

Received: 2014.12.10
Accepted: 2015.01.24
Published: 2015.02.16

Vincristine-Cyclophosphamide Combination Therapy Positively Affects T-Cell Subset Distribution in Systemic Lupus Erythematosus Patients

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Source of support: This work was supported in part by the Project for Scientific and Technological Social Development of Shanxi Province, China (grant no. 20120313025-4)

Background: This study aimed to analyze the T-cell subset distribution in systemic lupus erythematosus (SLE) patients and determine whether vincristine-cyclophosphamide combination therapy can positively affect their T-cell subset distribution to keep the disease in remission.


Material/Methods: Thirteen SLE patients with 'low activity' (SLE Disease Activity Index (SLEDAI)≤9), 17 SLE patients with 'high activity' (SLEDAI>9), and 15 healthy controls were recruited. SLE patients were treated with vincristine-cyclophosphamide combination therapy. CD3⁺, CD4⁺, and CD8⁺ T-cell percentages were analyzed by flow cytometry at baseline, 3 months, 6 months, 12–24 months, and >24 months.

Results: Significantly negative correlations were observed between the CD3⁺ and CD4⁺ T-cell percentages and SLEDAI scores at baseline ($r=-0.471$, $P=0.015$; $r=-0.473$, $P=0.015$, respectively). A significantly positive correlation was observed between CD4⁺ T-cell percentage and the complement component C3 at baseline ($r=0.612$, $P=0.002$). After 3 months of combination therapy, the CD3⁺ and CD4⁺ T-cell percentages were significantly higher than the high activity baseline ($P<0.01$, $P<0.05$, respectively). After 6 months, the CD3⁺, CD4⁺, and CD8⁺ T-cell percentages were all significantly higher than the high activity baseline ($P<0.01$, $P<0.05$, $P<0.05$, respectively).

Conclusions: T-cell subset distributions vary across different levels of SLE disease activity with higher CD3⁺ T-cell and CD4⁺ Th cell percentages favoring lower SLE activity. As CD3⁺ T-cell and CD4⁺ Th cell percentages negatively correlate with SLEDAI, vincristine-cyclophosphamide combination therapy appears to positively affect the T-cell subset distribution in SLE patients to keep the disease in remission by increasing their CD3⁺ T-cell and CD4⁺ Th cell percentages.

MeSH Keywords: **Leukemia, T-Cell • Lupus Vulgaris • Vincristine**

Full-text PDF: <http://www.medscimonit.com/abstract/index/idArt/893271>

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Background

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by immune system dysfunction and the production of autoantibodies that lead to inflammation and tissue damage [1]. This multisystem disorder results in considerable morbidity and mortality [2]. In SLE, T-lymphocytes (T-cells) promote inflammation through producing co-stimulatory signals and cytokines that activate dendritic cells and B-cells [3,4]. Activation of auto-reactive T-cells leads to abnormalities in CD4⁺ and CD8⁺ T-cells, which are critical drivers of the B-cell-dependent autoantibody response [3,5–7]. Based on these previous findings, we speculate that an imbalance in the T-cell subset distribution eventually results in the development of SLE.

To that end, immunosuppressive or cytotoxic drugs that target T-cells can induce long-term remission in SLE patients [8]. In T-cell leukemia patients, long-term maintenance combination chemotherapy has been shown to be an effective, low-cost treatment regimen with limited adverse effects that can control disease in a stable manner over several years [9]. Based on these previous findings, we hypothesized that the combination of the cell cycle-specific drug vincristine with the non-cell cycle-specific drug cyclophosphamide would be effective in treating SLE via positively affecting their T-cell subset distribution.

Thus, the aims of the present study were to (i) analyze the T-cell subset distribution in SLE patients at baseline and (ii) determine whether vincristine-cyclophosphamide combination therapy can positively affect their T-cell subset distribution to keep the disease in remission.

Material and Methods

Recruitment of participants

This study was approved by the Ethics Committee (IRB) of the Second Hospital at Shanxi Medical College (Taiyuan, China). All subjects recruited for this study provided written informed consent prior to their participation.

Between October 2008 and October 2012, 30 SLE patients were consecutively recruited from the inpatient population of the Second Hospital at Shanxi Medical College. All SLE patients fulfilled the American College of Rheumatology (ACR) classification criteria for SLE [10]. The degree of SLE disease activity was assessed using the SLE Disease Activity Index (SLEDAI) [7]. Patients with any other autoimmune disease, those having received immunosuppressive therapies other than glucocorticoid therapy within the past 6 months, or those with WBC counts of less than $3.0 \times 10^9/L$ were excluded.

Fifteen healthy volunteers matched by race, sex, and age were recruited from the outpatient population of the same hospital during the same time period.

Study design

The 30 SLE patients were subdivided into 2 groups: those with low disease activity ('low activity', SLEDAI \leq 9, n=17 cases) and those with high disease activity ('high activity', SLEDAI $>$ 9, n=13 cases). All SLE patients were given vincristine (1 mg by intravenous drip) followed by intravenous cyclophosphamide over the next 24 hours (200–600 mg depending on SLE patient's WBC count; 200 mg for $3.0\text{--}4.0 \times 10^9/L$, 400 mg for $4.0\text{--}10 \times 10^9/L$, 600 mg for $>10 \times 10^9/L$). This therapeutic regimen was executed every three weeks, and prednisone therapy was permitted. According to the total duration of vincristine-cyclophosphamide combination therapy, all SLE patients were divided into 4 groups: a 3-month group, a 6-month group, a 12–24-month group, and >24 -month group.

Flow cytometry

MultiTEST antibodies – CD3 (FITC), CD8 (PE), CD45 (PerCP), and CD4 (APC) – were purchased from BD Biosciences. Peripheral blood samples (2 ml) from each subject were collected in EDTA-coated tubes at baseline and at various time points (i.e., 3 months, 6 months, etc.) during vincristine-cyclophosphamide combination therapy. For immunofluorescence staining, fresh EDTA-treated whole blood samples were kept at room temperature immediately after collection, and 50 μ l was placed in a TruCount tube. Then, 20 μ l CD3/CD8/CD45/CD4 was added to each sample. After incubation at 4°C for 30 min in the dark, stained cells were washed with FACS buffer (phosphate-buffered saline (PBS), 0.2% fetal bovine serum (FBS), 0.09% Na₃N) and then incubated once again for 15 min in the dark. Negative controls were performed simultaneously using FITC-labelled mouse anti-human IgG1 mAb and APC-labelled mouse anti-human IgG1 mAb. All samples were analyzed on a FACScanto (BD Bioscience, San Jose, CA, USA), and the resulting data was analyzed with MultiSET software.

Statistical analyses

All statistical analyses were performed with SPSS 17.0 software (IBM, USA). Data are presented as means \pm standard deviations (SDs). An independent Student's *t*-test was used for comparisons between 2 groups, and 1-way analysis of variance (ANOVA) was used for comparisons among 3 or more groups. Correlation analysis was performed using Spearman's rank correlation coefficient. A *P*-value of less than 0.05 was considered statistically significant.

Table 1. Clinical characteristics of study participants at baseline.

Parameter	SLE patients (n=30)		Healthy control (n=15)
	High activity (n=13)	Low activity (n=17)	
Male/female (n)	3/10	2/15	5/10
Age (years, mean ±SD)	31.85±9.55	25.12±9.55	31.73±11.50
SLEDAI (mean ±SD)	14.69±2.53	5.08±2.25	ND
Anti-dsDNA-positive (n)	8/13	7/17	ND
C3 (median ± range)	0.43 (0.79–1.52)	0.56 (0.16–0.38)	ND
C4 (median ± range)	0.04 (0.79–1.52)	0.10 (0.16–0.38)	ND

ND – not detected.

Table 2. T-cell subset percentages across experimental groups at baseline (mean ±SD).

Subset	SLE Patients (n=30)		Healthy control (n=15)
	High activity (n=13)	Low activity (n=17)	
T%	63.00±15.15*	73.47±9.21	73.47±8.00
Th%	23.54±7.74*	29.71±7.89*	40.73±5.95
Ts%	32.15±11.77	36.65±11.87*	28.67±7.21

* Significant difference compared with the healthy control group ($P<0.05$).

Results

T-cell subset analysis at baseline

Patient characteristics are summarized in Table 1, and the percentages of T-cell subsets in each experimental group are shown in Table 2. At baseline, the CD3⁺ T-cell percentage was significantly lower in the high activity group than healthy controls ($P=0.004$), while there was no significant difference in the CD3⁺ T-cell percentage between the low activity group and healthy controls ($P>0.05$; Figure 1A). Furthermore, at baseline, the CD4⁺ helper T-cell (Th cell) percentage was significantly lower in the low activity group than healthy controls ($P<0.001$), and the CD4⁺ Th cell percentage was significantly lower in the high activity group than the low activity group ($P<0.001$; Figure 1B). Finally, at baseline, the CD8⁺ suppressor T-cell (Ts cell) percentage was significantly higher in the low activity group compared with healthy controls ($P<0.05$), while there was no significant differences in the CD8⁺ Ts cell percentage between the high activity and low activity groups nor between the high activity group and healthy controls ($P>0.05$; Figure 1C).

Correlation analysis at baseline

Correlation analysis was then applied to evaluate the relationship between T-cell subsets, SLEDAI scores, and the complement component C3 at baseline. A significantly negative correlation

was observed between the CD3⁺ T-cell percentage and SLEDAI score at baseline ($r=-0.471$, $P=0.015$; Figure 2A). A significantly negative correlation was observed between the CD4⁺ Th cell percentage and SLEDAI score at baseline ($r=-0.473$, $P=0.015$; Figure 2B). Moreover, a significantly positive correlation was observed between CD4⁺ Th cell percentage and C3 at baseline ($r=0.612$, $P=0.002$; Figure 2C). However, no correlations were observed among CD8⁺ Ts cell percentage, SLEDAI score, or C3 at baseline ($P>0.05$; data not shown).

Combination therapy alters T-cell subset distribution in SLE patients

The low activity and high activity patients in each treatment group (i.e., 3-month, 6-month, etc.) were pooled together and compared against the high activity baseline group and low activity baseline group in order to simplify the comparative analysis. After 3 months of combination therapy, the CD3⁺ T-cell percentage was significantly higher than the high activity baseline ($P=0.000$; Figure 3). After 6 months of combination therapy, the CD3⁺ T-cell percentages remained significantly higher than the high activity baseline ($P<0.01$). The CD3⁺ T-cell percentages across all combination therapy time groups showed no significant differences with the low activity baseline ($P>0.05$).

After 3 months of combination therapy, the CD4⁺ Th cell percentage was significantly higher than both the high activity and

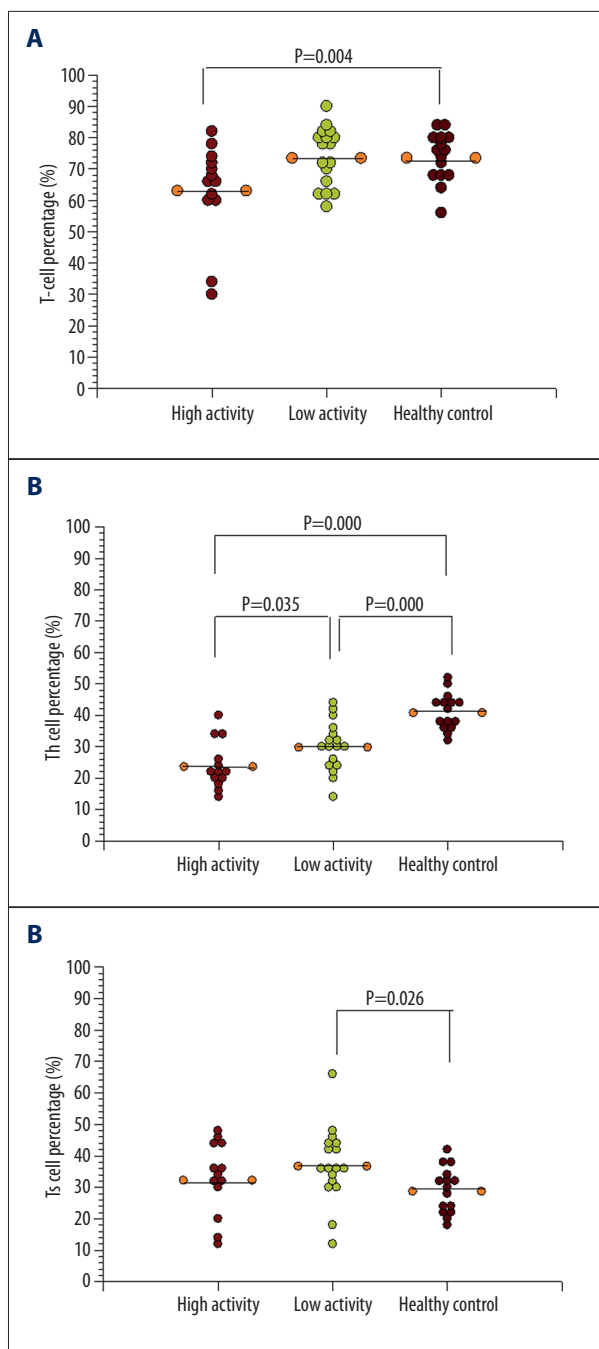


Figure 1. T-cell percentages across experimental groups at baseline. **(A)** The CD3⁺ T-cell percentage was significantly lower in the high activity group compared with the control group ($P=0.004$), while there was no percentage change observed in the low activity group ($P>0.05$). **(B)** The CD4⁺ helper T-cell (Th cell) percentage was significantly lower in both SLE groups as compared with the control group ($P<0.001$), and the Th cell percentage was significantly lower in the high activity group compared with the low activity group ($P<0.05$). **(C)** The CD8⁺ suppressor T-cell (Ts cell) percentage was significantly higher in the low activity group compared with the control group ($P<0.05$).

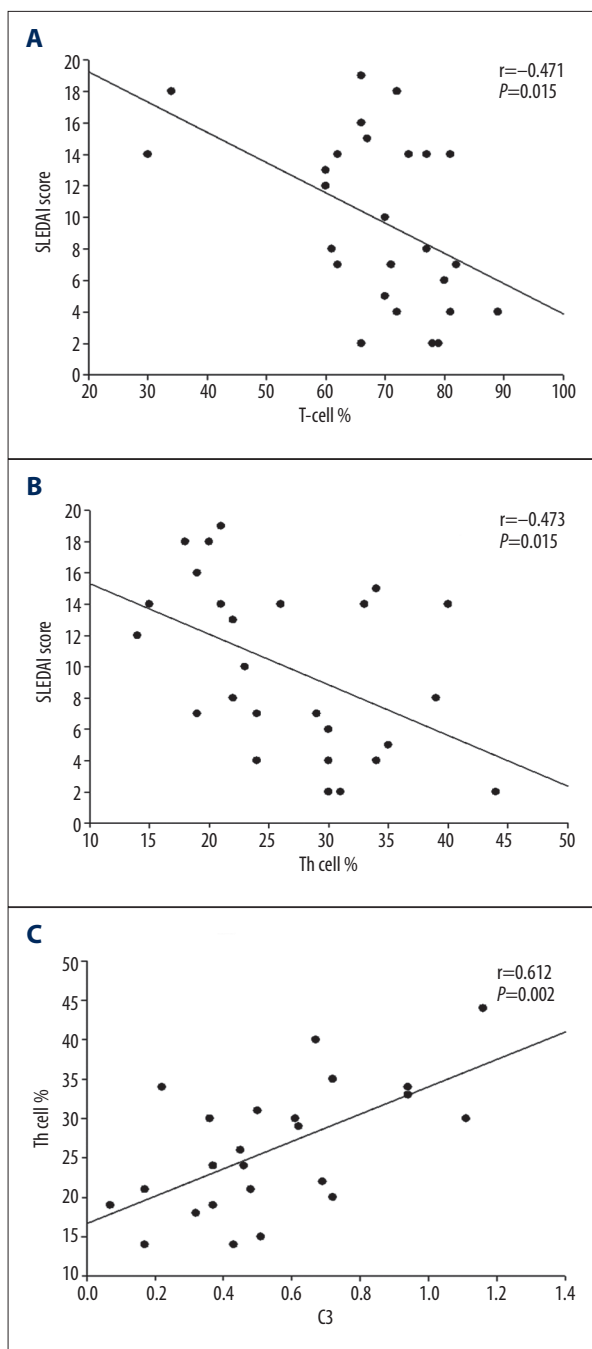


Figure 2. Correlations between T-cell subset percentages, SLEDAI score, and the complement component C3. **(A)** A significant correlation was found between CD3⁺ T-cell percentage and SLEDAI score ($r=0.471$, $P=0.015$). **(B)** A significant correlation was found between CD4⁺ helper T-cell (Th cell) percentage and SLEDAI score ($r=0.473$, $P=0.015$). **(C)** A significant correlation was found between the complement component C3 and CD4⁺ Th cell percentage ($r=0.612$, $P=0.002$).

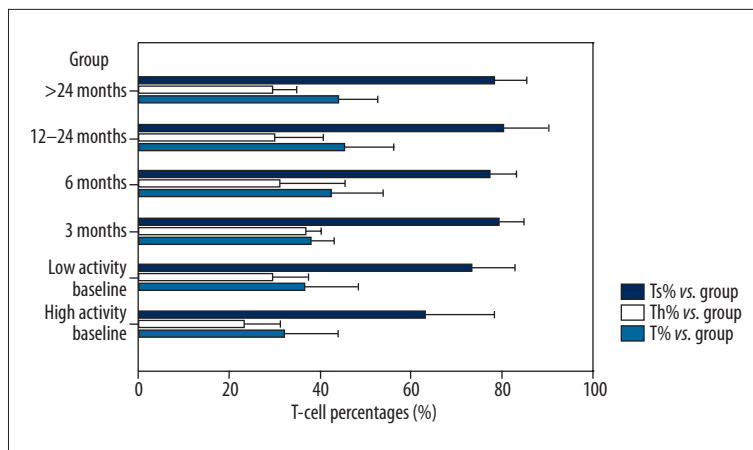


Figure 3. T-cell percentages over the duration of vincristine-cyclophosphamide combination therapy. Percentages of CD3⁺ T-cells, CD4⁺ helper T-cells (Th cells), and CD8⁺ suppressor T-cells (Ts cells) at 3 months, 6 months, 12–24 months, and >24 months of vincristine-cyclophosphamide combination therapy.

low activity baselines ($P < 0.05$). After 6 months of combination therapy, the CD4⁺ Th cell percentage showed no significant difference with both the high activity and low activity baselines ($P > 0.05$), but did present a non-significant upward trend.

After 3 months of combination therapy, the CD8⁺ Ts cell percentage showed no significant difference with both the high activity and low activity baselines ($P > 0.05$). After 6 months of combination therapy, the CD8⁺ Ts cell percentage was significantly higher than the high activity baseline ($P < 0.05$). After 12 months of combination therapy, the CD8⁺ Ts cell percentage was also significantly higher than the low activity baseline ($P < 0.05$).

Discussion

Dysfunction in T-cell activation and proliferation plays an important part in SLE development through promoting lymphocytic organ infiltration and aiding B-cells in producing auto-antibodies, a process which eventually leads to tissue destruction [11]. Specifically, changes in the T-cell subset distribution is a hallmark of SLE; for example, McInerney et al. [12] showed that 45% of the SLE patient population has markedly depressed CD4⁺ Th cells levels, and Matsushita et al. [5,13] showed that SLE patients have an increase in CD8⁺ Ts cell counts accompanied by a decrease in CD4⁺ Th cell counts. Here, we analyzed the T-cell subset distributions of both high and low activity SLE patients relative to healthy control individuals at baseline (prior to vincristine-cyclophosphamide combination therapy) in order to determine whether T-cell subset distributions vary across different levels of SLE disease activity. In the high activity group, the percentages of CD3⁺ T-cells and CD4⁺ Th cells were significantly lower than healthy controls at baseline; however, the CD8⁺ Ts cell percentage was not significantly different than healthy controls at baseline. In contrast, in the low activity group, the CD3⁺ T-cell percentage was not significantly different than healthy controls at baseline, but the CD4⁺ Th

cell percentage was significantly lower, while the CD8⁺ Ts cell percentage was significantly higher, than healthy controls at baseline. Based on this data, significantly negative correlations were observed between the CD3⁺ T-cell and CD4⁺ Th cell percentages and SLEDAI score at baseline. These combined results indicate that T-cell subset distributions vary across different levels of SLE disease activity with higher CD3⁺ T-cell and CD4⁺ Th cell percentages favoring lower SLE activity.

A complex regulatory interplay exists between CD8⁺ Ts cells and CD4⁺ Th cells in SLE. For example, Filaci et al. demonstrated that only CD8⁺ Ts cells from SLE patients in remission and healthy individuals can function as effective suppressors of CD4⁺ Th cells, while CD8⁺ Ts cells from active SLE patients fail to suppress committed CD4⁺ Th cells [14,15]. Moreover, CD4⁺2H4⁺ Th cells – which are suppressor/inducer CD4⁺ Th cells that promote CD8⁺ Ts cell-based inhibition of B-cells – have been previously shown to have significantly lower proportions in SLE patients. Based on this previous evidence from SLE patients, the lower CD4⁺ Th cell percentages observed with higher SLE activity in the current study may actually indicate lower CD4⁺2H4⁺ Th cell levels and, in turn, lower CD8⁺ Ts cell-based inhibition of B-cells. If so, this hypothesis would correctly explain the deficient CD8⁺ Ts cytotoxic capacity as well as the B-cell hyperactivity observed in highly active SLE [16–19]. In order to test this hypothesis, further research should focus on measuring and correlating the levels of CD4⁺2H4⁺ Th cells, CD8⁺ Ts cells, and B-cell autoantibody production as a function of SLE activity.

With respect to vincristine-cyclophosphamide combination therapy, vincristine blocks T-cell mitosis in metaphase, while cyclophosphamide targets rapidly-proliferating T-cells irrespective of cell cycle phase [20,21]. Therefore, vincristine and cyclophosphamide can be applied in combination to accomplish a synergistic cytotoxic effect. Specifically, as vincristine activity begins to decline after a few hours, T-cells that have accumulated in metaphase re-initiate their cell cycle, and cyclophosphamide is then available to kill these T-cells during DNA replication [22–27]. Here,

we found that after the first 3 months of vincristine-cyclophosphamide combination therapy, the CD3⁺ T-cell and CD4⁺ Th cell percentages both increased relative to the high activity baseline, which appropriately correlates with reduced SLE disease activity as discussed above. After 6 months of combination therapy, the CD3⁺, CD4⁺, and CD8⁺ T-cell percentages all increased relative to the high activity baseline. As CD3⁺ T-cell and CD4⁺ Th cell percentages negatively correlate with SLEDAI, vincristine-cyclophosphamide combination therapy appears to positively affect T-cell subset distribution to control SLE disease activity by increasing their CD3⁺ T-cell and CD4⁺ Th cell percentages.

Conclusions

T-cell subset distributions vary across different levels of SLE disease activity with higher CD3⁺ T-cell and CD4⁺ Th

cell percentages favoring lower SLE activity. Vincristine-cyclophosphamide combination therapy appears to positively affect the T-cell subset distribution in SLE patients to keep the disease in remission by increasing their CD3⁺ T-cell and CD4⁺ Th cell percentages. Further studies are needed to examine how the distributions of CD4⁺ Th, CD8⁺ Ts cells, and other lymphocytes are maintained in SLE and to investigate the T-cell-specific cytotoxic mechanisms underlying vincristine-cyclophosphamide combination therapy in SLE.

Conflict of interest

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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