Therapeutic Potential of Targeting Tfr/Tfh Cell Balance by Low-Dose-IL-2 in Active SLE: A Post-Hoc Analysis from a Double-Blind RCT Study

Miao Miao  
Peking University People's Hospital  https://orcid.org/0000-0001-8927-4128

Xian Xiao  
Peking University People's Hospital Department of Rheumatology and Immunology

Jiayi Tian  
Peking University People's Hospital

Yunzhi Zhufeng  
Peking University People's Hospital

Ruiling Feng  
Peking University People's Hospital

Ruijun Zhang  
Peking University People's Hospital

Jiali Chen  
Peking University People's Hospital

Xiaoying Zhang  
Peking University People's Hospital

Bo Huang  
Peking University People's Hospital

Yuebo Jin  
Peking University People's Hospital

Xiaolin Sun  
Peking University People's Hospital

Jing He (✉️ hejing1105@126.com)  
Peking University People's Hospital

Zhanguo Li  
Peking University People's Hospital

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Abstract

**Objective:** To investigate the regulation of T follicular regulatory (Tfr) and T follicular (Tfh) cell subtypes by low-dose IL-2 in systemic lupus erythematosus (SLE) in a randomized, double-blind, placebo-controlled clinical trial.

**Methods:** A post-hoc analysis was performed in a randomized cohort of SLE patients (n=60) receiving low-dose IL-2 therapy (n=30) or placebo (n=30), along with standard of care treatment. The primary end point was attainment of SLE responder index-4 (SRI-4) at week 12 in the trial. Twenty three healthy controls were enrolled for T cell subsets detection at the same time with the trial. The t-stochastic neighbor embedding (tSNE) analysis of CD4 T subsets based on immune cells flow cytometry markers was performed to distinguish Tfh, Tfh1, Tfh2, Tfh17 and Tfr cell subsets.

**Results:** Compared with HC, the frequency of Tfr (CXCR5⁺PD-1lowTreg and CXCR5⁺PD-1highTreg) cells was significantly reduced, while the pro-inflammatory Tfh cells were increased in patients with SLE. The imbalanced Tfh cell was associated with several pathogenic factors (anti-dsDNA antibodies (r=0.309, P=0.027) and serum IL-17 (r=0.328, P=0.021)) and SLE Disease Activity Index (SLEDAI) score (r=0.273, P=0.052). Decreased CXCR5⁺PD-1lowTreg/Tfh and CXCR5⁺PD-1lowTreg/Tfh17 were both associated with increased immunoglobulin M (IgM) (r=-0.448, P=0.002 and r=-0.336, P=0.024 respectively). Efficacy of low-dose IL-2 therapy was associated with a restored Tfr/Tfh cell balance.

**Conclusion:** These data supports the hypothesis that promotion of Tfr associated with decreased disease activities, and that low-dose IL-2 therapy can recover Tfr/Tfh immune balance.

**Trial registration number** ClinicalTrials.gov Registries (NCT02465580).

**Key Messages**

1. Deregulation between Tfr and Tfh subsets associated with severity of SLE.
2. Low-dose IL-2 therapy was efficient in patients with SLE.
3. Low-dose IL-2 therapy elevates the Tfr/Tfh ratio, which might be a novel concept to design the therapeutic regimen.

**Introduction**

Systemic lupus erythematosus (SLE) is characterized by the breakdown of immune tolerance leading to auto-reactive immune responses and consequently, tissue and organ damages. Over the past decades, extensive studies on regulatory T (Treg) cells have revealed that these cells can maintain tolerance and regulate immune responses [1, 2], while T follicular helper cells (Tfh) play an important role in the production of autoantibodies and pro-inflammatory cytokines in SLE [3–5]. Moreover, the imbalance of the immune response between pro-inflammatory and anti-inflammatory cells is central in SLE pathogenesis.
T follicular regulatory (Tfr) cells share features with Tfh and conventional Treg cells, and can inhibit Tfh cells and germinal center (GC) responses with a significant impact in humoral immunity [6–8]. Previous studies have suggested that Tfr cells can be identified as CXCR5⁺PD-1⁺ Treg and CXCR5⁺PD1⁺ Treg according to the surface marker CXCR5 and programmed cell death protein 1 (PD-1) [9–13]. The function of the two subsets of Tfr remains unclear. Imbalance or disfunction of Tfr subsets may directly or indirectly affect B cells, leading to expansion of overactive B cells which contributes to various immune-related clinical diseases [14, 15]. So far, the role of balance between Tfh and Tfr subsets in SLE is still controversial due to the heterogeneity of the disease, cohort size, and methods of studies [16, 17].

Efficacious treatments, including low-dose Interleukin 2 (IL-2), might promote Tfr cell responses, and inhibit Tfh cell development in SLE [18, 19]. However, it is not well understood how these circulating Tfh-like cell subsets, including Tfr and Tfh subsets, are involved in the disease. To date, the balance of these new subsets has not been addressed, neither how these cells respond to SLE treatment. Here, we identified an imbalanced profile of Tfh cell subsets in SLE, including newly described anti-inflammatory CXCR5⁺PD-1⁻ Treg, CXCR5⁺PD-1⁺ Treg and the pro-inflammatory Tfh and Tfh17; and investigated the change of these subtypes by low-dose IL-2 treatment in a randomized, double-blind, placebo-controlled study in SLE.

Participants And Methods

Participants

This was a post-hoc analysis of data from an RCT clinical study (NCT02465580) of low-dose IL-2 in SLE patients. Full details of study designs and inclusion/exclusion criteria for each completed study have previously been published [1, 2]. Studies were conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonization Guidelines for Good Clinical Practice. Besides, 23 healthy controls (HC) were enrolled during the same time of RCT study. Table 1 summarized baseline characteristics. Written informed consents were obtained from these healthy controls. The experimental protocol followed the guidelines of the Declaration of Helsinki and was approved by the Human Ethics Committee of Peking University People's Hospital (Beijing, China).
Table 1
Baseline characteristics of SLE patients and healthy controls (HC) in this study

| Characteristics                        | SLE (n = 60) | HC (n = 23) | P value |
|----------------------------------------|-------------|-------------|---------|
| Age, year, mean ± SD                   | 30.84 ± 9.48| 29.83 ± 9.72| 0.474   |
| Female/Male                            | 56/4        | 21/2        | > 0.99  |
| Duration, months, mean ± SD            | 65.15 ± 58.65| -           | -       |
| Medications                            | -           | -           | -       |
| Prednisone dose, mg/day, median (range)| 13.5 (0, 50) | -           | -       |
| Hydroxychloroquine                     | 57 (95)     | -           | -       |
| Cyclophosphamide                       | 4 (6.67)    | -           | -       |
| Azathioprine                           | 5 (8.33)    | -           | -       |
| Cyclosporine                           | 5 (8.33)    | -           | -       |
| Mycophenolate Mofetil                  | 17 (28.33)  | -           | -       |
| Tacrolimus                             | 2 (3.33)    | -           | -       |
| Leflunomide                            | 4 (6.67)    | -           | -       |
| Thalidomide                            | 1 (1.67)    | -           | -       |
| Methotrexate                           | 1 (1.67)    | -           | -       |
| Interleukin-2                          | 30 (50)     | -           | -       |

For a continuous variable, median (range) or mean ± SD. For a categorical variable, count (percentage). SLE, Systemic lupus erythematosus.

Flow Cytometric Analysis

Single-cell suspensions from peripheral blood in SLE patients and HC were analyzed by multicolor flow cytometry (FACSria II; BD Biosciences, Franklin Lakes, NJ, USA). t-stochastic neighbor embedding (tSNE) analysis of CD4 T subsets based on immune cells flow cytometry markers was shown in Fig. 1. Data were also analyzed using FlowJo v10 software (Tree Star, Ashland, OR, USA) (Figure S1). The absolute number of CD4 T cell subsets was calculated by multiplying proportion of CD4 T cell subsets in lymphocytes by absolute lymphocyte number determined with an automated hematology analyzer. Detailed protocol of trial has been published online [18].

Cytometric Beads Array (cba) Analysis Of Serum Cytokines
Serum levels of IL-2, IL-17 and other inflammatory cytokines were determined by human Th1/Th2/Th17 14-plex (QuantoBio, Beijing, China) according to the manufacturer’s instructions.

**Statistical analysis**

Data were expressed as the median and range for non-normally distributed data, while mean ± standard deviation (SD.) for normally distributed data. The Student’s unpaired or paired t test was performed to compare two groups for parametric data, and the Mann-Whitney U test or Wilcoxon rank sum test was performed for nonparametric data. Relationships between variables were analyzed by Spearman’s rank test. Statistical analyses were performed using SPSS v.22.0 or R v.3.6.3 software. Two-sided P values < 0.1 were considered statistically significant.

**Results**

**Characteristics of SLE patients**

Given recent studies showing the imbalances in the effector and regulatory Tfh cells compartment in SLE patients [3, 4], and in light of our finding that low-dose IL-2 treatment significantly influences Tfh subtypes, we recruited a small cohort of healthy controls (HC) (n=23, Table 1) for comparative analysis.

The demographic and clinical manifestations of these patients were shown in Table 1. There was no significant difference between patients and HCs regarding age or gender. 98% of the SLE patients were positive in anti-dsDNA tests, 42% had renal involvement and 48% had skin manifestations.

**Imbalanced Tfr/Tfh in SLE**

As shown in Table 2, regulatory T cells including Treg, CXCR5⁺PD-1lowTreg, CXCR5⁺PD-1highTreg cells were significantly reduced in SLE patients than those in HC (P=0.087, P=0.033, P<0.001, P<0.001, respectively). In contrast, the effector Tfh cells were significantly increased in SLE patients than in HC (p=0.081). Besides, Tfr subsets: Tfh subsets ratios in SLE were dramatically decreased, including CXCR5⁺PD-1lowTreg/Tfh (P=0.043), CXCR5⁺PD-1highTreg/Tfh (p<0.001), CXCR5⁺PD-1lowTreg/Tfh17 (P=0.052) and CXCR5⁺PD-1highTreg/Tfh17 (P<0.001).

**High Tfh associated with decreased serum IL-2**

As show in Figure 2, an expanded inflammatory Tfh cell compartment was correlated with higher serum IL-17 levels (r=0.328, P=0.021) and increased frequency of Tfh17 correlated with lower serological IL-2 (r=-0.295, P=0.04) (Figure 2). There was a reduction of CXCR5/PD-1 double positive subset (CXCR5⁺PD1highTreg), which was related with IL-10 elevation (r=0.243, P=0.093, Figure 3).
Table 2. Difference of CD4 T subsets between HC and SLE, and between active and remission group.

| Variables                  | HC (n=23) | SLE Active (n=60) | Remission (n=59) | P, HC VS. Active | P, Remission VS. Active |
|----------------------------|-----------|-------------------|------------------|-----------------|------------------------|
| Proportion (percentage in lymphocyte, %) |           |                   |                  |                 |                        |
| Treg                       | 1.22 (1, 1.47) | 0.95 (0.65, 1.53) | 1.6 (0.97, 2.49) | 0.087           | 0.001                  |
| CXCR5⁺PD⁻¹lowTreg          | 0.09 (0.04, 0.14) | 0.06 (0.03, 0.1)  | 0.13 (0.06, 0.25) | 0.033           | <0.001                 |
| CXCR5⁺PD⁻¹highTreg         | 0.09 (0.07, 0.13) | 0.02 (0.01, 0.04) | 0.11 (0.06, 0.19) | <0.001          | <0.001                 |
| Tfh                        | 0.28 (0.17, 0.43) | 0.39 (0.24, 0.56) | 0.19 (0.09, 0.37) | 0.081           | 0.002                  |
| Tfh1                       | 0.61 (0.38, 0.99) | 0.2 (0.08, 0.36)  | 0.17 (0.09, 0.28) | <0.001          | 0.42                   |
| Tfh2                       | 1.06 (0.58, 1.5)  | 0.42 (0.21, 0.6)  | 0.36 (0.19, 0.68) | <0.001          | 0.367                  |
| Tfh17                      | 0.45 (0.15, 0.65) | 0.47 (0.27, 0.87) | 0.44 (0.2, 0.83) | 0.345           | 0.851                  |
| Absolute number (cells/L)  |           |                   |                  |                 |                        |
| Treg                       | 25.51 (19.98, 30.78) | 11.94 (6, 20.81) | 20.51 (9.17, 29.06) | 0.035           | 0.014                  |
| CXCR5⁺PD⁻¹lowTreg          | 2.98 (1.71, 3.1)  | 0.67 (0.24, 1.21) | 1.33 (0.8, 3.54) | 0.037           | 0.005                  |
| CXCR5⁺PD⁻¹highTreg         | 2.99 (1.18, 3.57) | 0.24 (0.06, 0.56) | 1.5 (0.71, 2.27) | 0.001           | <0.001                 |
| Tfh                        | 7.29 (4.34, 8.92) | 4.6 (2.58, 10.28) | 2.84 (0.81, 6.04) | 0.625           | 0.008                  |
| Tfh1                       | 14.26 (12.37, 18.22) | 2.11 (0.95, 5.47) | 1.92 (0.68, 4.28) | 0.002           | 0.427                  |
| Tfh2                       | 19.31 (14.66, 24.22) | 4.43 (2.11, 11.2) | 4.04 (1.73, 11.88) | 0.002           | 0.751                  |
| Tfh17                      | 8.82 (7.25, 12.22) | 5.39 (2.33, 14.38) | 4.67 (2.47, 10.36) | 0.642           | 0.664                  |
### Ratios

|                      | Treg/Tfh | CXCR5⁺PD⁻₁lowTreg/Tfh | CXCR5⁺PD⁻₁highTreg/Tfh | Treg/Tfh17 | CXCR5⁺PD⁻₁lowTreg/Tfh17 | CXCR5⁺PD⁻₁highTreg/Tfh17 |
|----------------------|----------|------------------------|-------------------------|------------|--------------------------|--------------------------|
|                      | 3.69 (3.04, 8.49) | 0.3 (0.21, 0.57) | 0.46 (0.21, 0.69) | 3.87 (2.27, 5.71) | 0.25 (0.17, 0.34) | 0.3 (0.14, 0.64) |
|                      | 2.49 (1.89, 4.5)  | 0.16 (0.08, 0.31) | 0.05 (0.01, 0.1) | 2.29 (1.23, 4.21) | 0.11 (0.05, 0.22) | 0.03 (0.01, 0.09) |
|                      | 8.93 (4.04, 18.29) | 0.61 (0.4, 1.32)  | 0.54 (0.24, 1.3)  | 3.89 (2.01, 6.62) | 0.28 (0.18, 0.52) | 0.23 (0.12, 0.45) |
|                      | 0.002     | <0.001                 | <0.001                 | 0.047      | <0.001                   | <0.001                   |

SLE, systemic lupus erythematosus. HC, healthy controls. Data are median (IQR).

### Imbalanced Tfh and Tfr cell association with disease activity in SLE

Treg cells were decreased and associated with elevated ESR (r=-0.382, P<0.01) and Safety of Estrogens in Lupus Erythematosus National Assessment version of the SLE Disease Activity Index (SLEDAI) (r=-0.245, P=0.089, Figure 2). At the same time, the decrease of CXCR5⁺PD⁻₁lowTreg was associated with increased ESR and anti-dsDNA antibodies production (Figure 2, 3). In contrast, an increased inflammatory Tfh cell compartment was found and was correlated with elevated SLEDAI, titer of anti-AnuA, anti-dsDNA antibodies, serum IL-17 and decreased C3 (Figure 2, 3).

Upon further analysis of the correlation between regulatory and effector subsets, we found decreased Treg/Tfh ratio in severe patients with higher SLEDAI score, higher titers of anti-AnuA and anti-dsDNA antibodies (Figure 3). In addition, there was a reduced CXCR5⁺PD⁻₁lowTreg/Tfh in this group of severe patient.

Figure 3 showed that the frequency of Tfh1 and Tfh2 was positively correlated with the number of total B cells and switched memory B (CD19⁺IgD⁻CD27⁺) cells. CXCR5⁺PD⁻₁lowTreg/Tfh17 was negatively correlated with switched memory B cells and plasma B cells (r=-0.341, P=0.027, Figure 3). And decreased CXCR5⁺PD⁻₁lowTreg/Tfh and CXCR5⁺PD⁻₁lowTreg/Tfh17 were both associated with increased serum level of IgM.

### Low-dose IL-2 therapy increased Tfr/Tfh ratio in SLE patients

In this RCT of low-dose IL-2 therapy in SLE, low-dose IL-2 significantly increased Tregs [10]. With effective treatment, British Isles Lupus Assessment Group (BILAG), SLEDAI, SLE Responder Index-4 (SRI-4),
physician’s global assessment (PGA), myositis, fever, alopecia, vasculitis, arthritis, oral ulcer and rash were all improved at week 12 (Figure 4) [10]. After 3 cycles of low-dose IL-2 therapy, the frequency of CXCR5*PD-1lowTreg cells and CXCR5*PD-1highTreg cells in lymphocyte was significantly increased at week 12 compared to placebo control (0.06 (0.03, 0.1) vs. 0.13 (0.06, 0.25), \( p<0.001 \) and 0.02 (0.01, 0.04) vs. 0.11 (0.06, 0.19), \( P<0.001 \) respectively) (Table 2, Figure 4). Similarly, the absolute number of these Treg cells (CXCR5*PD-1highTreg and CXCR5*PD-1lowTreg) were significantly increased after the treatment of low-dose IL-2 (0.67 (0.24, 1.21) vs. 1.33 (0.8, 3.54), \( P=0.005 \) and 0.24 (0.06, 0.56) vs. 1.5 (0.71, 2.27), \( P<0.001 \) respectively) (Table 2, Figure 4). Besides, compared to baseline, Tfr subsets: Tfh subsets ratios in SLE were dramatically increased, including CXCR5*PD-1lowTreg/Tfh (\( P<0.001 \)), CXCR5*PD-1highTreg/Tfh (\( P<0.001 \)), CXCR5*PD-1lowTreg/Tfh17 (\( P<0.001 \)) and CXCR5*PD-1highTreg/Tfh17 (\( P<0.001 \)).

**Discussion**

Increasing evidence indicates that Tfh cells are important in the pathogenesis of SLE. Tfh cells are recognized as a distinct T-cell subset, which provides help for GC formation, B-cell affinity maturation, and immunoglobulin class switching, as an indispensable part of adaptive immunity. Our previous work showed that patients with SLE have an increased number of peripheral Tfh cells, which positively correlates with autoantibody titers (anti-dsDNA antibodies) and disease activity, as measured by the SLEDAI. Others have reported that the aberrant expression of Tfh cells is a common feature in mouse models of SLE, suggesting its contribution in the development of autoimmune diseases [4, 5].

Besides Tfh cells, a subset of Treg cells, named Tfr cells, have been identified. These cells share common characteristics with Tfh and conventional Treg cells, and can inhibit GC responses, regulating the number of Tfh and GC B cells [6–8]. Therefore, it is generally believed that Tfr cells constrain the B-cell “help” provided by Tfh cells to maintain immune homeostasis. An aberrant or disordered Tfh/ Tfr balance may result in the break of tolerance, excessive B-cell proliferation, antibody production, and the development of autoimmune diseases. A recent study showed the importance of the Tfr/ Tfh balance in autoimmune responses in BXD2 mice, which display spontaneous autoreactive GC formation [21]. In addition, intravenous immunoglobulin administration to mice with collagen-induced arthritis augments the number of Tfr cells and represses the subsequent maturation of GC B cells [22], which also supports the idea of a critical role for Tfr cells in autoimmune diseases.

In our study, we found a deficiency of Tfr cell subsets, including CXCR5*PD-1lowTreg, and CXCR5*PD-1highTreg; and increased Tfh cells in the peripheral blood of SLE patients. The shifted balance between circulating CXCR5*PD-1lowTreg and Tfh cells correlated not only with reduced serum IL-2, IL-10 and increased IL-21 levels in patients; but also with clinical SLE parameters, e.g. ESR, anti-dsDNA antibodies and disease activity (SLEDAI Scores). These findings are consistent with previous studies in vitro, in which Tfh and CXCR5*PD-1lowTreg or CXCR5*PD1highTreg cells can antagonize B cell function, production of high-affinity antibodies and the memory B cell differentiation [22]. CXCR5*PD-1lowTreg cells
play an important immunosuppressive function by curbing self-reactive auto-antibodies development within the GC during an inflammatory immune response [5]. Therefore, deregulation of the Tfr and Tfh cell compartments is associated with disease severity, B cell frequency and antibody production in SLE.

There have been several relatively successful attempts to reduce the severity of SLE in humans via blockade of Tfh-cell differentiation and activity. Studies using monoclonal antibodies against ICOSL inhibited the development of Tfh and GC B cells resulting in decreased anti-dsDNA antibodies and improved kidney function in both human and mouse [23]. For years, SLE therapy has relied on broad spectrum immunosuppressants; however, a growing body of work shows that a targeted increase of regulatory T cells may be a more attractive therapy [18, 19, 24, 25].

IL-2 is essential for the development and maintenance of Treg cells, which prevent the development of autoimmune disease. Low-dose IL-2 can promote Tregs by activating the transcription factor STAT5, which binds to the Foxp3 locus and promotes Foxp3 expression without activation of effector T cells. More recently, IL-2 has been shown to be essential for the inhibition of Tfh cell development. Thus, in this study, we asked if low-dose IL-2 therapy might also elevate the Tfr/Tfh ratio, exploring a novel concept for rational therapeutic design.

Our previous studies had proven a deficient Treg cell compartment and decreased IL-2 levels in circulation of SLE, and the efficacy of low-dose IL-2 treatment. But there was no study addressing the impact of low-dose IL-2 on Tfr : Tfh balance. After effective therapy, especially low-dose IL-2 therapy, the imbalanced Tfr and Tfh subsets were reversed accompanying improvement of disease activity. Furthermore, Tfr subsets were all increased regardless of output measurement; proportion and absolute number. Although we didn’t see a significant change in Th17 frequency, the ratios of CXCR5⁺PD-1<sub>low</sub>Treg/Tfh17 and CXCR5⁺PD-1<sub>high</sub>Treg/Tfh17 were significantly decreased compared to those in healthy controls. Besides, we didn’t see any obvious difference between CXCR5⁺PD-1<sub>low</sub>Treg and CXCR5⁺PD-1<sub>high</sub>Treg, perhaps reflecting a functional overlap of these two subsets.

**Conclusions**

In summary, our findings indicate that imbalance of Tfh and Tfr is important for SLE severity; and low-dose IL-2 ameliorates lupus autoimmunity favoring Tfr cell expansion. Our study added to these findings by demonstrating that low-dose IL-2 therapy selectively activates and expands Tfr cells, while demonstrating clinical efficacy in SLE. Further studies are needed to better understand how to explore Tfh cell or Tfr cell signatures to stratify patients, and guide the design of novel treatment regiments for SLE in future clinical trials.

**Abbreviations**

Tfr: T follicular regulatory; Tfh: T follicular; IL-2: interleukin-2; SLE: systemic lupus erythematosus; tSNE: t-stochastic neighbor embedding; HC: healthy controls; IL-17: interleukin-17; SLEDAI: SLE Disease Activity
Declarations

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Author’s contributions

JH had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. JH, XS, ZL and MM conceived of and designed the study. MM, XX, JT, YZ, RZ, RF, JC, XZ, BH and YJ acquired the data. MM, XX, JT, YZ and RF analyzed and interpreted the data. All authors were involved in drafting the article or making critical revisions for important intellectual content, and all authors read and approved the submitted manuscript.

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Ethics approval and consent to participate

This study was approved the Human Ethics Committee of Peking University People's Hospital (Beijing, China) and all subjects provided written informed consent.

Consent for publication

Not applicable.

Disclosure statement

The authors have declared no conflicts of interest.

Data availability statement

Data are available upon reasonable request.

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Figures
Figure 1

tSNE analysis of CD4 T subsets based on immune cells flow cytometry markers. tSNE, t-stochastic neighbor embedding.
Figure 2

The correlations between CD4 T cell subsets and clinical characteristics in SLE patients. SLEDAI, SLE Disease Activity Index. ESR, erythrocyte sedimentation rate. C3, complement 3. C4, complement 4. IFN-α, interferon-α. IL-2, Interleukin-2. IL-21, Interleukin-21. IL-7, Interleukin-7. IFN-γ, interferon-γ. IL-17, Interleukin-17. IL-10, Interleukin-10. TGF-β, tumor necrosis factor-β. **, P<0.01. *, P<0.05. ▲, P<0.1. #, absolute number. %, percentage in lymphocyte.
Figure 3

Correlations between CD4 T cell subsets, B cells and immunoglobulins in SLE. B, B cell. Plasma B, Plasma B cell. Trans B, class-switched memory B cells. IgA, immunoglobulin A. IgG, immunoglobulin G. IgM, immunoglobulin M. ANuA, anti-nucleosome antibodies. dsDNA, Anti-double stranded DNA antibodies. *, P<0.05. **,P<0.01. ▲, P<0.1. #, absolute number. %, percentage in LY.

Supplementary Files

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