6.35
SLEDAI (mean; std. dev.)	6.92; 5.61
Renal involvement (no.)	7 (33%)
Neurological involvement (no.)	11 (52%)
Hematological involvement (no.)	15 (71%)

### Cell preparation, culture conditions, and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were obtained by buoyant density-gradient centrifugation on Lymphoflot (Biotest, Germany). The cells were then stimulated with 25 ng/ml phorbol 12-myristate 23-acetate (PMA) and 1 mg/ml of ionomycin (Ion) (Sigma-Aldrich) in the presence of 10 mg/ml of brefeldin A (BFA, protein transport inhibitor) for 4 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. For analysis of Tregs (CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>+</sup> T cells) as well as CTLA-4<sup>+</sup> Tregs, unstimulated PBMCs were aliquoted into tubes directly after isolation for further staining performed as follows: the cells were stained with anti-CD4/PerCP (BD Pharmingen), anti-CD25/FITC (BD Pharmingen) or CTLA-4/FITC monoclonal antibodies (MoAbs) (BD Pharmingen).

For intracellular staining of cells, including Tregs, the surface-labeled cells were fixed and permeabilized with BD Permeabilizing Solution 2 (Becton Dickinson) according to the manufacturer's instructions with subsequent incubation with anti-IFN- $\gamma$ /FITC (BD Pharmingen), anti-IL-17/PE (BD Pharmingen), anti-IL-10/PE, anti-human FoxP3/PE MoAbs (BD Pharmingen) or isotypic controls for 30 min at room temperature in the dark. Directly after immunostaining, the cells were washed and analyzed by flow cytometry using a FACScan cytometer (Becton Dickinson) equipped with Cell Quest software (BD Bioscience Pharmingen). At least 10,000 events per sample were analyzed in each experiment.

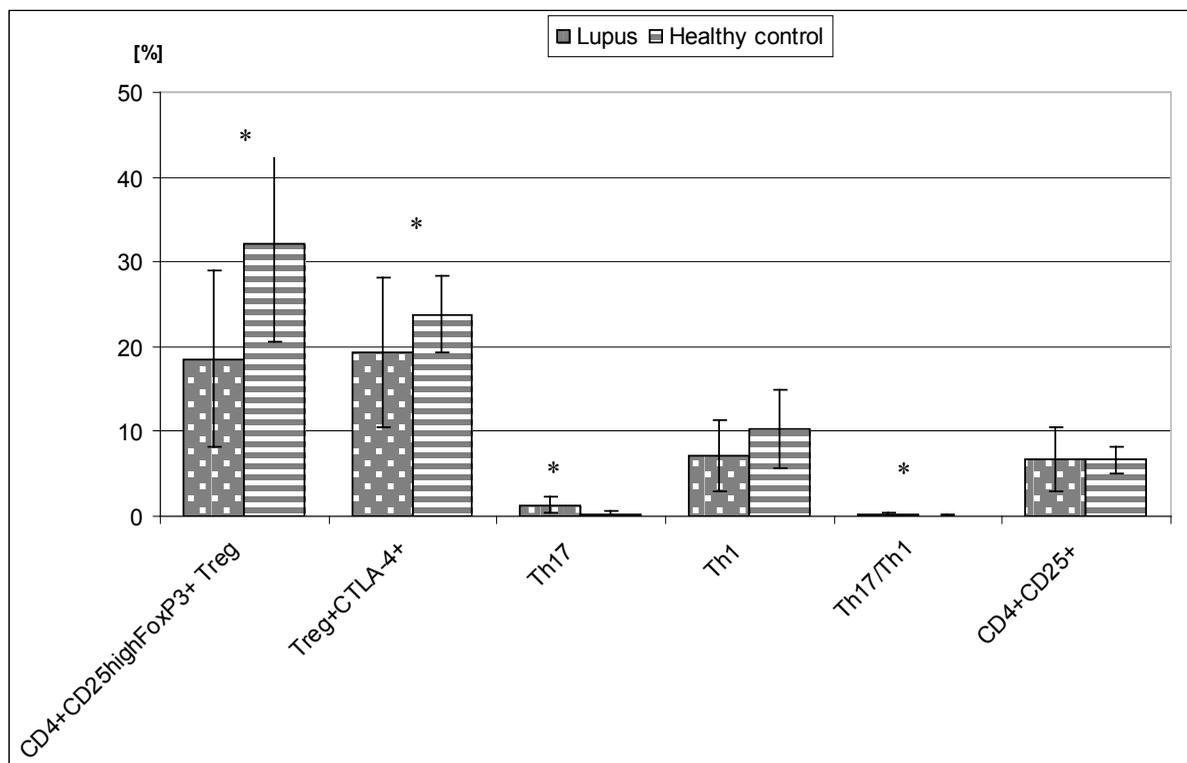
### STATISTICAL ANALYSIS

The statistical package STATISTICA was used for conducting statistical analysis. A significance level of  $p = 0.05$  was assumed. For comparison of two independent groups a Mann-Whitney U test was used. The choice of non-parametric test was made due to the small number of observations in compared groups.

### RESULTS

We assessed different subpopulations of Treg cells with the phenotype described as follows: CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup>CTLA-4<sup>+</sup> (defined as functional Tregs). We observed statistically significant differences ( $p < 0.01$ ) between the percentages of CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>+</sup> Treg cells in SLE patients (18.57%) and healthy controls (32.08%). Similarly, proportions of functional CTLA-4<sup>+</sup> Treg cells, known to exert suppressive potential, were markedly lower in SLE patients than in healthy controls; the respective values were as follows: 19.3% vs. 23.82% ( $p = 0.03$ ) (Figure 1, Table 2).

In contrast, the frequency of Th17 cells with the phenotype CD4<sup>+</sup>IL-17<sup>+</sup> was significantly higher in SLE patients than in controls – 1.36% vs 0.19% ( $p < 0.01$ ). Although the percentage of Th1 cells in SLE patients was lower than that observed in healthy controls (7.13% and 10.23%,



**Fig. 1.** Mean percentage of various phenotypes of T cells in lupus patients and control subjects. Statistically significant differences at the level  $p < 0.05$  (marked with asterisks) were present between CD4+CD25highFoxP3+ Treg, CTLA-4+ Treg and Th17 cells and Th17/Th1 ratio

**Table 2.** Mean frequency of different T cell populations in patients with SLE in comparison to healthy controls

	SLE group	Control group	Std. dev. SLE	Std. dev. controls	p-level
CD4+CD25highFoxP3+ Treg	18.57	32.08	10.44	11.54	< 0.01
CTLA-4+ Treg	19.30	23.82	8.81	4.51	0.03
Th17	1.36	0.19	0.98	0.45	< 0.01
Th1	7.13	10.23	4.27	4.59	0.08
Th17/Th1	0.25	0.07	0.19	0.15	< 0.01
CD4+CD25+	6.75	6.65	3.73	1.59	0.63

respectively), the difference was not statistically significant. Nevertheless, the ratio of Th17 cells to Th1 cells was significantly higher in SLE patients than in the control group – 0.25 and 0.07, respectively ( $p < 0.01$ ) (Figure 1).

We found that the frequencies of Treg and Th17 cells did not differ depending on the specific organ involvement, namely in lupus nephritis (LN), neuropsychiatric (NP) lupus or lupus with predominant hematological involvement.

## DISCUSSION

The interaction between T regulatory cells and proinflammatory Th17 cells is involved in the pathogenesis

of different autoimmune diseases. The balance between Treg/Th17 in SLE has been investigated for several years, giving inconclusive results. These discrepancies throughout the literature may be due to the heterogeneity of the disease, studies in patients with different levels of disease activity, the possible impact of immunosuppressive treatment, and other factors.

Due to the quiescent course of SLE in the patients enrolled in the study, no obvious changes in the frequency of circulating Treg cells were expected. Therefore, we decided simultaneously to estimate the magnitude of the subpopulation of Tregs co-expressing the inhibitory CTLA-4 molecule defined as functionally active Tregs. Our results demonstrated for the first time that patients

with SLE exhibit significantly decreased percentages of Treg cells in peripheral blood despite the non- or low-active course of the disease. Furthermore, suppressive activity of Tregs in patients studied seemed to be limited, as estimated by the decreased population of functional CTLA4<sup>+</sup> Treg cells, indicating that Treg-mediated peripheral tolerance in SLE might be broken even in the non-active disease. Regulatory T cells in SLE patients have been previously investigated, and the results are still confusing. Most authors report a reduced number and impaired function of Treg cells in SLE [18], while some others found increased numbers of Treg cells, but with impaired suppressor function or the resistance of effector T cells to suppressive action of Tregs [15]. The inconsistencies may be due to differences in both the phenotype assigned to Tregs and the degree of SLE activity in those studies.

Moreover, our present data showing the expanded Th17 cell population in low-active SLE seem to be complementary to previous results. Yang et al. [18] found that the Th17 population was enriched in total PBMC and Th17 cells infiltrated involved organs of patients with active SLE, such as skin and lungs. Nonetheless, the percentage of Th17 cells was reported to be higher only in circulation of active lupus patients compared to healthy individuals and patients with non-active SLE. Accordingly, elevated levels of IL-17 were found in sera from active SLE patients in the above experiment; in addition, they demonstrated that the percentage of Th17 cells correlated with the SLEDAI score. They also found that the number of Th17 cells increased with lupus flares, especially in patients with vasculitis, and decreased after the treatment [18]. The organ involvement found has been attributed to IL-17-induced expression of adhesion molecules on the endothelial cells, thus promoting vascular inflammation in SLE [18].

A growing body of evidence emphasizes a strong association of Th17/Treg imbalance with an active course of SLE [11]. Furthermore, the proportion of Treg cells and Th17 cells was shown to be strongly and negatively interrelated [18]. Yang et al. also reported a reversal of Treg cell population by the treatment, while the flares were associated with a reduction in Treg numbers. In addition, dysregulation of the balance between Th17 and Treg cells was found to be more pronounced in patients with organ involvement, especially in those with lupus nephritis (LN). Xing et al. [17] found that patients with LN were characterized by decreased numbers of Tregs described as CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells as well as a significant increase in the frequency of Th17 cells in comparison with healthy controls and SLE patients without nephritis. In addition, the level of FoxP3 expression was reduced in LN. Furthermore, in the LN group, the Th17/Treg ratio was significantly increased and correlated with disease activity as measured by SLEDAI, and those Th subsets were also strongly and negatively correlated

with each other. In consequence, serum levels of IL-17 were increased in patients with LN in comparison with active SLE without nephritis and healthy controls [17].

In turn, Ma et al. [11] reported that while Treg and Th17 alone were not correlated with SLE, the ratio of Treg/Th17 in peripheral blood cells of SLE patients with active disease was significantly reduced and inversely correlated with the disease activity and anti-dsDNA antibodies. It is worthy of note, in addition, that two months of prednisone treatment corrected the Treg/Th17 imbalance and resulted in a decrease of SLEDAI scores, which, in consequence, may improve outcomes of SLE patients. This notion was consistent with the previous finding that corticosteroids enhanced Foxp3 expression and function of Foxp3<sup>+</sup> Treg cells [11]. However, to date, there is a lack of reports focusing on Th17/Treg balance in SLE patients with non- or low-active disease. In this context, our data showing the alterations of PB Th17 and Treg cell populations in clinically quiescent SLE seem to be a novel finding. However, we did not observe an association of either Th17 cell expansion or Treg decrement with organ involvement, which was in contrast to past reports [14]. This may be explained by the relatively small number of patients enrolled in the current study and low activity of the disease.

The mechanisms underlying the altered Th17/Treg balance in lupus patients, including those with non-active disease, are still unclear. Since IL-6 promotes Th17 differentiation, its elevated serum levels in SLE patients should be taken into consideration as one of the key factors of pathogenic importance [18,13]. In addition, Th17 expansion might result from the elevated levels of IL-23 previously reported in lupus patients [18]. Our study showed, in addition, that the enriched Th17 population in SLE was accompanied by unchanged frequency of Th1 cells, recently suggested to exert anti-inflammatory activity in autoimmune diseases [6,7,8,12]. Our observation on the elevated Th17/Th1 ratio in SLE is in line with the previous report by Shah [13]. Given that Th17 was the only Th cell population recently demonstrated to be resistant to Treg-mediated suppression in autoimmune disorders, our finding of the increased ratio of Th17 cells to both Treg as well as Th1 cells indicates that in low-active lupus as well, the PB Th cell balance is shifted from anti-inflammatory towards pro-inflammatory conditions. Hence, we suggest that the immune dysregulation observed in our study may precede clinical and laboratory symptoms of the disease activity.

The shortcoming of our study was the relatively small group of patients and the fact that they already had an established disease and were under immunosuppressive treatment. Therefore the impact of medication on the study results should be considered. It would be interesting to carry out a longitudinal observation to determine how disease flares and remissions influence the Th lymphocyte profile in the patients examined.

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The authors have no potential conflicts of interest to declare.