

# CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cells and CD4<sup>+</sup>CD69<sup>+</sup> T cells in peripheral blood of patients with cutaneous lupus erythematosus

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## Abstract

Lupus erythematosus (LE) is a chronic autoimmune disease characterized by functional alterations of T and B lymphocyte subsets. The cutaneous forms of lupus erythematosus comprise subacute cutaneous LE (SCLE) and discoid LE (DLE). Recent studies have shown that the activation process of lymphocytes in patients with systemic disease manifestations is disturbed. The aim of this study was to investigate the levels of CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cells and CD4<sup>+</sup>CD69<sup>+</sup> cells in peripheral blood of patients with cutaneous lupus erythematosus (CLE). Using flow cytometry to determine cell populations, we characterized CD4<sup>+</sup>CD25<sup>high</sup> cells and CD4<sup>+</sup>CD69<sup>+</sup> cells in patients with CLE (DLE – 5 patients, SCLE – 5 patients), SLE (n = 5), and healthy controls (n = 15). We compared CD4<sup>+</sup>CD25<sup>high</sup> cells and CD4<sup>+</sup>CD69<sup>+</sup> cells from the patients with CLE versus SLE and healthy controls. CD4<sup>+</sup>CD25<sup>high</sup> cells was significantly increased in patients with CLE when compared to the patients with SLE (p < 0.05). While the percentage of CD4<sup>+</sup>CD25<sup>high</sup> cells in CLE patients was similar to that in the control subjects. CD4<sup>+</sup>CD69<sup>+</sup> cells was decreased in the patients with CLE and control subjects when compared to the patients with SLE (p < 0.05). Our results indicate that patients with systemic disease manifestations had lower levels of circulating CD4<sup>+</sup>CD25<sup>high</sup> cells and higher levels of CD4<sup>+</sup>CD69<sup>+</sup> cells than patients with cutaneous forms of lupus erythematosus.

**Key words:** lupus erythematosus, CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cells, CD4<sup>+</sup>CD69<sup>+</sup> T cells.

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## Introduction

Lupus erythematosus (LE) is an autoimmune disease with a broad spectrum of clinical symptoms. Many factors are involved in the pathogenesis, including environmental factors, genetic factors, hormones, hyperactivated B and T cells, and abnormal immunoregulation. The disease can be manifested in a cutaneous form – cutaneous lupus erythematosus (CLE) or as a systemic form – systemic lupus erythematosus (SLE). There are a variety of clinical manifestations that patients with CLE may demonstrate.

Cutaneous lupus erythematosus includes different subsets: localized discoid lupus erythematosus (L-DLE), disseminated discoid lupus erythematosus (D-DLE), and subacute cutaneous lupus erythematosus (SCLE) [1]. Patients with localized DLE have only a 5% chance of having SLE, while those who have generalized DLE have a 20% chance of having SLE, and those who have SCLE – a 50% chance [2].

In spite of profound research, the pathogenesis of cutaneous form versus systemic lupus erythematosus remains largely unexplored. In recent years, CD4<sup>+</sup>CD25<sup>+</sup> regulatory

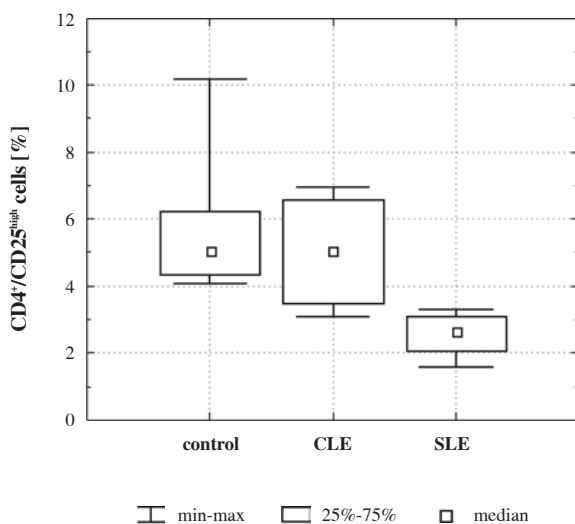
T cells (Tregs) have been thought to play an important role in the development of autoimmune diseases [3-7]. In a recent report it was found that in SLE patients levels of Tregs in the peripheral blood inversely correlated with the disease activity; while the percentage of CD4<sup>+</sup>CD69<sup>+</sup> cells correlated with the disease activity [8]. These observations inspired our interest in conducting a similar research on patients with cutaneous lupus erythematosus with CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells and CD4<sup>+</sup>CD69<sup>+</sup> cell subsets. In our research we sought to answer the following questions: (1) could clinical manifestation of skin symptoms lead to a general imbalance in the immune system, and (2) whether cutaneous form developing into systemic disease is preceded by an earlier immune system dysfunction without clinical evidence of a systemic disease.

## Materials and Methods

### Patients and control subjects

The present study included 15 healthy controls, 5 SLE patients and 10 patients with the following subtypes of CLE: subacute CLE (SCLE – 5 patients), discoid LE (DLE – 5 patients).

None of the CLE patients fulfilled 4 or more criteria of the American College of Rheumatology criteria for the classification of SLE [9,10]. SLE patients fulfilled ACR criteria for SLE. The age of studied populations ranged from 34 to 67 years (mean age 47.3). All of them were females. Blood samples were obtained from patients after informed consent was provided.



**Fig. 1.** Percentages of circulating CD4<sup>+</sup>CD25<sup>high</sup> T cell populations in patients with CLE (cutaneous lupus erythematosus), SLE (systemic lupus erythematosus) and control subjects.

### Flow cytometric analysis

EDTA-anticoagulated peripheral blood cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody (clone L120), phycoerythrin (PE)-conjugated anti-CD8 antibody (clone RPA-T8), phycoerythrin (PE)-conjugated anti-CD25 antibody (clone 2A3), and phycoerythrin-cyanine 5 (PE-Cy5)-conjugated anti-CD69 antibody (clone MOPC-21) (Becton Dickinson, USA) for 15 min. Appropriate isotype antibodies were used as negative controls. Red cells were lysed in a lysing solution (BD FACS Lysing Solution, Becton-Dickinson, USA) for 10 min, remaining leucocytes were washed in phosphate-buffered saline (PBS) and fixed with 1% formalin. Routinely,  $2 \times 10^5$  cells were analyzed by flow cytometry (FACSscan, BD, USA). Percentages of CD4<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD69<sup>+</sup> cells were calculated in the population of cells gated on the lymphocyte base FSC/SCC diagram.

### Statistical analysis

The Mann-Whitney test was used to compare the levels of CD4<sup>+</sup>CD25<sup>high</sup> cells and CD4<sup>+</sup>CD69<sup>+</sup> cells in the studied patients and healthy controls. Results were considered significant at a p value < 0.05.

Since distributions of CD25 and CD69 lymphocytes differed significantly from normal distribution in some of the analyzed groups (Shapiro-Wilk's test,  $p < 0.05$ ), non-parametric tests were applied. Significance of differences found in the 3 analyzed groups (CLE, SLE and the controls) was evaluated by using Kruskal-Wallis test, followed by Mann-Whitney test.

## Results

### Percentages of CD4<sup>+</sup>CD25<sup>high</sup> T cells in CLE patients

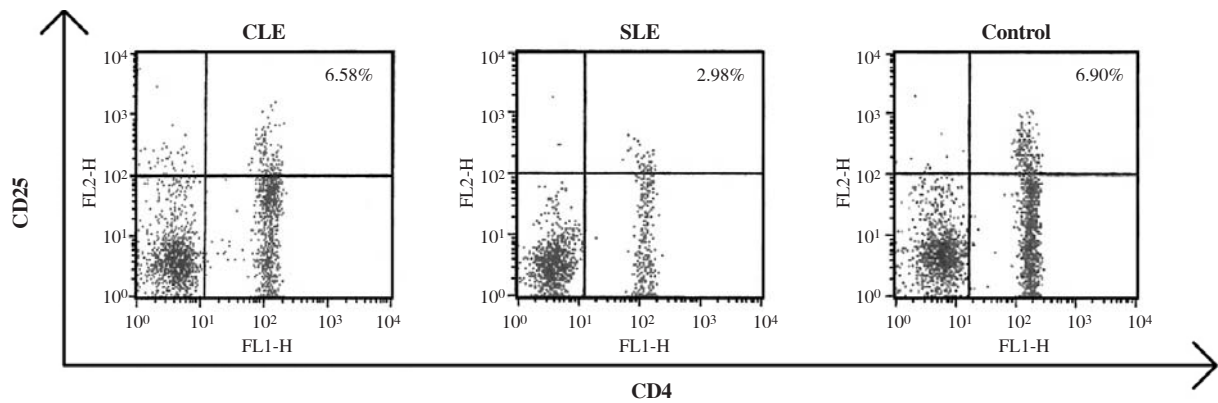
Flow cytometry analysis revealed that the percentage of CD4<sup>+</sup>CD25<sup>high</sup> T cells was higher in CLE patients than in SLE patients [median (interquartile range), 5.06% (3.07-6.95%) vs. 2.51% (1.59-3.31%)]. The difference between CLE patients and SLE patients was statistically significant  $p = 0.00002$ .

The percentage of CD4<sup>+</sup>CD25<sup>high</sup> T cells in CLE patients was similar to that in the control subjects [5.06% (3.07-6.95%) vs. 5.52% (4.06-10.17%)]. The difference between CLE patients and the control subjects was not statistically significant ( $p > 0.05$ ) (Fig. 1).

The representative data of CD4<sup>+</sup>CD25<sup>high</sup> T cell populations in patients with CLE (cutaneous lupus erythematosus), SLE (systemic lupus erythematosus) and control subjects are shown in Fig. 2.

### Percentages of CD4<sup>+</sup>CD69<sup>+</sup> T cells in CLE patients

Patients with CLE had a significantly decreased percentage of CD4<sup>+</sup>CD69<sup>+</sup> T cells when compared to SLE

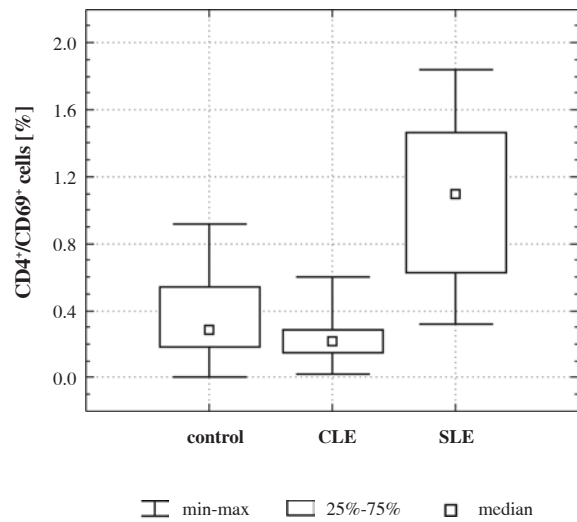


**Fig. 2.** Representative CD4<sup>+</sup>CD25<sup>high</sup> cell populations analyzed by flow cytometry in the patients with CLE (cutaneous lupus erythematosus), SLE (systemic lupus erythematosus) and the control subjects.

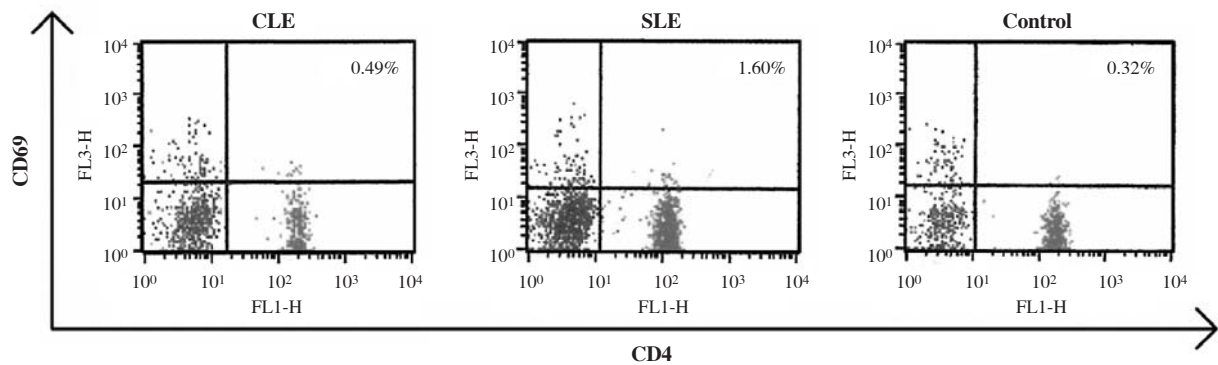
patients [median (interquartile range), 0.23% (0.02-0.6%) vs. 1.07% (0.32-1.84%)]. The difference between CLE patients and SLE patients was statistically significant  $p = 0.00002$ .

The percentage of CD4<sup>+</sup>CD69<sup>+</sup> T cells in CLE patients was similar to that in the control subjects [0.23% (0.02-0.6%) vs. 0.34% (0-0.92%)]. The difference between CLE patients and control subjects was not statistically significant ( $p > 0.05$ ) (Fig. 3).

The representative data of CD4<sup>+</sup>CD69<sup>+</sup> cells populations in patients with CLE (cutaneous lupus erythematosus), SLE (systemic lupus erythematosus) and control subjects are shown in Fig. 4.



**Fig. 3.** Percentages of circulating CD4<sup>+</sup>CD69<sup>+</sup> cell populations in patients with CLE (cutaneous lupus erythematosus), SLE (systemic lupus erythematosus) and the control subjects.



**Fig. 4.** Representative CD4<sup>+</sup>CD69<sup>+</sup> cells populations analyzed by flow cytometry in patients with CLE (cutaneous lupus erythematosus), SLE (systemic lupus erythematosus) and the control subjects.

that the Tregs play a significant role in the development of systemic autoimmune diseases, including SLE [14, 15]. Recently, Lee et al. [16] have reported that the number of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in the peripheral blood of pediatric patients with active SLE was significantly decreased. Liu et al. obtained similar results: they observed lower levels of Tregs in the peripheral blood of patients with SLE. This, however, was not observed in patients with rheumatoid arthritis [17]. In our study, we compared the levels of Tregs in the peripheral blood of patients with cutaneous lupus erythematosus (CLE) versus SLE patients and healthy controls.

The present study has shown that the patients with SLE have significantly lower levels of Tregs than the patients with cutaneous form of lupus erythematosus (Fig. 1). A similar phenomenon took place when comparing SLE patients to the control group. Franz et al. [18] have reported that the number of Tregs was decreased, but only at the site of inflammation in skin lesions of patients with CLE, and not in the peripheral blood. Such connection observed by Sakaguchi et al. was supported in the research conducted on mice [11]. It showed that mice in the CD25<sup>+</sup> cell-depleted group developed multiorgan autoimmune disease. Some of them developed antibodies to dsDNA and glomerulonephritis, typical features of lupus. In the course of another study, it has been shown that an early thymectomy in mice (the third day after birth) caused a significant decrease in Treg levels and an increase in the antibody production [19].

The cited research studies indicated that Tregs may be responsible for the development of autoimmune disease to various degrees. Tregs certainly play a crucial role in the development of lupus erythematosus. Yet, its pathomechanism has not been entirely explained. In recent years, some researchers pointed out that patients with active SLE had lower levels of Tregs and the levels of Tregs inversely correlated with the disease activity. One of the characteristic features of T lymphocytes activation is the presence of CD69 antigen on their surface. The CD69 antigen is a type II integral membrane protein with a C-type lectin-binding domain. This antigen participates in the transmission of an activation signal, which leads to the synthesis of different cytokines, including IL-2, IFN- $\gamma$  as well as the receptor for IL-2. Crispin et al. assayed CD4<sup>+</sup>CD69<sup>+</sup> cells in SLE patients. They observed a positive correlation between the CD4<sup>+</sup>CD69<sup>+</sup> cell levels and the disease activity [20]. In our study we also compared CD4<sup>+</sup>CD69<sup>+</sup> cell levels in CLE patients to patients with SLE and healthy controls. The obtained results showed a statistically significant difference between the levels of CD4<sup>+</sup>CD69<sup>+</sup> cells observed in patients with SLE as compared to CLE patients. CD4<sup>+</sup>CD69<sup>+</sup> lymphocyte subset in CLE patients, however, was comparable to the percentage obtained in the control group. These results confirmed the earlier predictions regarding the role CD4<sup>+</sup>CD69<sup>+</sup> lymphocytes in autoaggression, and activating process of these T-cells towards to disease.

Recent studies [21-25] and our study indicate a significant role of Tregs in inhibition of the development of autoimmune diseases as well as lack of such inhibition in case of large deficiency of this lymphocyte subpopulation. When the immune system balance is disturbed, T-cells subsets, such as CD4<sup>+</sup>CD69<sup>+</sup>, become more active and may trigger the disease process through the transmission of an activation signal, for e.g. the synthesis of proinflammatory cytokines.

In conclusion, our observations suggest that monitoring these two subsets of T cells may be applied as a prognostic marker, contributing to the defining the mechanisms of induction of systemic, autoimmune reactions in patients with cutaneous lupus erythematosus.

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